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VOLUME 1

Extremophiles Handbook

Springer

Extremophiles Handbook

Koki Horikoshi (Ed.)

Extremophiles Handbook

With 238 Figures and 120 Tables

Springer

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ISBN 978-4-431-53897-4
DOI 10.1007/978-4-431-53898-1

This publication is available also as:
Electronic publication under ISBN 978-4-431-53898-1 and
print and electronic bundle under ISBN 978-4-431-53899-8

Library of Congress Control Number: 2010938941

Springer Tokyo Dordrecht Heidelberg London New York

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

Printed on acid-free paper

Preface

▶ Man is a pliable animal, a being who gets accustomed to everything.

Feodor Mikhailovitch Dostoievsky

Until quite recently, almost all biologists believed that life could survive only within a very narrow range of temperature, pressure, acidity, alkalinity, salinity and so on, in so-called moderate environments. So when microbiologists looked around for interesting bacteria and other life-forms, they attempted to isolate microorganisms only from moderate environments. Nature, however, contains many extreme environments, such as acidic or hot springs, saline lakes, deserts, alkaline lakes and the ocean bed. All of these environments were thought to be too harsh for life to survive.

However, in recent times, huge numbers of microorganisms have been found in such extreme environments. Moreover, some of them cannot survive in so-called "moderate" environments. For example, thermophilic bacteria, high temperature-loving bacteria, grow in environments with extremely high temperatures, but will not grow at 20 to 40°C. Some alkali-loving bacteria cannot grow in a nutrient broth at pH 7.0, but flourish at pH 10.5. For thermophiles, the "moderate" environments would be very cold. Thus, our idea of what constitutes an extreme environment is based on our experience of our own environment.

The progressive discoveries in recent years of life in environments controlled by these and other extreme conditions such as radiation, hyper acidity and salinity, intense pollution, or very low nutrient availability, has revealed that extremophilic organisms are both abundant and diverse. It is important to distinguish between true extremophiles that flourish only in extreme environments and those organisms that, for various reasons, can merely tolerate or survive at environmental extremes. Clearly we have been too anthropocentric in our thinking.

It should be our task to save and conserve such organisms for the benefit of all humankind and the sustainability of our planet. Finding new life-forms will definitely contribute to the development of basic science and new biotechnologies. Basic science is the one common language of all human beings. We have just started to communicate with nature through basic science.

Science is just a sheet of white paper. If Monet placed his colors on this paper, the paper would become a painting. If Beethoven wrote on the paper, the paper would become music. We should therefore extend our consideration to other environments in order to investigate new extremophiles through this book Extremophiles Handbook.

The purpose of this book is to bring together the rapidly growing and often scattered information on microbial life in the whole range of extreme environments and to evaluate it in relation not only to the biodiversity, biochemistry, physiology and ecology that it comprises, but also to assess how we can gain clues to the origin of life and aid the search for astrobiology, and finally to explore the biotechnological potential of these fascinating organisms.

I used to say "Microorganisms can do anything you want, if you know how to ask them". The readers may find the solutions from our Extremophiles Handbook.

Koki Horikoshi, Editor-in-Chief
August 2010

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Koki Horikoshi has published 4 books for alkaliphiles: Alkalophilic Microorganism, 1982 (Springer-Verlag); Microorganisms in Alkaline Environments, 1991 (VCH Publisher); Alkaliphiles, 1999 (Harwood Academic Publishers); Alkaliphiles: Genetic properties and applications of enzymes, 2006 (Springer-Verlag).

He has served on the managing committees of several scientific societies and was a founder of International Society of Extremophiles as the first President. In 1999, he launched one important journal "Extremophiles" from Springer-Verlag. And he served as the Chief Editor for 13 years. By these works, ISE offered the award for lifetime achievement in 2010.

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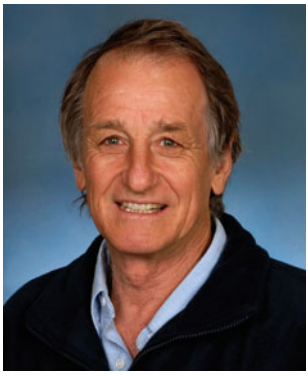

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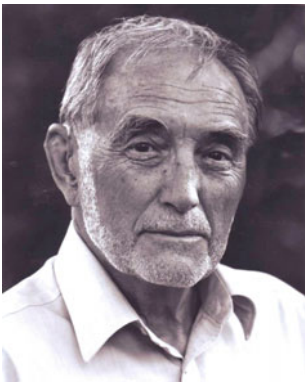

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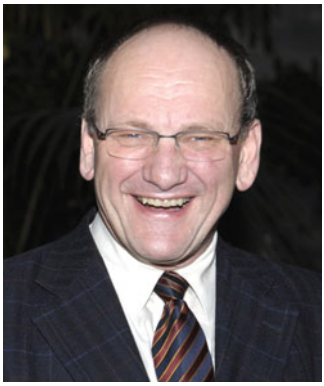

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Prologue

1.1 Prologue: Definition, Categories, Distribution, Origin and Evolution, Pioneering Studies, and Emerging Fields of Extremophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_1.1,

> *I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.*
>
> Isaac Newton

What are Extremophiles?

Extremophiles are organisms that are adapted to grow optimally at or near to the extreme ranges of environmental variables. Most extremophiles are microorganisms that thrive under conditions that, from a human perspective, are clearly hostile. RD MacElroy first coined the term "extremophile" in a 1974 paper entitled "Some comments on the evolution of extremophiles," but definitions of *extreme* and *extremophile* are of course anthropocentric; from the point of view of the organism per se, its environment is that to which it is adapted and thence is completely normal. 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 *extremotrophs* (Mueller et al. 2005). This distinction between extremophily and extremotrophy is not merely a semantic one and it highlights a number of fundamental issues relating to the experimental study such as (1) inappropriate methods that may have been used to isolate putative extremophiles, (2) claims of extremophily that may not have been tested rigorously, (3) putative extremophily that may be compromised by subsequent serial cultivation under laboratory conditions, and (4) inadequate attempts to determine whether organisms are adaptable to only small differences in environmental variables (see Bull, Chap. 12.1 Actinobacteria of the Extremobiosphere). Note also that many species can survive extreme conditions in a dormant state but are not capable of growing or reproducing indefinitely under those conditions.

An extremophile is an organism that thrives under extreme conditions; moreover the term most frequently is used to refer to organisms that are unicellular and prokaryotic. Because many extremophiles are members of the Domain *Archaea* and most known archaea are extremophilic, on occasion, the terms have been used interchangeably. However, this is a very misleading conception because many organisms belonging to the *Bacteria* and *Eukarya* have extremophilic or extremotrophic life cycles. Additionally, not all extremophiles are unicellular. Most extremophiles are microorganisms, for example, the presently known upper optimum growth temperature is 113°C for archaea (upper known maximum is for a black smoker strain at 121°C), 95°C for bacteria, and 62°C for single-celled eukaryotes, in contrast to multi-cellular eukaryotes that have rarely been shown to grow above 50°C. Members of the *Archaea* are uniquely hyperthermophilic, that is, they exhibit optimum growth at 80°C and above, but organisms having other expressions of hyper-extremophily have evolved in each of the domains.

The study of extremophilic and extremotrophic eukaryotic organisms has been relatively neglected in comparison to their prokaryotic counterparts. Nevertheless, extremophily is being increasingly reported among algae and fungi, for example. The case of halophilic green algae such as *Dunaliella* species has been known for several decades but more recently discovered examples of unicellular eukaryotic extremophiles/extremotrophs include *Cyanidiales* algae (Toplin et al. 2008) that show obligate acidophily and moderate thermophily. Similar adaptations to extreme environments can be seen among unicellular and mycelial fungi. Members of the black yeast genus *Hortaea* include extreme halophiles and extreme acidotrophs and to date represent the most salt- and acid-tolerant eukaryotic biota on Earth. Moreover, quite a wide

taxonomic range of yeast-like and mycelial fungi have evolved potent radiation resistance (see Grishkan, Chap. 10.2 Ecological Stress: Melanization as a Response in Fungi to Radiation; Dadachova and Casaadevall, Chap. 10.3 Melanin and Resistance to Ionizing Radiation in Fungi). Further discussion of eukaryotes in extreme environments can be found in Weber et al. (2007).

From the mid-1970s onward, increasing numbers of novel extremophilic and extremotrophic organisms have been isolated as researchers have acknowledged that extreme environments are capable of sustaining life. Studies on extremophiles have progressed to the extent that there are now regular international extremophile symposia, as well as dedicated scientific societies and journals such as *Extremophiles – Microbial Life Under Extreme Conditions* and *Archaea*.

Categories of Extremophiles and Extremotrophs

The literature contains several terms that are used to describe extremophiles and extremotrophs and sub-definitions exist for organisms that present moderate, extreme, hyper-extreme, and/or obligate extremophily. Among the terms that are frequently used, and to be found in this book, are the following (further values for minimum, optimum, and maximum growth characteristics can be found in Bull, Chap. 12.1 Actinobacteria of the Extremobiosphere):

Acidophile: an organism with a pH optimum for growth at, or below 3–4
Alkaliphile: an organism with optimal growth at pH values above 10
Endolith: an organism that lives inside rocks
Halophile: an organism requiring at least 1 M salt for growth
Hyperthermophile: an organism having a growth temperature optimum of 80°C or higher
Hypolith: an organism that lives inside rocks in cold deserts
Metalotolerant: organisms capable of tolerating high levels of heavy metals, such as copper, cadmium, arsenic, and zinc
Oligotroph: an organism capable of growth in nutritionally deplete habitats
Piezophile: an organism that lives optimally at hydrostatic pressures of 40 MPa or higher
Psychrophile: an organism having a growth temperature optimum of 10°C or lower, and a maximum temperature of 20°C
Radioresistant: organisms resistant to high levels of ionizing radiation
Thermophile: an organism that can thrive at temperatures between 60°C and 85°C
Toxitolerant: organisms able to withstand high levels of damaging agents, such as organic solvents
Xerophile: an organism capable of growth at low water activity and resistant to high desiccation

These anthropocentric definitions that we make of extremophily and extremotrophy focus on a single environmental extreme but many extremophiles may fall into multiple categories, for example, organisms living inside hot rocks deep under the Earth's surface. The phenomena of polyextremophily and polyextremotrophy refer to organisms adapted to more than two extreme conditions but have received comparatively little detailed study. Examples of adaptation to multiple extremes can be found throughout this book and, again, while most attention is given to prokaryotic organisms, there are dramatic instances of polyextremophily among eukaryotes. A case in point is the unicellular eukaryotic red alga *Cyanidioschyzon* (order *Cyanidiales*), a strain of which is acidophilic (pH 0.2–3.5), moderately thermophilic (38–57°C), has a high tolerance of arsenic, and the capacity for its biotransformation

(Qin et al. 2009); these authors consider that algae play a significant role in arsenic cycling in marine and freshwater geothermal environments.

Distribution of Extremophiles and Extremotrophs

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, and volcanoes and other geothermal phenomena such as marine hydrothermal vent systems. The latter, for example, are distributed globally and consist of very contrasting black smokers and carbonate chimneys. Such tectonic manifestations variously produce extreme heat, pH, dissolved gasses, and metals.

A high proportion of the Earth's surface contains water in solid form (sea ice, ice caps and sheets, glaciers, snowfields, permafrost) forming the cryosphere, 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. In regions where precipitation is very low (or zero) and also unpredictable, deserts develop, the aridity of which is defined as hyper- (annual precipitation to annual evaporation <0.05) or extreme hyper-arid (<0.002). Highly saline lakes and pans often develop under these circumstances; they also arise more frequently in endorheic basins that are drainage basins with no outflow of water. Given that the average depth of the world's oceans is ca. 3,800 m, high pressure generates yet another extreme environment. Oligotrophic environments are defined as those presenting very low nutrient concentrations; they include oceans deplete 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).

The foregoing descriptions include most of the world's dominant ecosystems, all of which have evolved as the results of natural processes over geological time scales. In more recent times, similar or significantly different extreme conditions have been imposed as a consequence of human insult of the environment, for example, soil salinity as a result of deep well drilling for irrigation water, radioactivity contamination from power plant failure, persistently high xenobiotic chemical challenge as a result of industrial pollution, and agrochemical use. Thus the totality of these global and local, natural and anthropogenic "extreme" environments has provided a remarkable panoply of opportunities for the evolution of the organisms that are treated in this handbook.

A few additional points need to be made before we leave this topic. First, 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 (Kelley et al. 2001). Such is the combination of extreme conditions that characterize the marine deep biosphere that Sass and Parkes (see Chap. 9.1 Sub-seafloor Sediments - An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)) prompt the thought that only organisms found therein might be extremophiles sensu stricto! Second, as we have stated several times, extreme environments almost invariably are affected by two or more extreme conditions. Third, in describing extremophilic and extremotrophic organisms, care must be taken to discriminate between the mere presence of an organism (or its phylogenetic signature) and those that are growing or

metabolically active in an extreme environment. Fourth, certain extremophilic organisms can be recovered from "normal" environments. Fifth, is our current knowledge of extremophile diversity comprehensive? The results of recent analyses made by Pikuta et al. (2007) point to a lack of evidence for the existence of acidopsychrophiles, acidohalophiles, and thermohalophiles – a challenge that should not be neglected by microbiologists! And finally, the question of conservation of what might be termed extremophile "hot spots." Priority-setting systems were established originally for defining and conserving regions or localities that possessed unusually high levels of animal and plant diversity. A similar strategy seems desirable for conserving representative habitats that are dominated by extremophilic and extremotrophic organisms, and paradoxically, would include heavily degraded environments that are providing conditions for ongoing extremophile evolution.

Concerning Origins and Evolution of Extremophily

It may appear overambitious to introduce the evolution of extremophiles into this chapter given the uncertainty and disparate hypotheses and opinions attending discussions of the origin of life, the last common ancestor, and the origin of eukaryotes. It is not our intention to examine these issues in detail but rather to attempt a realistic framework – incomplete and speculative as parts of it may be – that might encourage discussion and further researches.

A pertinent starting point for this topic is the stimulating article published recently by Martin and his colleagues (Martin et al. 2008). These authors consider the case for hydrothermal vents as systems where life might have originated and, ipso facto, where extremophiles could be expected to have had their genesis. Such vent systems are found in abundance worldwide and are presumed to have existed as soon as liquid water accumulated on Earth (ca. 4.2 Ga). Martin et al. are at pains to differentiate black smoker and carbonate chimney vents: black smokers arise at diverging plate boundaries above magma chambers, they are highly acidic (pH 2–3), very hot (up to 405°C), with vent fluids rich in Fe and Mn, and CO_2, H_2S, H_2, and CH_4; carbonate chimneys in contrast are found off-axis (away from diverging boundaries), are highly alkaline (pH 9–11), moderately hot (up to 90°C) and rich in H_2, CH_4, and low-molecular-weight hydrocarbons. The process of serpentinization (see Takai, Chap. 9.2 Physiology) results in the geochemical production of hydrogen at both types of vent systems; at the carbonate chimneys hydrogen can reduce CO_2 to methane, also geochemically. The conditions that arose in the Hadean oceans could have been conducive for supporting chemolithotrophic life prior to the much later generation of photosynthetic carbon. The microbiota of present-day, actively venting carbonate chimneys is dominated by anaerobic methanogens that are replaced by anaerobic methanotrophs in cooler, less active vents. Present-day black smoker communities contain a variety of hyperthermophilic archaea and bacteria, some of which also grow chemoautotrophically by gaining energy from the reduction of sulfate or CO_2 by H_2. Thus, the intriguing possibility posed by Martin et al. is that the contemporary microbiota of marine thermal vents "harbour relict physiological characteristics that resemble the earliest microbial ecosystem on Earth." Several authors have cautioned that present-day hyperthermophiles may reflect later evolutionary adaptations to altered conditions on Earth and that inferences made about distant ancestral life from contemporary hyperthermophiles may be inappropriate (see Glansdorff et al. 2008, for example).

Another conceptual step relevant to ideas about evolution and the physiological divergence of extremophilic and extremotrophic life has been made by Battistuzzi and Hedges (2009).

By combining data from phylogenetic (core protein, and small- and large-subunit rRNA genes) surveys, cytology, and environmental surveys, they have proposed that a large clade of prokaryotic organisms evolved on land during the mid-Arcaean era (ca. 3.18 Ga). This clade, termed the Terrabacteria, comprises two-thirds of all recognized species of prokaryotes and includes the *Actinobacteria, Chloroflexi, Cyanobacteria*, and *Firmicutes*. This evolutionary hypothesis claims to be consistent with a number of geological and biomarker calibration points and posits that members of the Terrabacteria include those showing salient adaptations to a wide spectrum of extreme environments shaped by factors such as desiccation, high salinity, and radiation exposure. Some lineages of the Terrabacteria are proposed to have subsequently re-invaded marine environments. A further informative approach to extremophile biology has been to plot the occurrence of known species in 2-dimensional matrices, pH versus temperature, pH versus salinity, etc. (Pikuta et al. 2007). While we have referred above to some of the results of these analyses, Pikuta and her colleagues, in scrutinizing their matrices, pose the interesting question as to the direction in which biological (and particularly extreme biological) changes to organisms may have occurred during the geochemical-geophysical evolution of Earth. Assuming a thermophilic beginning, acidophily probably arose at an early stage, while alkaliphily evolved only after certain mineral precipitation and sufficient buffer concentration of CO_2 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. From the analysis of a large and environmentally diverse collection of 16S rRNA gene sequences, Lozupone and Knight (2007) concluded that salinity was the major determinant of microbial community composition rather than extreme temperature, pH, or other environmental factor(s). It might be the case, therefore, that despite the powerful selective pressure of extreme temperatures and pH, the more general properties of such environments exemplified by salinity are the primary determinants of which lineages adapt and evolve.

It is clear that a cautious approach needs to be taken to the topic raised in this section and excellent research and scholarship notwithstanding, conjecture inevitably is a significant element in attempts to unravel questions of extremophile evolution. What is apparent is the stimulus that work and discussions of this kind will provide for further investigations. In addition to the work referred to above, the interested reader will discover a large relevant literature among which the papers of Sheridan et al. (2003), Cox et al. (2008), Glansdorff et al. (2008), and Cavalier-Smith (2010) are thought provoking.

Pioneering Studies

Breakthroughs in the discovery and physiological characterization of extremophilic organisms occurred in a number of laboratories throughout the world during the early and middle decades of the twentieth century. Here we consider a selection of major achievements that changed the course of much subsequent research with microorganisms.

Thermophiles

In June 1965, Thomas Brock, a microbiologist at Indiana University (he moved later to the University of Wisconsin), discovered a bacterium, *Thermus aquaticus*, in the thermal vents of

Yellowstone National Park that could survive at near-boiling temperatures (Brock and Freeze 1969). At that time, the upper temperature for life was thought to be 73°C. At one particular site, Octopus Spring, Brock had discovered large amounts of pink filamentous bacteria at temperatures of 82–88°C; here were organisms living at temperatures above the then "upper temperature for life." Attempts to cultivate these pink bacteria were initially unsuccessful, but during the next several years, as he continued broader work on the ecology of the Yellowstone springs, Brock isolated and collected many microbes from geothermal areas. Strain YT-1 of *T. aquaticus* collected from Mushroom Spring on September 5, 1966 was the first to be developed as the source of Taq polymerase that would become universally and routinely used in molecular biology. His group showed that *T. aquaticus* was widespread in hot-water environments and that enzymes from *T. aquaticus* were temperature tolerant in boiling water. During various travels to study thermal areas in other parts of the world, Brock isolated a number of other cultures of *T. aquaticus*, one interesting strain being recovered from the hot-water system on the Indiana University campus. Subsequently, he could show that *T. aquaticus* was widespread in artificial hot-water environments, and other workers have isolated it from hot tap water in other parts of the world.

Brock commented that for many years his Yellowstone work had seemed somewhat "exotic" to many microbiologists, perhaps because of the presumed restricted distribution of hot springs on Earth. This attitude changed after the discovery of the deep-sea vents, with their very high temperatures and their associated diverse and flourishing life forms. Now deep-sea thermal vents are known to be widespread in the oceans, just as the overall range of geothermal environments supporting life has been revealed. Seventeen years after Brock's discovery, Karl Stetter isolated, from a shallow marine vent, the first organisms that could grow optimally at temperatures greater than 100°C and so began the era of hyperthermophilic microbiology. Although the upper temperature for life remains an open question, there is now a great opportunity to pursue it by studying microorganisms living in the deep seas, the deep marine and terrestrial biosphere, and the myriad of other extremely hot locations.

Alkaliphiles

In 1956, Koki Horikoshi first encountered moderate alkaliphilic bacteria when working as a graduate student in the Department of Agricultural Chemistry, University of Tokyo on the lysis of *Aspergillus oryzae*. One day in November he found one cultivation flask in which the mycelia of *A. oryzae* had completely disappeared; the flask smelt of ammonia and the pH of its contents had increased to nine. The lytic microorganism isolated from the flask was *Bacillus circulans*, and strong endo-1,3-β-glucanase activity was detected in the culture fluid. However, this bacterium showed very poor growth in conventional media but on the addition of 0.5% sodium bicarbonate, good growth and enzyme production occurred.

Later, in 1968, during a visit to Florence and looking at Renaissance buildings so different from Japanese architecture, a voice whispered in his ear, "There might be a whole new world of microorganisms in different unexplored cultures" and memories of those experiments with *B. circulans* flashed into his mind. Could there be an entirely unknown world of microorganisms at alkaline pH? Hardly any microbiological work had been done in the alkaline region principally because alkaline foods are uncommon, except a few Chinese types. Upon his return to Japan, Horikoshi prepared an alkaline medium and inoculated it with small amounts of soil collected from various sites on campus of The Institute of Physical and Chemical Research

(RIKEN). To his surprise, after overnight incubation at 37°C, various microorganisms flourished in all test tubes. Horikoshi named these microorganisms that grow well in alkaline environments "alkaliphiles," and found subsequently that they were widely distributed throughout the earth (even at ca.10,900 m depth in the Mariana Trench in Pacific Ocean) and that they produced new products (Horikoshi 1971). Over the past four decades, studies of alkaliphiles have been comprehensive with one big question being "how do alkaliphiles tolerate extreme alkaline environments?" Alkaliphiles can keep the intracellular pH at about 7–8 in environments of pH 10–13. How pH homeostasis is maintained is one of the most fascinating aspects of alkaliphiles and is discussed in ❯ Chap. 2.2 Distribution and Diversity of Soda Lake Alkaliphiles of this handbook.

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit unique properties; about 35 new kinds of enzymes have been isolated and purified by Horikoshi's group and some produced on the industrial scale (see ❯ Chap. 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic Bacillus for the Production of Beta-Cyclodextrin and ❯ Chap. 2.11 Alkaline Enzymes in Current Detergency).

Deep-Sea Extremophiles

Claude ZoBell was instrumental in laying the foundations for modern marine microbiology, and during his long career at the Scripps Institution of Oceanography provided the first convincing evidence for the existence of indigenous marine bacteria, among them being ones that were growing in the deep seas. In a seminal paper of 1949, Zobell and Johnson reported bacteria in sediment sampled at 5,800 m off Bermuda that could grow under high hydrostatic pressures (500–600 atmospheres ≈50–60 MPa) that were equivalent to the in situ pressures of the deep-sea environment; they coined the term barophile to "characterize microbes which grow preferentially or exclusively at high hydrostatic pressures." Note that the term barophile has been replaced in more recent times by piezophile (Gk *piezein*, to press). Subsequently, ZoBell joined the famous Danish Galathea round the world deep-sea expedition, and for four months in 1951 he was on board during the Manila (Philippines) to Port Moresby (Papua New Guinea) leg of the cruise. Sediments taken from the Philippine Trench (10,120–10,190 m depth) were found to contain viable bacteria representing a variety of physiological types (ZoBell 1952) showing thereby that life existed in the deepest parts of the ocean. These pioneering studies, together with essential developments in marine engineering (notably submersibles, deep drilling, sampling equipment) have inspired successive generations of microbiologists and organizations to invest in deep-sea research, the results of much of which are described in this handbook.

Emerging Fields

Since the early pioneering days of extremophile discovery, a growing number of individuals and organizations have been drawn into this compelling field of research and new vistas continue to be opened up. In Japan, for example, a 15-year research program called DEEP STAR (Deep-sea Environment Exploration Program, Science and Technology for Advanced Research) was launched in October 1990 and directed by Horikoshi with a mission to expand the sources of microorganisms for study and application from the surface of the Earth to the deep sea. We illustrate these new developments by reference to just two examples but recognize

that several others deserve mention; in addition, opportunities for innovative biotechnological applications of extremophiles and extremotrophs are briefly introduced.

The Deep Biosphere

ZoBell also found microorganisms in sub-seafloor sediments, but culturable organisms were not recovered at depths greater than ca. 7 m. It was only after applying a portfolio of culture-dependent, culture-independent, and biogeochemcal techniques several decades later that the existence of deep submarine and subterranean microbial communities was established.

The modern era of deep biosphere studies began in the 1980s and was catalyzed particularly by concerns for the contamination of ground waters and by the launch of the Ocean Drilling Program. Early explorations of coastal sediments by the Savannah River Laboratory in South Carolina revealed a diversity and abundance of microorganisms at depths of 850 m. The first intensive study of deep submarine sediments, from the Peru Margin, at about the same time, confirmed the existence of prokaryotic communities several hundred meters below the sea floor. The extent of these deep sub-seafloor populations led Parkes and his colleagues to propose that approximately 10% of global biomass carbon might exist as prokaryotic organisms in these sediments down to a depth of 500 m (Parkes et al. 1994). The deepest submarine sediments proven to sustain prokaryotic organisms are greater than 1,600 m. The physiology and ecology of these organisms are discussed in Chap. 9.1 Sub-seafloor Sediments - An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology) of this handbook. Some of the more recent investigations of the deep subterranean biosphere are ongoing in Sweden and South Africa. Research at the Äspö Hard Rock Laboratory, for example, has led to the proposal that a H_2-driven deep biosphere has developed in crystalline bedrocks at depths greater than 1,200 m (Pedersen 1997). Subsequently, the study of a fracture zone 2,825 m below land surface (Mponeng gold mine, S. Africa) has produced clear evidence for a microbial community sustained by geochemically derived H_2 and sulfate (Lin et al. 2006) and dominated by one *Firmicutes*-related phylotype. Here the remarkable conclusion is that these organisms might have been sustained by lithotrophic metabolism for millions of years without inputs from photosynthesis.

Hyper-Arid Environments

Approximately 15% of the land surface of Earth is desert, a biome that is found in all of the geographic realms. Deserts are classified as subtropical (e.g., Sahara, the largest non-polar desert), cool coastal (e.g., Namibian), cold winter (e.g., Gobi), and polar. Hyper-aridity and extreme hyper-aridity were defined above (see "Distribution of Extremophiles and Extremotrophs") on the basis of precipitation: evaporation ratios and deserts of this type, exemplified by the world's driest (the cool coastal Atacama Desert of northern Chile), also may experience high salinity, intense atmospheric radiation, and very low nutrient availability. Such is the nature of the extreme arid region of the Atacama that its soils have been proposed as a model for those of Mars, and taken to be the dry limit for microbial survival in the extremobiosphere (Navarro-Gonzalez et al. 2003). However, subsequent investigations made in the same hyper-arid core of this Desert have shown that amplifiable DNA and a variety of culturable bacteria can be recovered from this harsh environment (see Chap. 12.1 Actinobacteria of the Extremobiosphere, this handbook for details).

The microbiology of high-elevation (>5,000 m) hyper-arid and periglacial landscapes in Peru and Chile/Argentina, some of which could be appropriate analogs of Martian polar regions, have been explored recently by Steve Schmidt's group (Schmidt et al. 2009; Costello et al. 2009). These multi-extreme environments harbor diverse populations of bacteria, fungi, and micro-metazoans, many members of which are novel taxa; in addition, metabolic activities are sustained even under conditions of extreme diurnal fluctuations of soil temperature (−12°C to 27°C).

Biotechnology

The exploration of the extremobiosphere and the surge of interest in biotechnology occurred during roughly the same period; so it is not surprising that extremophiles and extremotrophs became prime targets in search and discovery programs aimed at new natural products, new biocatalysts, and other goods and services. The application of these organisms opened up an exciting phase of biotechnology innovation.

The information summarized in *Table 1.1.1* provides a snapshot of biotechnologically interesting compounds and activities produced by extremophilic and extremotrophic microorganisms. This is not an exhaustive compilation and it is also important to view these products as a potential resource since only relatively few of them have been brought to market at this stage. From a commercial perspective, enzymes (extremozymes) from extremophiles have made the most impact so far. As an example, alkaline proteases, derived from alkaliphilic species, constitute an important group of enzymes that find applications primarily as protein-degrading additives in detergents. Given the robust nature of alkaliphiles, these enzymes can be subjected to harsh operational environments, including elevated temperature, high pH, surfactants, bleach chemicals, and chelating agents, where applications of many other enzymes are limited because of their low activity or stability. Enzyme production for detergents is a huge market constituting approximately 40% of the total enzymes produced worldwide. In addition to detergent enzymes, the application of extremophile products has been wide, ranging from large-scale processing (e.g., metal recovery, coal desulphurization, waste treatment, and paper bleaching) to smaller-scale, high-value-added products (e.g., food additives, optical switches, and photocurrent generators). Research on anti-infective agents from extremophiles/extremotrophs has been slower to develop but the potential in this area is now being appreciated with the discovery of new chemical entities and first-in-a-class modes of action as illustrated by the abyssomicins. A recent review by Wilson and Brimble (2009) describes the large chemical diversity found in extremophiles that probably have evolved in part "as unique defences against their environment, leading to the biosynthesis of novel molecules ranging from simple osmolytes and lipids to complex secondary metabolites."

The cost and effort involved in collecting samples from extreme environments both for fundamental research and for screening campaigns is far from being trivial. The search for extremophiles is an expedition in itself; researchers are found deep-sea diving in Hawaii and Japan, sending submersibles to the ocean depths, foraging in Yellowstone National Park and other regions of geothermal activity, ascending high mountains and volcanoes, and collecting ice core samples in polar and similar cryoenvironments. Although this matter is rarely commented upon by contributors to the *Extremophiles Handbook*, it is one that the reader might usefully ponder as she or he is brought close to the extremobiosphere.

Table 1.1.1

Products of extremophilic microorganisms

Environmental extreme	Therapeutics				Miscellaneous[a]	Biocatalysts			
	Antibacterial	Antifungal	Antiparasitic	Antitumor		Carbohydrases	Proteases	Lipases	Other
High temperature	✓	✓		✓	✓	✓	✓		
Low temperature	✓			✓		✓	✓	✓	✓
High pressure	✓	✓	✓	✓	✓				
High salinity	✓				✓	✓	✓	✓	
High alkalinity	✓		✓	✓	✓	✓	✓	✓	
High acidity	✓			✓	✓	✓		✓	✓

[a]Antifoulant, antifreeze, anti-inflammatory, antioxidant, bacteriorhodopsin, biopolymer, cyclodextrin, osmolyte, pigment, polyunsaturated fatty acid, receptor agonist, surfactant.

Purpose and Organization of the Handbook

The environmental limits to life on Earth are defined by the distribution of microorganisms as primary colonizers, and it is this marvel of evolutionary history that is the principal theme of this book. Environmental limits in this context describe the outermost boundaries of the physicochemical world as we know it, and, by extension, those organisms that grow under such extreme conditions are known as *extremophiles*.

Until quite recently, temperature, pH, and pressure extremes of the order of about −18°C to 121°C, 0–13, and up to 100 MPa respectively would have been thought as inimical to life. However, the progressive discoveries of recent years, of life in environments controlled by these and other extreme conditions such as radiation, hyper acidity and salinity, intense pollution, or very low nutrient availability, has revealed that extremophilic organisms are both abundant and diverse. It is important to distinguish between true extremophiles that flourish only in extreme environments and those organisms that, for various reasons, can merely tolerate or survive at environmental extremes. This distinction is not simply a matter of semantics but has major implications, for example, in understanding the behavior of extreme ecosystems and for the biotechnological exploitation of these organisms. The relevance of molecular biological approaches in revealing hidden organisms and metabolic potential within the communities of extreme ecosystems is undisputable, but in this post-genomics era, the importance of developing innovative isolation and cultivation methods for extremophiles remains an important objective in gaining a comprehensive picture of their physiology and ecology.

The purpose of the *Extremophiles Handbook* is to bring together the rapidly growing and often scattered information on microbial life in the whole range of extreme environments and to evaluate it in relation not only to the biodiversity, biochemistry, physiology, and ecology that it comprises, but also to assess how we can gain clues to the origin of life and the search for astrobiology, and finally to explore the biotechnological potential of these fascinating organisms. In some cases, the reader will find little or no information on certain aspects of extremophile biology; ecology, for example, often stands out as a void in our understanding. We have asked contributors to mention such information gaps in their accounts of particular extremophile groups as a means of directing further research into extremophily.

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Part 2

Extremophiles: Alkaliphiles

2.1 Introduction and History of Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.1,

Introduction and History of Alkaliphiles

Why Did I Study Alkaliphiles?

In 1956, I encountered an alkaliphilic bacterium, although not alkaliphilic in the true sense of the word. I was a graduate student in the Department of Agricultural Chemistry, University of Tokyo, working on autolysis of *Aspergillus oryzae*, which was the research theme for my doctoral thesis.

One day in November, I found one cultivation flask in which mycelia of *Asp. oryzae* was completely disappeared. Last night when I saw flasks, the mold flourished in all culture flasks. I still remembered spectacular pictures of how bacteria thrived and vividly moved. No mycelium could be seen under microscope.

The microorganism isolated from the flask was *Bacillus circulans* and strong endo-1,3-β-glucanase activity was detected in the culture fluid. This enzyme lyzed *Asp. oryzae*. It was the first time that mold cells had been found to be lyzed by bacteria and these results were published in Nature (Horikoshi and Iida 1958). However, this bacterium showed very poor growth in the absence of mycelia of *Asp. oryzae* and production of endo-1,3-β-glucanse was very low. Therefore, purification of endo-1,3-β-glucanase could be done only in culture fluid in the presence of mycelia of *Asp. oryzae*. I did not realize at the time that the culture fluid had alkaline pH value. A few years later, I attempted production of endo-1,3-β-glucanase in conventional media. I tested many cultivation media containing various nutrients. An addition of 0.5% sodium bicarbonate to conventional nutrient culture broth gave good growth and production of the enzyme. Autolysis of *Asp. oryzae* changed the culture medium from weakly acidic to alkaline pH. In this way, I discovered that such a change in pH value accelerated bacterial growth and enzyme production.

In 1968, I visited Florence, Italy, and saw the Renaissance buildings, which are so very different from Japanese architecture. No Japanese could have imagined this Renaissance culture, although both cultures date back almost to the same time: between fourteenth century and fifteenth century (*Fig. 2.1.1*). Then suddenly I heard a voice whispering in my ear,

> There could be a whole new world of microorganisms in different unexplored cultures.

Memories of experiments on *B. circulans* done almost 10 years ago flashed back into my mind. Could there be an entirely unknown domain of microorganisms existing at alkaline pH? The acidic environment was being studied, probably because most food is acidic. Very little work had been done in the alkaline region. Almost all biologists believed that life could survive only within a very narrow range of temperature, pressure, acidity, alkalinity, salinity, and so on, in so-called moderate environments. Therefore, when microbiologists looked around for interesting bacteria and other life-forms, they attempted to isolate microorganisms only from moderate environments.

Science, just as much as the arts, relies upon a sense of romance and intuition. Upon my return to Japan, I prepared two alkaline media containing 1% sodium carbonate "Horikoshi-I and Horikoshi-II" (as shown in *Table 2.1.1*) put small amounts of soil collected from various area of the Institute of Physical and Chemical Research (RIKEN), Wako, Japan, into 30 test tubes and incubated them overnight at 37°C.

To my surprise, various microorganisms flourished in all 30 test tubes. I isolated a great number of alkaliphilic microorganisms and purified many alkaline enzymes. Here was a new

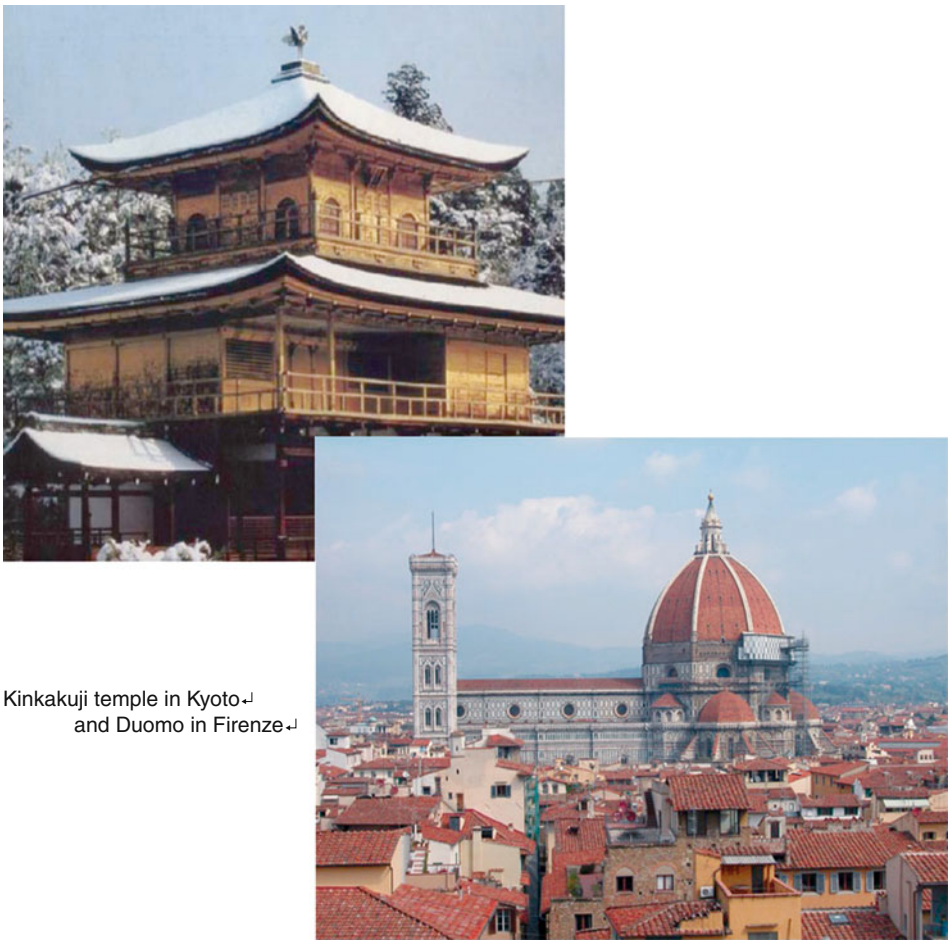

Fig. 2.1.1

Two photos of Kinkauji (Golden Temple) in Kyoto, Japan and Duomo in Firenze, Italy. The Golden Temple was built at the end of the fourteenth century and The Duomo was built at the middle of the fifteenth century

alkaline world that was utterly different from the neutral world discovered by Pasteur. This was my first encounter with alkaliphiles. The first paper concerning an alkaline protease was published in 1971(Horikoshi 1971).

Then in 1972, I was talking with my father-in-law, Shigeo Hamada about alkaliphilic microorganisms. He had been in London after World War 1 as a businessman and quidnunc about everything. He showed interest in alkaliphiles. These microorganisms were unique, required high alkalinity, and they could produce alkaline enzymes such as alkaline proteases and alkaline amylases. As I was speaking, he said

- Koki wait a minute, I have an interesting present for you.

Table 2.1.1
Basal media for alkaliphilic microorganisms

	Horikoshi-I (g/l)	Horikoshi-II (g/l)
Glucose	10	–
Soluble starch	–	10
Polypeptone	5	5
Yeast extract	5	5
KH_2PO_4	1	1
Mg_2SO_4 $7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar for plats	20	20

He brought out a sheet of old newspaper, *Nikkei Shimbun*, dated June 11, 1958. A short column with one electron micrograph was like a punch to my head. As I had been in Purdue University, Indiana, USA as a graduate student, I could not see this newspaper at all!

The article stated:

> In Japan, since ancient times, indigo has been naturally reduced in the presence of sodium carbonate. Indigo from indigo leaves can be reduced by bacteria that grow under high alkaline conditions. Indigo reduction was controlled only by the skill of the craftsman. Takahara and his colleagues isolated the indigo-reducing bacterium from a indigo vat.

I carefully checked scientific papers from Chemical Abstracts in the library of RIKEN. Only 16 scientific papers were discovered (Johnson 1923; Downie and Cruickshank 1928; Vedder 1934; Jenkin 1936; Bornside and Kallio 1956; Chesbro and Evans 1959; Kushner and Lisson 1959; Takahara and Tanabe 1960; Chislett and Kushner 1961a,b; Takahara et al. 1961; Takahara and Tanabe 1962; Wiley and Stokes 1962; Wiely and Stokes 1963; Barghoorn and Tyler 1965; Siegel and Giumarro 1966).

I felt a sense relief glancing at these scientific papers, because alkaline-loving microorganisms remained little more than interesting biological curiosities. No industrial application of these microorganisms was attempted, until my papers were published in 1988. I named these microorganisms that grow well in alkaline environments "alkaliphiles" and conducted systematic microbial physiological studies on them. It was very surprising that these microorganisms, which are completely different from any previously reported, were widely distributed throughout the globe even in the deepest point of the Mariana Trench in the Pacific Ocean as shown in *Fig. 2.1.2*. Here was a new alkaline world that was utterly different from the neutral world. Using such simple media, I found thousands of new microorganisms (alkaliphiles) that grow optimally well at pH values of 10, but cannot grow at neutral pH value of 6.5 (*Fig. 2.1.3*). Many different kinds of alkaliphilic microorganisms have been isolated including bacteria belonging to the genera *Bacillus*, *Micrococcus*, *Pseudomonas*, *Actinobacteria*, and eukaryotes, such as yeasts and filamentous fungi.

Then over the past 4 decades, my coworkers and I have focused on the enzymology, physiology, ecology, taxonomy, molecular biology, and genetics of alkaliphilic microorganisms

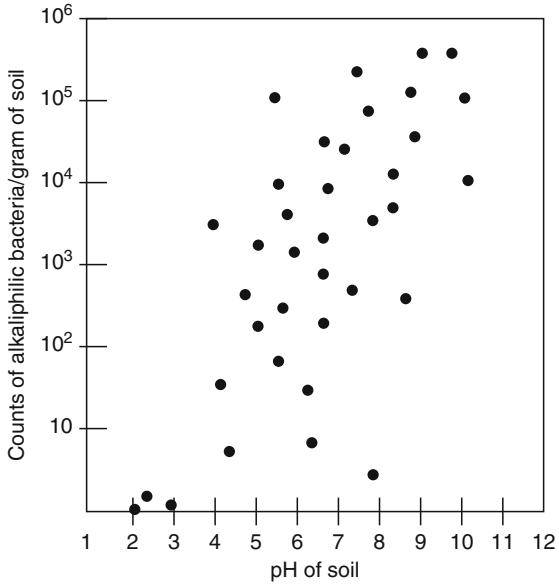

Fig. 2.1.2
Distribution of alkaliphilic bacteria in soil

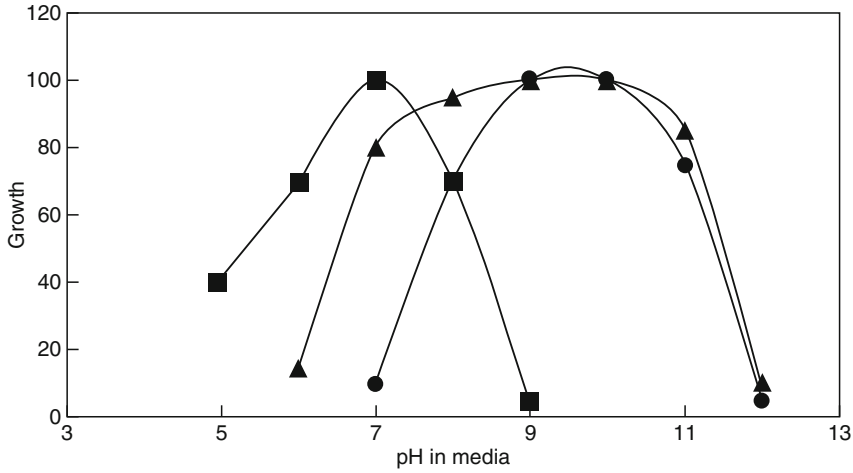

Fig. 2.1.3
pH dependence of microorganisms. The typical dependence of the growth of neutrophilic bacteria (*Bacillus subtilis*), obligate alkaliphilic bacteria (*Bacillus pseudofirmus* 2b-2), and facultative alkaliphilic bacteria (*Bacillus halodurans* C-125) are shown by *solid squares*, *solid circles*, and *solid triangles*, respectively

to establish a new microbiology of alkaliphilic microorganisms. Another big question arises: "Why do alkaliphiles require alkaline environments?" The cell surface of alkaliphiles can keep the neutral intracellular pH values in alkaline environments of pH 10–13. How the pH homeostasis is maintained is one of the most fascinating aspects of alkaliphiles. In order to

understand this simple but difficult question, we carried out several basic experiments to establish gene recombination systems. The whole genome sequence of alkaliphilic *Bacillus halodurans* C-125 was completed in 2000 by my coworkers (Takami et al. 2000). This was the second whole genome sequence of spore-forming bacteria thus far reported. This sequence work revealed interesting results. Many genes were horizontally transferred from different genera, and different species as well. Small fragments of enzyme genes were inserted in opposite directions, or separated by insertion fragments. Therefore, these bacteria could not produce some enzymes. However, we still have not found crucial gene/s responsible for alkaliphily in the true sense. Recent results indicate that many gene products synergistically cooperate and exhibit alkaliphily or adaptation to alkaline environments.

It noteworthy to write that alkaliphiles gave a great impact on industrial applications. Biological laundry detergents contain alkaline enzymes, such as alkaline cellulases and/or alkaline proteases from alkaliphilic Bacillus strains. Another important application is the industrial production of cyclodextrin with alkaline cyclomaltodextrin glucanotransferase. This enzyme reduced the production cost and opened new markets for cyclodextrin use in large quantities in foodstuffs, chemicals, and pharmaceuticals. Industrial applications of these microorganisms have also been investigated extensively, and some enzymes, such as alkaline proteases, alkaline amylases, alkaline cellulases, and alkaline xylanases have been put to use on an industrial scale. Subsequently, many microbiologists have published numerous papers on alkaliphilic microorganisms in various fields. At the beginning of our studies, very few papers were presented, but now a plethora of scientific papers and patents have been published. It is not clear which field our studies of alkaliphiles will focus on next, but the author is convinced that alkaliphiles will provide much important information. A series of our work established a new microbiology of alkaliphilic microorganisms, and the studies performed spread out all over the world. Although there are many things we have to solve, it is clear that alkaliphilic microorganisms are one of the best DNA resources, especially in the field of physiology and biotechnology. Further information is mentioned in the following sections: Distribution, diversity, taxonomy, ecology, adaptation mechanism, bioenergetics, genetics, evolution, and industrial applications.

If the readers would like to know details on alkaliphiles by 2005, they can see the following four monographs: Horikoshi and Akiba (1982) and Horikoshi (1991, 1999, 2006).

History of Alkaliphiles Before 1968

Before publication of our reports, very few microorganisms growing at high pH values had been discovered. The earliest papers on microorganisms in alkaline environments dealt with *Nitrosomonas* and *Nitrobacter* species, and with *Streptococcusfaecalis* (Meek and Lipman 1922; Downie and Cruickshank 1928). Later, Gibson (1934) found a strain of *Bacillus pasteurii* that grew well at about pH 11. In 1934, Vedder reported that he had isolated *Bacillus akalophilus* from feces of normal human on a cholera culture medium (highly alkaline hemoglobin medium, containing sodium carbonate, pH value about 10). The isolated strain grew well at pH 8.6–11, but did not grow at "ordinary" pH. So they proposed a new name "*Bacillus alcalophilus n.* sp." This strain is motile and hydrolyze albumin (gelatin, hemoglobin) in this alkaline medium. Twenty-five years later, Kushner and Lisson (1959) and Chislett and Kushner (1961a, b) reported the discovery of alkalitolerant strains. The strain *Bacillus circulans* Jordan capable of growing at pH values up to 11.0 was isolated, presumably as a contamination, in the

course of training experiments. They reported that the alkali-resistant *B. circulans* showed little loss of resistance after many transfers on neutral medium.

In Japan, since ancient times, indigo has been naturally reduced in the presence of sodium carbonate. Indigo from indigo leaves can be reduced by bacteria that grow under alkaline conditions. We call this process "indigo fermentation." The most important element in this fermentation process is control of the pH value at the range of 10–11, although no one realized this until the pioneering work of Takahara and coworkers (1960, 1961, 1962). Indigo reduction was controlled only by the skill of the craftsmen. They isolated the indigo-reducing bacterium, *Bacillus sp.* No. S-8, from an indigo ball, fermented indigo plant *Polygonmu tinctorium Ait.* They then improved the indigo fermentation process by adding alkaliphilic *Bacillus sp.* No. S-8 during fermentation under alkaline conditions. Thus, indigo dyeing is probably the first industrial application of alkaliphilic bacteria in the world.

Further microbiological studies, however, were not conducted until the author's systematic investigations. No industrial application was attempted at all before 1969 (Horikoshi 1971).

Cross-References

- 9.2 Physiology
- 9.3 Biochemistry

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2.2 Distribution and Diversity of Soda Lake Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.2,

Genesis of Soda Lakes

Soda lakes and soda deserts are the most stable naturally occurring alkaline environments on earth, where pH values of 10 and above are common. As the name implies, these environments are characterized by large amounts of soda (sodium carbonate, or complexes of this salt), formed by evaporative concentration (Grant 1992; Jones and Grant 2000). Other salts, especially sodium chloride may also concentrate leading to the formation of alkaline saline lakes. Although soda lakes have a worldwide distribution (*Table 2.2.1*), they are mainly confined to subtropical latitudes in continental interiors or rain-shadow zones.

Following a very early study by of the Wadi Natrun in Egypt (1898), the likely explanations for a general mechanism promoting alkalinity have been reviewed by a number of authors, notably the work by Eugster and colleagues (Eugster and Hardie 1978; Hardie and Eugster 1970) reviewed by Jones et al. (1994). The simplest theory would suppose the contribution of Na_2CO_3 via vulcanism – at least one active volcano generates a soda-rich lava flow in the southern path of the Rift Valley (Eugster 1970). However, this seems to be an exceptional case and there seems to be universal agreement that the most important feature of such areas is that the surrounding geology is dominated by high Na^+, low Mg^{2+}/Ca^{2+} silicates. The amount of Ca^{2+} in the surrounding geology (and to a lesser extent Mg^{2+}) is critical in determining the final pH of brine. Waters high in Na^+, Cl^-, and HCO_3^-/CO_3^{2-} evaporate down and if the concentration of HCO_3^-/CO_3^{2-} greatly exceeds that of any Ca^{2+} and Mg^{2+}, these cations precipitate as insoluble carbonates leaving behind an alkaline brine that develops as a consequence of a shift in the $CO_2/HCO_3^-/CO_3^{2-}$ equilibrium toward CO_3^{2-}, causing the development of a soda (Na_2CO_3) lake with pH values usually between 10 and 11, occasionally >pH12 (Jones et al. 1994). In the presence of significant amounts of Ca^{2+} (and Mg^{2+}), buffering occurs by the constant removal of alkaline carbonates (one of the main buffering systems in the marine environment), leading to a neutral hypersaline lake like the Great Salt Lake, or in the case of very high Mg^{2+} levels a slightly acid, hypersaline lake like the Dead Sea where acidity is generated by the chemistry of precipitation of Mg minerals like sepiolite. *Figure 2.2.1* depicts alternative pathways of brine formation dependant on original ion composition. The precise ion composition depends on the local geology.

The best-studied area where soda lakes have formed is the Kenyan–Tanzanian Rift Valley. The Great Rift Valley running through East Africa is an arid tropical zone where tectonic activity has created a series of shallow depressions. These shallow depressions are often closed basins with no obvious outflow where ground water and streams flowing from the surrounding highlands on the margins of the Rift Valley collect to form semipermanent standing bodies of water. In these zones with high rates of evaporation exceeding inflow, salts accumulate by evaporative concentration (Jones et al. 1994). Here, surrounding high Na^+ trachyte lavas are deficient in both Ca^{2+} and Mg^{2+}. As a result of evaporation in this arid tropical zone, saturation of the alkaline earth cations is rapidly achieved. Concentration of ions leads to Na^+, $C1^-$, and HCO_3/CO_3^{2-} as the major ions in solution. Total salinities vary with season and range from around 5%(w/v) total salts in the more dilute lakes such as Lake Elmenteita made up mainly from roughly equal amounts of NaCl and Na_2CO_3 at a pH of about 11, to saturated lakes like Lake Magadi at >30% (w/v) total salts, again dominated by NaCl and Na_2CO_3 at pH values approaching 12.

About one third of all salt and soda lakes in the world are to be found in an area that stretches from Tibet, throughout Qinghai province to the Inner Mongolia Autonomous Region, although many are unnamed. These lakes are situated in cryoarid zones that experience

Table 2.2.1

Worldwide distribution of soda lakes and soda deserts

North America	
Canada	Lake Manito
United States	Alkali Valley, Albert Lake, Lake Lenore, Soap Lake, Big Soda Lake, Owens Lake, Mono Lake, Searles Lake, Deep Springs, Rhodes, Marsh, Harney Lake, Summer Lake, Surprise Valley, Pyramid Lake, Walker Lake
Central America	
Mexico	Lake Texcoco
South America	
Venezuela	Langunilla Valley
Chile	Antofagasta
Europe	
Hungary	Lake Feher
Yugoslavia	Pecena Slatina
Russia	Kulunda Steppe, Tanatar Lakes, Karakul, Araxes plain, Chita, Barnaul, Slavgerod
Asia	
Turkey	Lake Van
India	Lake Loner, Lake Sambhar
China	Qinhgai Hu, Sui-Yuan, Heilungkiang, Kirin, Jehol, Chahar, Shansi, Shensi, Kansu
	Lake Chahannor; Lake Zabuye; Bange Lake; Lake Baer; Lake Wudunao; Lake Hamatai
Africa	
Libya	Lake Fezzan
Egypt	Wadi Natrun
Ethiopia	Lake Aranguadi, Lake Kilotes, Lake Abiata, Lake Shala, Lake Chilu, Lake Hertale, Lake Metahara
Sudan	Dariba lakes
Kenya	Lake Bogoria, Lake Nakuru, Lake Elmentieta, Lake Magadi, Lake Simbi, Lake Sonachi
Tanzania	Lake Natron, Lake Embagi, Lake Magad, Lake Manyara, Lake Balangida, Bosotu Crater, Lakes, Lake Kusare, Lake Tulusia, El Kekhooito, Momela Lakes, Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Ndutu, Lake Rukwa North
Uganda	Lake Katwe, Lake Mahega, Lake Kikorongo, Lake Nyamunuka, Lake Munyanyange, Lake Murumuli, Lake Nunyampaka
Chad	Lake Bodu, Lake Rombou, Lake Dijikare, Lake Momboio, Lake Yoan
Australia	
Australia	Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup

little rainfall and these largely ephemeral sites are charged by occasional rainfall leaching through the surface topography additionally influenced by the evaporative effect of constant wind (Mianping et al. 1993).

There is no doubt that in common with the majority of saline sites, soda lakes harbor significant populations of microorganisms. Some of the first studies in modern times were those of (Isachenko 1951) on the Kulunda Steppe Region. Microorganisms that inhabit soda

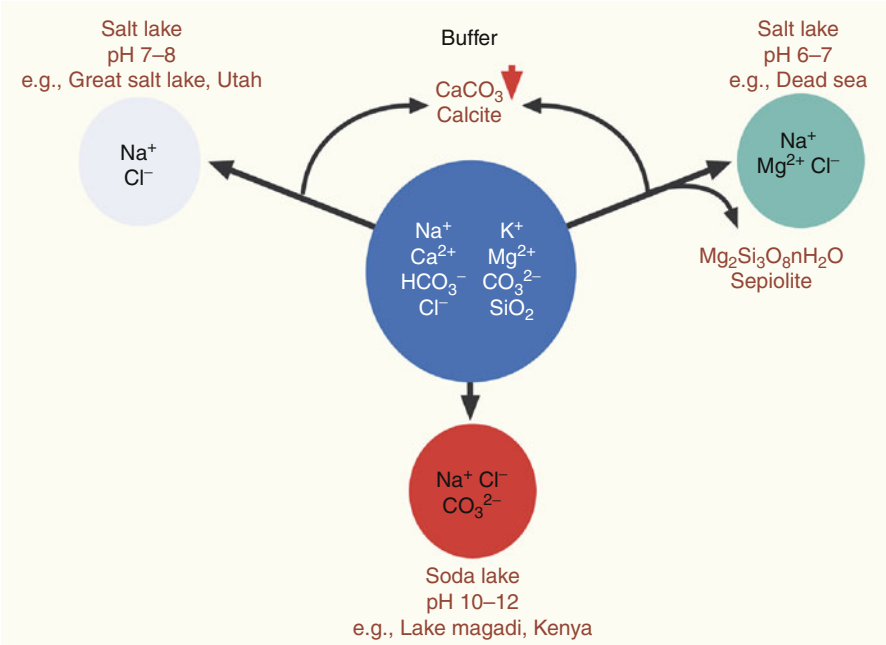

Fig. 2.2.1

Schematic representation of the genesis of hypersaline brines. The *center box* indicates the leaching of minerals by CO_2-charged waters. Alkaline lake development is dependent on low levels of Ca^{2+} and Mg^{2+}. Neutral lakes develop where Ca^{2+} and Mg^{2+} levels are high. High Mg^{2+} lakes are more acidic due to reactions involving sepiolite precipitation

lakes have to be of necessity alkali-loving or at least alkali-tolerant. The term "alkaliphile" is usually reserved for microorganisms growing optimally or very well at pH values above 9, often with pH optima for growth around 10, showing little, or no growth, at near neutral pH values. Microorganisms found in hypersaline soda lakes generally have an additional requirement for high levels of NaCl – as such, they are known as "haloalkaliphiles."

Soda Lake Chemoorganotrophs

Soda lakes have high levels of organic matter that supports dense populations of organotrophic prokaryotes. The brines may be various shades of green or red because of the massive blooms of microorganisms. This coloring is a reflection of the very high primary productivities associated with these lakes. The almost unlimited supply of CO_2 combined with high ambient temperatures and high daily light intensities in the tropics contribute to making the East African soda lakes among the most productive of the naturally occurring aquatic environments in the world (Melack and Kilham 1974).

The photosynthetic primary productivity, mainly the result of the dense populations of cyanobacteria, (up to 13,000 cyanobacterial filaments ml^{-1}) (Grant 1992) supports the rest of the microbial community. These blooms of cyanobacteria are usually dominated by *Spirulina*

(Arthrospira) spp., but in different lakes and also depending on seasonal factors, *Cyanospira* spp. and unicellular forms, which might be *Synechococcus* or *Chroococcus*, may also be common (Jones and Grant 2000). Unicellular *Eurhalothece* types may also be present in hypersaline sites (Mikhodiuk et al. 2008) as well as *Microcoleus chthonoplastis* (Kompantseva et al. 2005) Cyanobacteria are the principal food of the immense flocks of flamingos that inhabit the Rift Valley, one of the most studied soda lake areas.

Not only are the cyanobacteria critical for the fixation of CO_2 and thus generation of carbon for secondary heterotrophs, they also contribute toward fixation of nitrogen in these environments and are also producers of O_2. Daytime rates of oxygen production >2 g O_2 m^{-2} h^{-1} have been recorded from *Spirulina* spp. (Melack and Kilham 1974). However, during a 12-month survey of aerobic heterotrophic bacterial numbers in relation to cyanobacterial numbers no correlation was found between secondary productivity and the timing of a cyanobacterial bloom (Grant et al. 1990; Jones et al. 1994). In fact, bacterial numbers were remarkably constant, although the dominant types varied. These bacteria may be readily isolated on appropriate media (Grant and Tindall 1980). Viable counts of aerobic organotrophs from a range of dilute lakes indicate 10^5–10^6 cfu ml^{-1} (Grant et al. 1990).

The soda lake microbial community contains alkaliphilic representatives of all the major trophic groups of bacteria and archaea. Between these groups, there is cycling of carbon, sulfur, and nitrogen under aerobic and anaerobic conditions present in the lakes. There seems little doubt that in East Africa at least, *Arthrospira platensis*, and *Cyanospira rippkae* are responsible for photosynthetic primary production in dilute lakes. There is also an unquantified contribution to primary productivity made by anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* (Grant 1992) also seen in other geographical areas (Imhoff et al. 1979; Kompantseva et al. 2005), such as the Transbaikal region and the Wadi al Natrun lakes in Egypt. *Rhodobaca* spp. (Boldareva et al. 2008) and *Rubribacterium* sp. (Boldareva et al. 2009) have also been recorded. It is probable that hypersaline lakes during periods of dilution in any rainy season contain related cyanobacteria and similar anoxygenic phototrophs that may be responsible for primary productivity (Grant 2004).

A survey of those examples of alkaliphiles and haloalkaliphiles brought into culture reveals a remarkable diversity of types, with aerobic alkaliphiles represented in many of the major taxonomic groups of bacteria. Hypersaline environments in particular are relatively low in oxygen due to reduced oxygen solubility (2 ppm in saturated NaCl, compared with 7 ppm in seawater) hence suitable for anaerobic anoxygenic phototrophs and the brines also harbor substantial populations of anaerobic heterotrophs.

The first comprehensive culture survey of soda lake environments that attempted phylogenetic placement of the isolates was that carried out by (Duckworth et al. 1996) who isolated several hundred strains of aerobic, heterotrophic alkaliphilic, and haloalkaliphilic organotrophs from a range of soda lakes in the East African Rift Valley. Phylogenetic analysis revealed many proteobacteria, notably halomonads, plus high and low G + C Gram-positives. Some of these have now been published as novel types, including *Halomonas magadiensis* (Duckworth et al. 2000), *Cellulomonas bogoriensis* (Jones et al. 2005), *Alkalimonas delamerensis* (Ma et al. 2004a), *Dietzia natronolimnaea* (Duckworth et al. 2004). The halomonads (members of the Halomonadaceae like *H. magadiensis*) are probably the most important group of bacterial heterotrophs in both alkaline and neutral hypersaline environments, although other proteobacteria related to pseudomonads and vibrios are also present (Duckworth et al. 1996). Heterotrophic Gram-positive bacteria of both the high G + C (Firmicutes) and low G + C (Actinobacteria) lineages were also readily isolated from hypersaline brines in this study.

Especially abundant were members of the low G + C lineage associated with the diverse *Bacillus* spectrum. There were also high G + C relatives of streptomycetes. A more recent culture survey of Lonar soda lake in India revealed a remarkably similar range of aerobic alkaliphiles except that low G + C Gram positives were in a majority. Halomonads were also significant among the Gram negatives (Joshi et al. 2008). A number of other soda lakes at widely different geographical sites have yielded a number of new species including *Streptomyces sodiiphilus* from a Chinese lake (Li et al. 2005), *Heliorestis convoluta* from the Wadi al Natrun (Asao et al. 2006), *Roseinatronobacter monicus* from Mono Lake in the USA (Boldareva et al. 2007), further validly named new *Halomonas* spp. from China and Kenya (Boltianskaia et al. 2007), *Bacillus aurianticus* from a Hungarian soda lake (Borsodi et al. 2008), *Nesterenkonia aethopica* from an Ethiopian soda lake (Delgado et al. 2006), and *Alkalilimnicola ehrlichii* from Mono Lake (Hoeft et al. 2007).

In recent years, the microbial population of several of the Inner Mongolian soda lakes has been subject to quite detailed aerobic culture-based analyses and a similar picture of the heterotrophic aerobes has emerged with examples of proteobacteria such as newly described *Marinospirillum* spp. (Zhang et al. 2002), *Alkalimonas* spp. (Ma et al. 2004a), and *Aquisalimonas* spp. (Márquez et al. 2007). Again, particularly abundant were members of the high G + C *Bacillus* spectrum with new *Bacillus* spp. and the description of many new genera in addition such as *Gracilibacillus* (Carrasco et al. 2006), *Salsuginibacillus* (Carrasco et al. 2007), *Aquisalibacillus* (Márquez et al. 2007), *Sediminibacillus* (Carrasco et al. 2008b), *Amphibacillus haojiensis* (Zhao et al. 2004), and *Halolactibacillus* (Cao et al. 2008). Remarkably, many of these new species and genera from around the world are related to unnamed groups described in the early paper by (Duckworth et al. 1996) on the aerobes in the Rift Valley.

Isolates from soda lakes have proven to have commercial potential in that they secrete many extracellular hydrolytic enzymes, including proteinases, cellulases, and lipases capable of functioning at high pH and possibly high temperature and salt concentration. As such, they are of interest to the detergent industry in particular. Currently, two different cellulases derived from Gram-positive soda lake isolates are marketed for use in laundry and textile processes.

As noted, chemoorganotrophic populations are biochemically very active, hydrolyzing many different polymers, producing sugars and amino acids. These may be used as substrates for the fermentation of simple compounds by anaerobic fermenters. Fatty acids produced by anaerobes may be consumed by other groups such as the alkaliphilic soda lake acetogenic bacteria, including *Natroniella acetigena*, *Thermosyntropha lipolytica* (Svetlitshnyi et al. 1996), and *Tindallia magadiensis* (Zavarzin et al. 1999). Zavarzin, Zhilina, and colleagues have carried out exceptional pioneering studies and continue ongoing work on soda lake anaerobes from a variety of lakes in the former USSR. Viable counts on anoxic soda lake sediments indicate they contain $>10^6$ cfu ml^{-1} (37°C) chemoorganotrophic alkaliphilic anaerobes especially members of the *Halanaerobiales* and organisms related to other clostridial groups in the low G + C diversion of the Gram-positive bacteria (Jones et al. 1998). These bacteria too, are likely to have a worldwide distribution, for example, *Spirochaeta* spp. have been found at Lake Magadi (Kenya) and Lake Khatyn (Central Asia) (Zhilina et al. 1996). These organisms utilize a wide variety of pentoses, hexoses, and disaccharides producing acetate, lactate, ethanol, and H_2. Obligately anaerobic isolates from the moderately saline lakes were associated with the *Clostridium* group XI. They are phenotypically quite diverse, fermenting a variety of simple sugars or amino acids to acetate and propionate or butyrate and a maximum salt tolerance ranging from 4–12% (w/v). Different isolates were obtained from the hypersaline habitats at Lake Magadi. Phylogenetic analysis also placed these isolates within the *Clostridium* spectrum

but as a separate, well-defined group representing a new genus of obligately anaerobic haloalkaliphiles (Jones et al. 1998).

New species of *Tindallia* have been reported from Lake Texcoco (Alazard et al. 2007); a clostridial cluster XI relative *Anaerovirgula multivorans* from Owens lake in the USA; *Natronobacillus azotoifigens* (also a N_2 fixer) from several sites including Siberia, Mongolia, and Libya (Sorokin et al. 2008a); *Anaerobacillus alkaliliazotrophicus* from Lake Khadyn in Russia (Zavarzina et al. 2009); and *Clostridium alkalicellum*, *Natrinincola ferrireducens*, and *Alkaliphilus peptidofermentans* from Verkhnee soda lake in Russia (Zhilina et al. 2005a, Zhilina et al. 2009a, Zhilina et al. 2009b).

Recent culture studies by Wiegel and colleagues on the Egyptian soda lakes of the Wadi al Natrun have provided new information on the diversity of anaerobes at these sites (Mesbah et al. 2007), including several new genera (*Natronovirga, Natrananaerobius*) and the creation of a new family *Natranaerobiaceae* to accommodate these. The majority of these isolates exhibit the additional extremophile trait of moderate thermophily, a reflection of thermal sites in some soda lake environments. These "polyextremophiles" are, however, beyond the scope of this chapter and are considered elsewhere in this volume.

Conventional wisdom would suggest that in soda lakes the importance of sulfate-reducing bacteria is likely to be considerable. Geochemists have often implied the presence of microbially mediated sulfate-reducing activity in soda lakes in order to explain the appearance of the black lacustrine sediments or the relative depletion of sulfate in the brines of Lake Magadi for example, but until recently microbiological evidence has been lacking. The black color of the sediments of the Kenyan soda lakes would indicate the presence of sulfide (Jones et al. 1998) but there is often no detectable smell of H_2S presumably because the high pH (>pH 11) retards the escape of S^{2-} as volatile H_2S. A number of initially elusive soda lake sulfate-reducing bacteria have now been brought into culture, including *Desulfononatronospira thiodismutans* (Sorokin et al. 2008a), *Desulfonatronum cooperatum* (Zhilina et al. 2005), *Desulfonatronovibrio hydrogenovorans* (Zhilina et al. 1997), *Desulfonatronum lacustre* (Pikuta et al. 1998), and recent isolates (*Dethiobacter* and *Desulfurivibrio* spp.) reported by Sorokin et al. (2008).

Figure 2.2.2 indicates that bacteria isolated from soda lakes are restricted to the most studied evolutionary lines of bacteria, probably a reflection of the culture techniques applied to date. It is probably no accident that the halophilic character coincides with the same lines, a reflection of the usual association between high pH and elevated salt levels in soda lakes.

More recently, studies have tried to analyze the total prokaryote biotope in soda lake environments by extracting DNA and amplifying total 16S rRNA genes, followed by the sequence analyses of the diversity of 16S rRNA clone libraries. Whereas in almost all studies, the bacterial community in both the water and sediments of the lakes was dominated by clones affiliated with the low G+C Gram-type-positive group, Firmicutes-related clones, Proteobacteria, and *Bacteroidetes*, molecular analyses has revealed unexplored biodiversity as judged by the large number of novel bacteria and archaeal 16S rRNA gene sequences that have been detected in East African sites (Grant et al. 1999; Rees et al. 2004), Inner Mongolian sites (Ma et al. 2004b; (Pagaling et al. 2009)), Lake Van in Turkey (Lopez-Garcia et al. 2005), and the Wadi al Natrun (Mesbah et al. 2007).

A detailed biogeographical study of clone sequences from a range of Inner Mongolian lakes and one Argentinean lake has attempted to correlate 16S rRNA sequence diversity with lake chemistry and geography, coming to the conclusion that over short geographical distances, lake chemistry is the determining factor, whereas over large geographical distances, geographical separation becomes important (Pagaling et al. 2009).

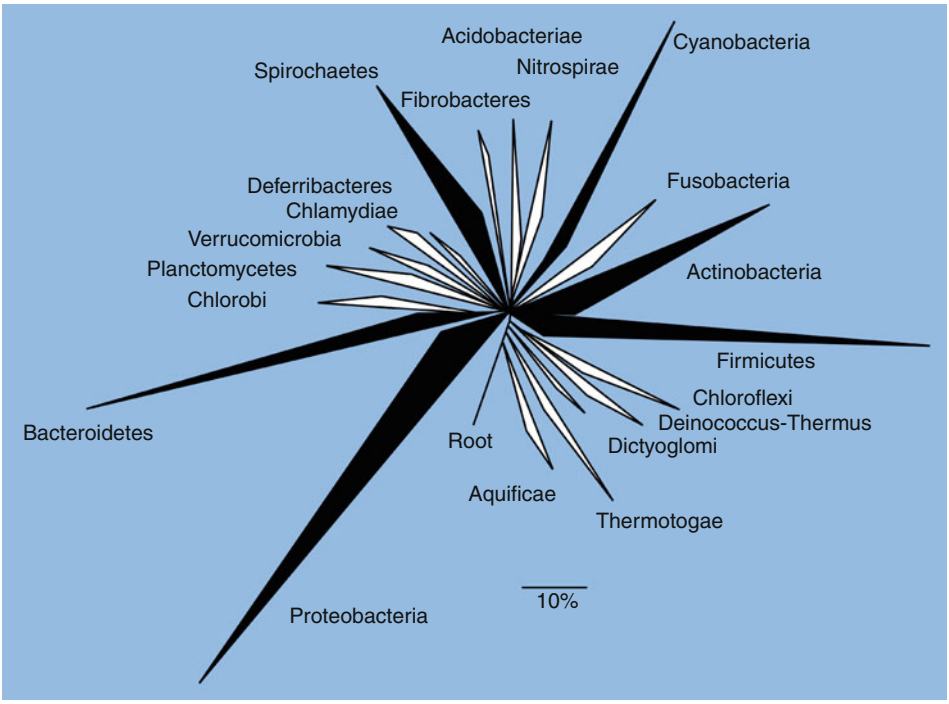

Fig. 2.2.2

16S rRNA gene sequence tree showing the major bacterial lines of descent. Lines of descent with known soda lake isolates are shown *shaded*

Soda Lake Haloarchaea

The extremely halophilic, aerobic archaea that require least 1.5 M NaCl for growth are placed in the order Halobacteriales, family Halobacteriaceae, class Haloarchaea (Grant et al. 2001). The current classification of this family is based mainly on three taxonomical characters: 16S rRNA gene sequence, polar lipid composition, and DNA–DNA hybridization (Grant et al. 2001; Oren et al. 1997). Initially classified solely on morphological and biochemical criteria, the group initially comprised only two original genera, *Halobacterium* and *Halococcus*, subsequently expanded to six genera following the adoption of polar lipid analysis as a discriminatory tool (Grant and Larsen 1989). The advent of 16S rRNA gene sequence analyses revolutionized the understanding of the systematics of the group. At the time of writing, the aerobic, extremely halophilic archaea are classified within 28 different genera. Type species of these genera are *Halobacterium salinarum* (Elazari-Volcani 1957), *Halococcus morrhuae* (Kocur and Hodgkiss 1973), *Natronobacterium gregoryi* (Tindall et al. 1984), *Haloarcula vallismortis* (Torreblanca et al. 1986), *Haloferax volcanii* (Torreblanca et al. 1986), *Natrononococcus occultus* (Tindall et al. 1984), *Halobaculum gomorrense* (Oren et al. 1995), *Halorubrum saccharovorum* (McGenity and Grant 1995), *Natrialba asiatica* (Kamekura and Dyall-Smith 1995), *Natronomonas pharaonis* (Kamekura et al. 1997), *Halogeometricum borinquense* (Montalvo-Rodríguez et al. 1998), *Natrinema pellirubrum* (McGenity et al. 1998), *Haloterrigena turkmenica* (Ventosa et al. 1999), *Natronorubrum bangense* (Xu et al. 1999), *Halorhabdus utahensis* (Wainø et al. 2000),

Halobiforma haloterrestris (Hezayen et al. 2002), *Halomicrobium mukohataei* (Oren et al. 2002), *Halosimplex carlsbadense* (Vreeland et al. 2002), *Halalkalicoccus tibetensis* (Xue et al. 2005), *Natronolimnobius baerhuensis* (Itoh et al. 2005), *Halovivax asiaticus* (Castillo et al. 2007), *Halostagnicola larsenii* (Castillo et al. 2006), *Haladaptatus paucihalophilus* (Savage et al. 2007), *Halopiger xanaduensis* (Gutiérrez et al. 2007), *Haloplanus natans* (Elevi-Bardavid et al. 2007), *Haloquadratum walsbyi* (Burns et al. 2007), *Halosarcina pallida* (Savage et al. 2008), and *Natronoarchaeum mannanilyticus* (Shimane et al. 2010).

The haloalkaliphilic archaea are a distinct physiological group of haloarchaea due to their obligate alkaliphily (Grant and Larsen 1989). These archaea have been found in hypersaline alkaline habitats such as soda lakes and soda soils at many different geographical sites, for example, Lake Magadi in Kenya ((Tindall et al. 1980; Tindall et al. 1984); Mwatha and Grant,1993; (Duckworth et al. 1996; Kanai et al. 1995)), the Wadi Natrun in Egypt (Morth and Tindall 1985; Soliman and Trűper 1982), Owens Lake in California (Morth and Tindall 1985), soda lakes in China, Inner Mongolia, and Tibet (Feng et al. 2005; Itoh et al. 2005; Tian et al. 1997; Wang and Tang 1989; Xu et al. 1999; Xu et al. 2001; Xue et al. 2005), soda solonchak soils in Russia (Zvyagintseva and Tarasov 1987), and a soda lake in India (Upasani and Desai 1990).

As might be expected, the isolates from alkaline soda lakes, as well as having a requirement for high levels of salt, have an additional requirement for high pH in growth media (haloalkaliphilic), usually growing between pH 8.5 and 11.0 with an optimum at pH 9.5–10.0, whereas those haloarchaea from neutral sites generally have pH optima for growth between pH 6 and pH 8 (haloneutrophilic). The extremely alkaline conditions would appear to favour the use of sodium ions rather than protons as the coupling ion in energy generation, but analysis of the genome sequence of the haloalkaliphilic haloarchaeon *Natronomonas phaoronis* clearly identifies protons as the coupling ion between respiratory chain and ATP synthesis (Falb et al. 2005).

Haloalkaliphilic haloarchaea were initially assigned to the then new genera *Natronobacterium* and *Natronococcus* described by (Tindall et al. 1984) following a study of Lake Magadi in Kenya, after earlier reports (Soliman and Trűper 1982; Tindall et al. 1980) of red halophiles at Kenyan and Egyptian alkaline hypersaline sites. Apart from the obligate requirement for high pH shown by the haloalkaliphilic strains, unlike neutrophilic haloarchaea, these organisms also exhibited very low requirements for Mg^{2+}, a consequence of living in alkaline brines essentially devoid of Mg^{2+} that has been removed by precipitation as insoluble $MgSO_4$ as the alkaline brine developed. Several of the other haloarchaeal genera referred to earlier exclusively harbor haloalkaliphilic types (often genera with *Natrono* prefixes), whereas other genera, notably *Halorubrum*, *Natrialba*, and *Halobiforma* have haloalkaliphilic soda lake representatives together with haloarchaea that are have pH optima for growth in the neutral region derived from neutral sites. *Table 2.2.2* lists those validly published haloalkaliphilic species that have been isolated from soda lakes. It should be noted that several of the neutrophilic haloarchaea are capable of some growth at quite alkaline pH despite originating in neutral pH saline environments. The recently described *Natronoarchaeum mannanilyticum* is also clearly a haloalkaliphile despite having been isolated from salt made from seawater at a salt making works in Japan (Shimane et al. 2010).

The climax population in sodium-dominated hypersaline lakes at the point of halite (NaCl) precipitation is almost always comprised of haloarchaea. Dense blooms of these organisms color neutral (and alkaline) hypersaline lakes and salterns bright red due to carotenoid production (*Fig. 2.2.3*). In neutral hypersaline lakes, there is also some variable color contribution from bacteria of the genus *Salinibacter* and the eukaryotic alga *Dunaliella salina*

Table 2.2.2
Soda lake haloarchaea

Species	Original location	Reference
Natronococcus occultus	Lake Magadii, Kenya	(Tindall et al. 1984)
Natronococcus amylolyticus	Lake Magadii, Kenya	(Kanai et al. 1995)
Natronobacterium gregoryi	Lake Magadii, Kenya	(Tindall et al. 1984)
Natronomonas phaoronis	Lake Magadii, Kenya	(Tindall et al. 1984) Kamekura et al. 1997
Natrialba magadii	Lake Magadii, Kenya	(Tindall et al. 1984) (Kamekura et al. 1997)
Natrialba hulunbeirensis	Hulunbeir Province, Inner Mongolia	(Xu et al. 2001)
Natrialba chahannaoensis	Lake Chahannor, Inner Mongolia	(Xu et al. 2001)
Natronolimnobius baerhuensis	Lake Baer, Inner Mongolia	(Itoh et al. 2005)
Natronolimnobius innermongolicus	Lake Baer, Inner Mongolia	(Itoh et al. 2005)
Natronorubrum bangense	Bange Lake, Tibet	(Xu et al. 1999)
Natronorubrum tibetense	Bange Lake, Tibet	(Xu et al. 1999)
Halorubrum vacuolatum	Lake Magadii, Kenya	(Mwatha and Grant 1993) (Kamekura et al. 1997)
Halorubrum alkaliphilum	Xinjiang Province, China	(Feng et al. 2005)
Halorubrum luteum	Lake Chahannor, Inner Mongolia	(Hu et al. 2008)
Halorubrum tibetense	Lake Zabuye, Tibet	(Fan et al. 2004)
Halalkalicoccus tibetensis	Lake Zabuye, Tibet	(Xue et al. 2005)
Halobiforma nitratireducens	Lake Chahannor, China	(Hezayen et al. 2002)

(Elevi Bardavid et al. 2008), but there is no good evidence for the presence of these or similar organisms in alkaline hypersaline sites. Haloarchaeal blooms in neutral solar salterns are known to promote crystallization of halite and almost certainly also in alkaline salterns. It is possible that the cells may serve as templates in the nucleation of halite crystals and their subsequent development (Lopéz-Cortés and Ochoa 1998). In neutral salterns, observations of halite crystallization show that haloarchaea become entrapped within the crystals, leaving behind a so-called bittern brine dominated by $MgCl_2$ and KCl, which does not support significant growth of haloarchaea, although it is not actually toxic (Norton and Grant 1988).

Haloarchaea are organootrophic (Grant et al. 2001) and presumed to develop to high densities in hypersaline sites by deriving carbon and cell protein from phototrophic primary productivity by other salt-loving organisms. Despite apparently inhospitable conditions, salt and soda lakes are extremely productive environments (particularly soda lakes, presumably because of unlimited access to CO_2 for photosystems via the $HCO_3^-/CO_3^{2-}/CO_2$ equilibrium). Phototrophic productivity is probably greatest during periods of dilution, since most of the recorded phototrophs, with the possible exception of *Dunaliella* spp. are unable to grow significantly at saturation point for NaCl. In neutral environments, haloarchaea probably derive most of their carbon as glycerol from preceding blooms of *D. salina* that occur as halite

Fig. 2.2.3
Soda crust at lake Magadi, Kenya

saturation approaches. In alkaline sites, the role of *Dunaliella* is taken by haloalkaliphilic cyanobacteria, notably *Spirulina* and *Cyanospira* spp. (Grant and Jones 2000). Hypersaline environments are relatively low in oxygen due to reduced oxygen solubility (2 ppm in saturated NaCl, compared with 7 ppm in seawater) and the brines may also harbor substantial populations of anaerobic phototrophic bacteria of the genus *Halorhodospira* that may be significant producers of biomass from time to time (Grant 2004).

Direct molecular analysis of both alkaline and neutral hypersaline brines by 16S rDNA amplification of environmental DNA, preparation of gene libraries, followed by sequence determinations of individual 16S rRNA genes, has revealed novel haloarchaeal lineages that have yet to be brought into culture (Caton et al. 2009; Grant et al. 1999; Mwichia et al. 2010; Ochsenreiter et al. 2002; Valenzuela-Encinas et al. 2008) together with clones that affiliate with isolates in culture. In diverse alkaline saline environments including Lake Texcoco (Mexico), an unnamed soda lake in Nevada (USA) and Lake Elmenteita (Kenya), clones affiliated with the genera *Natronococcus*, *Halovivax*, *Halobiforma*, *Halalkalicoccus*, *Halorubrum*, *Natronomonas*, *Natronolimnobius*, and *Natrinema* were detected. However, a considerable percentage of the clones (often 50% or more), although clearly affiliated with the Halobacteriales, did not closely affiliate with any of the organisms in culture, indicating as yet unexplored haloarchaeal diversity.

Hydrolysis products of complex polymers probably derived from the anaerobic decomposition of cyanobacterial mats to acetic acid, hydrogen, and CO_2 (Grant et al. 1998; Zavarzin et al. 1999), in soda lakes in turn may be used by haloalkaliphilic methanogens, (also, strictly speaking, alkaliphilic haloarchaea!) although most of the methanogens isolated to date from hypersaline environments are methylotrophic, utilizing compounds such as methanol and methylamine (Oren 1999). This aspect of archaeal biodiversity in soda lakes is sadly underrepresented in the literature and is largely confined to a few studies on Russian

((Namsaraev et al. 1999a); b) and Indian lakes (Sukarasi et al. 2007). Isolates in culture include *Methanocalculus*, *Methanoculleus*, and *Methanosalsus* spp. from Lake Magadi (Kevbrin et al. 1997). Again, direct molecular analysis indicates other such groups (Sukarasi et al. 2007).

Soda Lake Chemolithotrophs

Aerobic chemolithotrophic bacteria utilizing reduced inorganic compounds as electron donors are important players in the element cycling in natural and technogenic environments. For the last 15 years, the culturable diversity of chemolithotrophs in soda lakes in Asia (southwest Siberia, Transbaikal region, Mongolia), Africa (Kenya), and the USA (Mono Lake, Soap Lake, Searles Lake, Owens Lake) has been studied. Lake pH values vary from 9 to 11, salinity from 5 to 500 g/l (w/v), and alkalinity from 0.005 to 4 M. The work has resulted in the description of novel haloalkaliphilic representatives of all functional groups of chemolithoautotrophs (Sorokin and Kuenen 2005a). One of the important features of these studies was the development of a mineral base medium suitable for the successful enrichment and isolation of a wide range of soda lake chemolithotrophs. The optimized mineral medium is based on the sodium bicarbonate/carbonate buffer with a pH of 10–10.1, variable NaCl concentration, and a total Na^+ content 0.3–4.0 M. Such media, even at minimal salt content, were able to maintain the pH above 9.2 during active growth of acid-producing chemolithotrophs.

Soda Lake Sulfur-Oxidizing Bacteria (SOB)

With thiosulfate as electron donor, wide distribution of obligately autotrophic haloalkaliphilic SOB in soda lakes of different geographic locations has been demonstrated with abundance ranging from 10^3 to 10^8 viable cells/cm^3 in sediments. More than 100 strains of haloalkaliphilic SOB actively growing at pH up to 10.3–10.5 have been obtained in pure culture. They are grouped into four new genera within the Gammaproteobacteria – *Thioalkalimicrobium*, *Thioalkalispira*, *Thioalkalivibrio*, and *Thioalkalibacter* (*Fig. 2.2.4*) ((Sorokin and Kuenen 2005b; Sorokin et al. 2006b); Banciu et al. 2008). The first three genera include obligate alkaliphiles and the latter contains facultatively alkaliphilic halophiles. Among these, the genus *Thioalkalivibrio* is most diverse phylogenetically and representatives are physiologically the best adapted for life in hypersaline brines. Several large geographical populations each containing about 20 isolates could be distinguished among the extremely salt-tolerant representatives of the genus (Foti et al. 2006): isolates from Kulunda Steppe and Mongolia are clustered together and also resemble the Mono Lake and Soap Lake *Thioalkalivibrio* strains, while most of the Wadi Natrun isolates cluster with *Tv. halophilus*. The basic properties of the soda lake SOB are summarized in *Table 2.2.3*.

On the basis of their salt tolerance/requirement, *Thioalkalimicrobium*, some of the *Thioalkalivibrio* isolates and *Thioalkalispira* belong to a moderately salt-tolerant type, growing up to 1.2–1.5 M total Na^+. In contrast, many of the *Thioalkalivibrio* isolates are able to grow in saturated soda brines (4 M Na^+). Overall, the cultivated forms of SOB from soda lakes cover a complete range of pH/salt concentration typical for their environment. Isolation and study of two facultatively alkaliphilic halophilic SOB species, *Thioalkalivibrio halophilus* and *Thioalkalibacter halophilus*, indicated *natronophily* – a preference of sodium carbonates over sodium chloride by the soda lake organisms. Since sodium carbonates are much weaker electrolytes

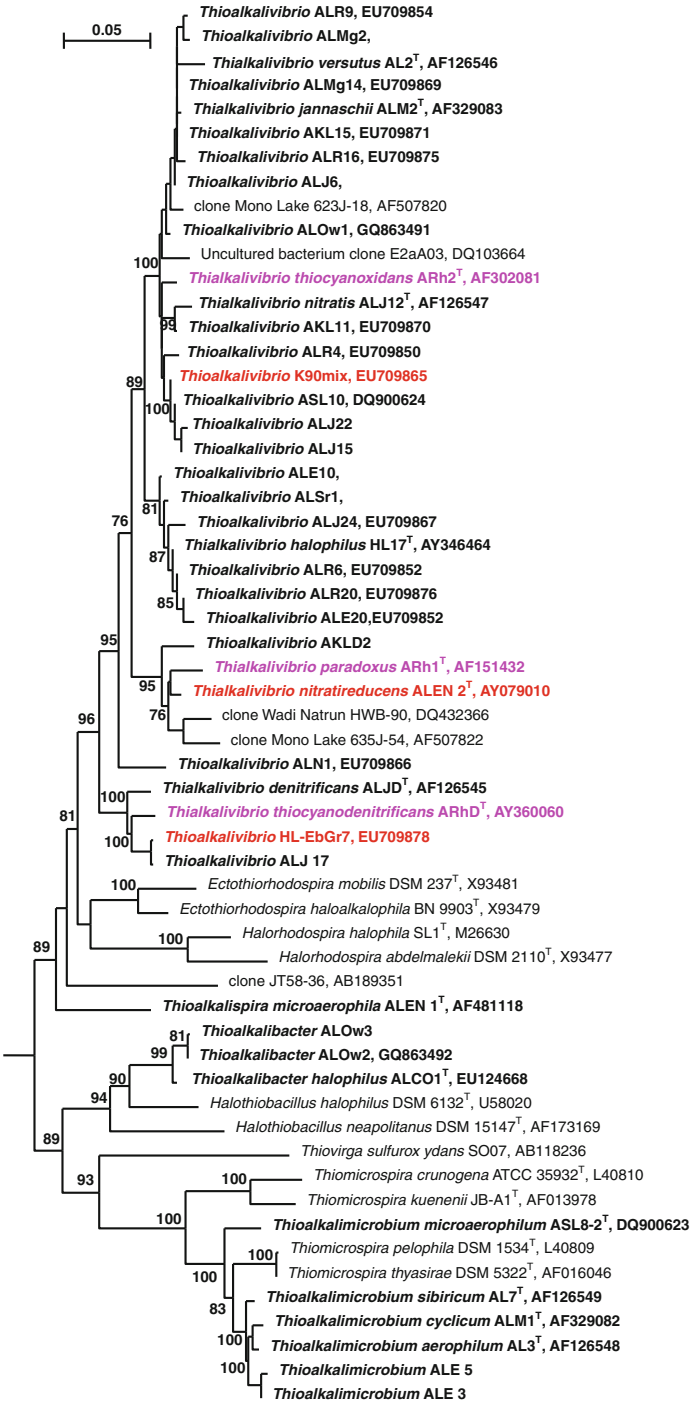

Fig. 2.2.4 (Continued)

Table 2.2.3

Basic properties of haloalkaliphilic SOB from soda lakes

Property	*Thioalkalimicrobium* spp.	*Thioalkalispira microaerophila*	*Thioalkalivibrio* spp.	*Thioalkalibacter halophilus*
Number of species	3	1	9	1
Closest relative	*Thiomicrospira pelophila*	No	*Ectothiorhodospira*	*Halothiobacillus*
Cell morphology	Rods and spirilla	Spirilla	Rods, vibrios, spirilla, and cocci	Rods
Sulfur compounds oxidized	HS^-, S_n^{2-}, $S_2O_3^{2-}$	HS^-, S_n^{2-}, $S_2O_3^{2-}$	HS^-, S_n^{2-}, $S_2O_3^{2-}$, S_8, $S_4O_6^{2-}$, SCN^-	HS^-, S_n^{2-}, $S_2O_3^{2-}$
Electron acceptors	O_2	O_2 microaerophile	O_2, NO_3^-, NO_2^-, N_2O	O_2
pH optimum	9.5–10.0	10.0	10.0–10.2	8.5
Upper salt limit	1.5 M Na^+	1.4 M Na^+	4.3 M Na^+	3.5 M Na^+
Maximal specific growth rate	0.33 h^{-1}	0.08 h^{-1}	0.25 h^{-1}	0.22 h^{-1}
Maximal growth yield with $S_2O_3^{2-}$	3.5 mg protein $mmol^{-1}$	5.8 mg protein $mmol^{-1}$	6.5 mg protein $mmol^{-1}$	3.5 mg protein $mmol^{-1}$
Rate of HS^- oxidation	Extremely high	Low	Low	High
Dominant compatible solute	Ectoine	nd	Glycine betaine	Ectoine
Yellow membrane pigment	−	+	+	−
Distribution	Asia, Africa, North America	Egypt	Asia, Africa, North America	Asia, North America

Fig. 2.2.4

Phylogenetic position of haloalkaliphilic sulfur-oxidizing bacteria (SOB) from soda lakes (in *bold*) within the Gammaproteobacteria based on 16S rRNA sequence analysis. In the cluster of the genus *Thioalkalivibrio* the unclassified strain abbreviation is as follows: ALMg, strains from Mongolia; ALJ, strains from Kenya; AKL, strains from Kulunda Steppe; ALE, strains from Wadi Natrun in Egypt; ASL, strain from Soap Lake (USA); ASLr, strain from Searles Lake (USA); ALN1, extremely haloalkaliphilic nitrate-reducing strain from Wadi Natrun; ALR, strains from a lab-scale bioreactor; HL-Ebgr7, strain from a full-scale bioreactor. In red are the genome sequenced strains and in pink are strains for which genome sequence is in progress. Numbers at the nodes indicate the percentage of bootstrap values for the clade of this group in 1,000 replications (the values for maximum-likelihood method are given in parentheses). Only values above 70% are shown. *Bar*, 5 substitution per 100 nt

than NaCl, their osmotic burden and water stress on cells must be significantly less than that of NaCl. Therefore, organisms living in soda must have an advantage in synthesizing less compatible solutes as compared to halophiles living in NaCl brines of the same sodium molarity. Analysis of compatible solute content in *Tv. halophilus* (Banciu et al. 2004) and *Thioalkalibacter* (Banciu et al. 2008) grown either in NaCl or in soda brines demonstrated that, indeed, the cells grown in NaCl brines contained roughly twice more osmolytes than the soda-grown cells, exactly corresponding to the difference in electrochemical and ionic properties of the two sodium salts (› *Table 2.2.4*).

Furthermore, the reaction of extremely salt-tolerant SOB on sodium salts with different electrolytic properties correlated with the anionic composition of their habitats. Strains isolated from sodium carbonate-dominated lakes, such as *Thioalkalivibrio versutus* ALJ15 (Kenya), respired more actively in sodium carbonate and sodium sulfate brines (weak electrolytes), while *Thioalkalivibrio halophilus* ALE 20 from a NaCl-dominated alkaline lake in Wadi Natrun functioned better in NaCl brines (› *Fig. 2.2.5*). Therefore, the preference of soda lake organisms for sodium carbonates, despite the high pH stress, has a rational explanation and must be taken into consideration as a specific biological phenomenon.

Use of thiocyanate ($N{\equiv}C{-}S^-$) as electron donor instead of thiosulfate at pH 10 has resulted in the isolation in pure culture of two different phenotypes of haloalkaliphilic SOB from various soda lakes described as *Thioalkalivibrio thiocyanoxidans* and *Thioalkalivibrio paradoxus* (Sorokin et al. 2001a; Sorokin et al. 2002). These bacteria degraded thiocyanate via cyanate ($N{=}C{=}O^-$) with final production of ammonia, CO_2, and sulfate. But, in contrast to anaerobic hydrolysis, suggested previously as a mechanism of the primary thiocyanate degradation in neutrophilic thiobacilli, in alkaliphilic SOB this reaction is obligately aerobic and the enzyme responsible for it appears to act as an thiocyanate-cytochrome *c* oxidoreductase with elemental sulfur as an immediate product. The novel enzyme is a soluble periplasmic ~60-kDa monomer with a unique primary peptide structure representing a novel class of proteins and a novel type of oxidoreductases (our unpublished results).

Both very active sulfide oxidation and the ability to utilize thiocyanate at highly alkaline pH make natronophilic SOB attractive for bio-based environmental technology (Janssen et al. 2009). For example, cyanide waste is only safe at high pH and is usually

Table 2.2.4

Synthesis of compatible solutes (% from dry cells) by facultatively alkaliphilic SOB during growth in NaCl or in soda brines

	Thioalkalivibrio halophilus HL17		*Thioalkalibacter halophilus* ALCO1	
	4 M Na^+	4 M Na^+	3 M Na^+	3 M Na^+
	NaCl	soda	NaCl	soda
Compatible solute	pH 7.5	pH 10	pH 7.5	pH 10
Glycine betaine	19.8	12.4	13.5	5.2
Ectoine	0	0	0.9	1.3
Total	19.8	12.4	14.4	6.5

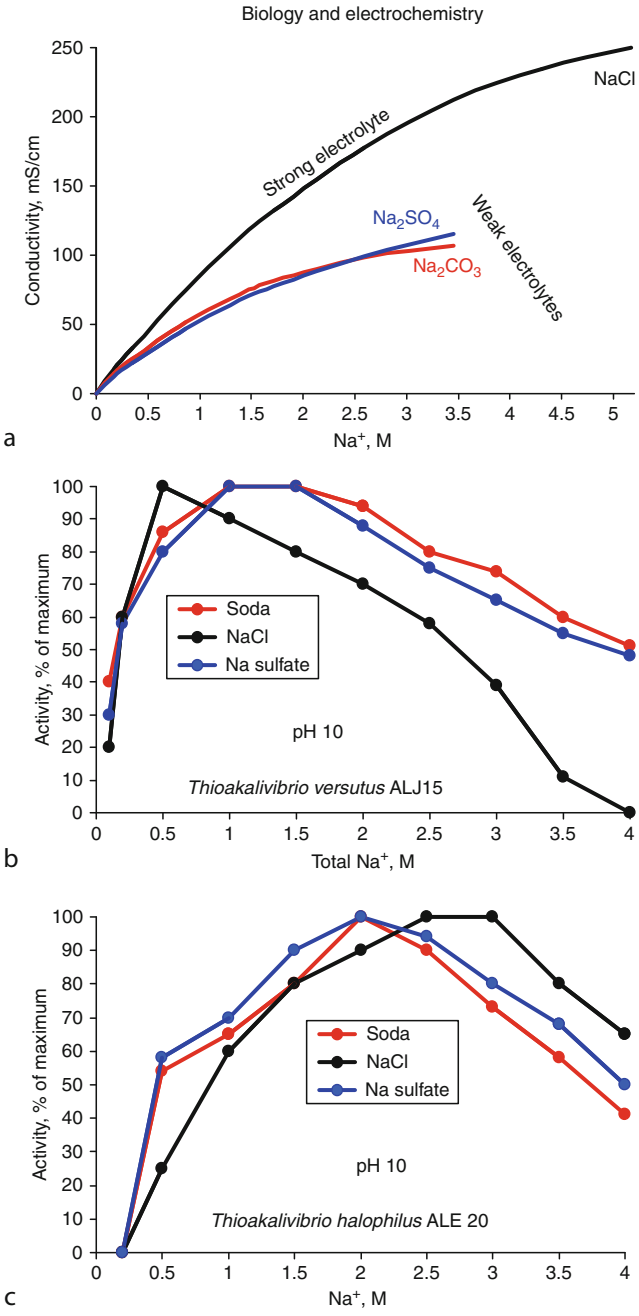

Fig. 2.2.5

Illustration of the presence of different "salt ecotypes" among the SOB isolates from alkaline lakes. (a) electrochemical difference in sodium salts indicated by conductivity dependence from concentration. (b and c) influence of sodium salts with different ionic properties on respiratory activity at pH 10 in two strains of extremely salt-tolerant strains of the genus *Thioalkalivibrio*

converted into less harmful, but still toxic, thiocyanate waste. The latter could be treated with alkaliphilic SOB.

Soda Lake Methanotrophs

Methanotrophic enrichments at pH 9–10 from soda lake sediments, even from the hypersaline ones, were positive only at a salt concentration below 1.5 M total Na^+, despite in situ experiments with $^{14}CH_4$ demonstrating measurable rates of methane oxidation in hypersaline soda lakes. In the hyposaline and moderately saline lakes, as was in the case of the northeastern Mongolia, the rates of methane oxidation were sufficient to balance the rates of methane formation, while at higher salinity the methane cycle was unbalanced (Sorokin et al. 2004). Molecular probing of the methanotrophic population in soda lakes of Central Asia demonstrated ubiquitous domination of type I methanotrophy belonging to the genus *Methylomicrobium* in the Gammaproteobacteria (Li et al. 2004).

A pure culture of an obligate methanotroph was obtained from a Kenyan soda lake after enrichment at 0.6 M total Na^+ and pH 10. Strain AMO 1 was identified as a member of the genus *Methylomicrobium* in the *Gammaproteobacteria* (Sorokin et al. 2000) and later classified as *M. kenyense* (Kalyuzhnaya et al. 2008). Other moderately alkaliphilic methanotrophic isolates from hyposaline alkaline lakes (Trotsenko and Khmelenina 2002) were all classified within the same genus (Kalyuzhnaya et al. 2008). On ultrastructure and biochemical properties, it is a typical representative of the type I methanotrophs with lamellar intracellular membrane structures and RuMP pathway of carbon assimilation, utilizing methane and methanol as carbon and energy source. Strain AMO 1 had a very narrow pH range for growth between 9 and 10.2 with an optimum at pH 9.9–10, although washed cells respired methane and methanol within much broader pH range from 6.0 to 11.0 with the same pH optimum. The total salt concentration in the form of sodium carbonate/bicarbonate and NaCl suitable for growth ranged from 0.2 to 1.2 M total Na^+, thus belonging to the low salt-tolerant alkaliphiles. The ecological role of the AMO-like alkaliphiles might not be limited only by methane oxidation, since the strain was found to possess an additional potential to oxidize ammonia and carbon disulfide (CS_2). Ammonia was converted to nitrite in presence of moderate concentrations of methane or methanol and only at very alkaline pH between 10 and 11. CS_2 was oxidized with relatively high rates of polysulfide formation, probably resulting from abiotic reaction of the true products sulfur and sulfide at high pH. Addition of low numbers of alkaliphilic SOB (a *Thioalkalimicrobium* strain) to the cells of AMO 1 increased the rate of CS_2 oxidation and changed the product to elemental sulfur, mimicking a possible variant of cooperation between these two groups of haloalkaliphiles.

Soda Lake Nitrifiers

The in situ activity of ammonia oxidation and the presence of 16S rRNA, *amo*A (ammonium monooxygenase), and *cbb*L (RuBisCO) gene sequences related to the *Nitrosomonas europea* lineage have been demonstrated in the chemocline of saline alkaline Mono Lake and Big Soda Lake (Cloern et al. 1983; Giri et al. 2004; Joye et al. 1999; Ward et al. 2000). Five stable enrichment cultures of the ammonia-oxidizing bacteria (AOB) growing at pH 10 have been obtained from sediments of the Mongolian soda lakes and one from the Wadi Natrun lake

using ammonium concentration <4 mM and salt concentration 0.6 M total Na^+. Nitrite production ceased at higher ammonium concentrations and salt content >1 M total Na^+ (Sorokin 1998; Sorokin et al. 2001b). Five strains obtained from the enrichments were very close genetically to each other and closely related to the known marine species *Nitrosomonas halophila*. In continuous culture with pH control, one of the strains was able to grow up to pH 11.4 – an absolute maximum not only among chemolithotrophs but also close to a proven maximum (11.5) for alkaliphilic heterotrophs (Sturr et al. 1994). It seems that the soda lake AOB have somehow managed to overcome a major problem of autotrophic metabolism at extremely high carbonate/bicarbonate ratio (e.g., $NaHCO_3$ limitation). Growth was possible only at low salt from 0.1 to 0.9 M Na^+ in purely sodium carbonate media without any Cl^-. Apart from high salt, ammonia toxicity was evident at pH above 10, isolates tolerating no higher than 8 mM of NH_4Cl and growing without lag phase only when ammonium concentration was <4 mM.

In contrast to ammonia, nitrite toxicity decreases with increasing pH, which made it possible to use 20 mM nitrite for the isolation of alkaliphilic NOB. Five pure cultures obtained from soda lakes and soda soils at 0.6 M Na^+ and pH 10 with nitrite represented a typical *Nitrobacter* phenotype, which was confirmed by phylogenetic analysis. However, despite very high sequence similarity to a type species *Nb. winogradskii*, there was very little DNA homology between the soda lake isolates and the type strain, which, together with distinct alkalitolerance justified the description of a new species *Nb. alkalicus* (Sorokin et al. 1998). In contrast to the soda lake AOB isolates, the NOB strains were facultatively alkaliphilic, only moderately alkalitolerant (pH maximum 10.2) and even less salt tolerant (up to 0.5 M Na^+). This is most probably due to a very low energy yield of the nitrite oxidation (the lowest among lithotrophs). Accordingly, it may be concluded that the nitrogen cycle in hypersaline soda lakes may be devoid of its oxidative part.

Soda Lake Hydrogenotrophs

Recent work with sulfidogenic populations in soda lakes (our unpublished results) demonstrated that, in contrast to neutral habitats, hydrogen is not a favorable substrate for sulfate-reducing haloalkaliphiles. Therefore, part of the hydrogen might escape from the anoxic sediments to the more oxidized water-sediment interface and thus become available for aerobic and denitrifying hydrogenotrophs. One such bacterium, strain AHO 1, was obtained from an aerobic enrichment inoculated with mixed sediment samples from Kenyan soda lakes at pH 10 and 0.6 M total Na^+ (Sorokin et al. 2000). The isolate AHO 1 was identified as a novel member of the alpha-3 subdivision of the Proteobacteria and was given a tentative name "*Natronohydrogenobacter thiooxidans*." It is a facultative autotroph growing chemolithoautotrophically with hydrogen, heterotrophically with various organic acids and sugars, and mixotrophically with hydrogen or sulfide as energy source and acetate as carbon source. Hydrogenotrophic growth was optimal at pH 9.5–9.8 and possible up to pH 10.25 at a salt concentration from 0.2 to 1 M total Na^+. An anaerobic enrichment under denitrifying conditions yielded a pure culture, strain AHN 1, which grew autotrophically with hydrogen and nitrate at pH 10 and salt concentration up to 1.5 M total Na^+. Strain AHN 1 is a partial denitrifier with a gap in the middle of its denitrification pathway, for example, it can reduce nitrate to nitrite (with hydrogen or acetate) and N_2O to dinitrogen (with acetate), but cannot grow anaerobically with nitrite. The strain was identified as a member of the genus *Alcalilimnicola* in the

Gammaproteobacteria (Sorokin et al. 2006a). Another haloalkaliphilic strain belonging to this cluster and described as *Alkalilimnicola ehrlichii* (Hoeft et al. 2007) was obtained from Mono Lake using somewhat "exotic" combination of substrates, previously unknown to sustain lithoautotrophic growth. Strain MLHE-1 was able to grow autotrophically with arsenite, hydrogen, sulfide, and thiosulfate coupled to nitrate reduction to nitrite (Oremland et al. 2002).

Soda Lake Carboxydotrophs

Early attempts to enrich for alkaliphilic aerobic carboxydotrophs from soda lakes were not successful. The most probable reason for this result was too high CO and O_2 concentrations (50% and 20% in the gas phase, respectfully). When those concentrations were reduced below 20% for CO and 5% for O_2, four positive enrichments were obtained at pH 10 and salt content from 0.2 to 2.0 M Na^+ from sediments of hypersaline soda lakes in Kulunda Steppe and a single culture from the Wadi Natrun hypersaline alkaline lakes. The enrichments resulted in isolation of five pure cultures of bacteria with a potential to grow chemolithoautotrophically with CO. Four of them were members of the genus *Alkalispirillum* and one belongs to the "sister" genus *Alkalilimnicola*, once again indicating the importance of this group of facultatively autotrophic haloalkaliphiles for the "aerobic filter" in soda lakes (Sorokin et al. 2010). Earlier, the potential to consume CO, but not to grow with it, and the presence of a typical aerobic CO-dehydrogenase (CODH) have been demonstrated in *Alkalilimnicola ehrlichii* (Hoeft et al. 2007). The ACO isolates were able to grow with CO within a relatively narrow alkaline pH range from 8.5 to 10.6 and up to a very high salt concentration (3.5 M total Na^+) possible only in the SOB group. Such a salt tolerance is probably possible due to a high energy yield of CO oxidation. Apart from CO, the strains utilized formate and oxidized sulfide also within the highly alkaline pH range but did not grow autotrophically with hydrogen, in contrast to most of the known aerobic carboxydotrophs. The CODH activity was detectable in the membranes and in the periplasme but conventional primers for the *cox*L forms I and II did not amplify the gene.

Overview of Lithotrophs in Soda Lakes

All known groups of aerobic chemolithoautotrophic bacteria that can be cultivated at least at pH 10 and sometimes higher have been found in soda lakes and cultivated using mineral medium highly buffered with sodium carbonate. The generalized scheme of their role in the soda lake element cycling is presented in *Fig. 2.2.6*. All of these use the Calvin–Benson cycle for inorganic carbon fixation in the form of HCO_3^-. The preference of sodium carbonate environment with its extremely high pH and alkalinity, that is, *natronophily* rather than haloalkaliphily, is actually the main property that discriminates these bacteria from their neutrophilic counterparts. Based on comparison of the pH profiles, three different pH-iono-philes can be discriminated among the salt-tolerant chemolithoautotrophs: NaCl-, $NaHCO_3$-, and Na_2CO_3-preferring types. The NaCl-type is usual for the marine environment, chloride-sulfate salt lakes, and solar salterns (e.g., *Nitrococcus halophilus* and *Halothiobacillus halophilus*); the $NaHCO_3$-type is probably adapted to increasing $NaHCO_3$ alkalinity in photosynthetic microbial mats of the tidal flats, with diurnal fluctuation of pH/HCO_3^- (e.g., *Nitrosomonas halophilus* Nm1 and *Thiomicrospira pelophila*, both from the North Sea). Neither of them, though, can tolerate Na_2CO_3-dominating environment with a pH $\geq$ 10, which

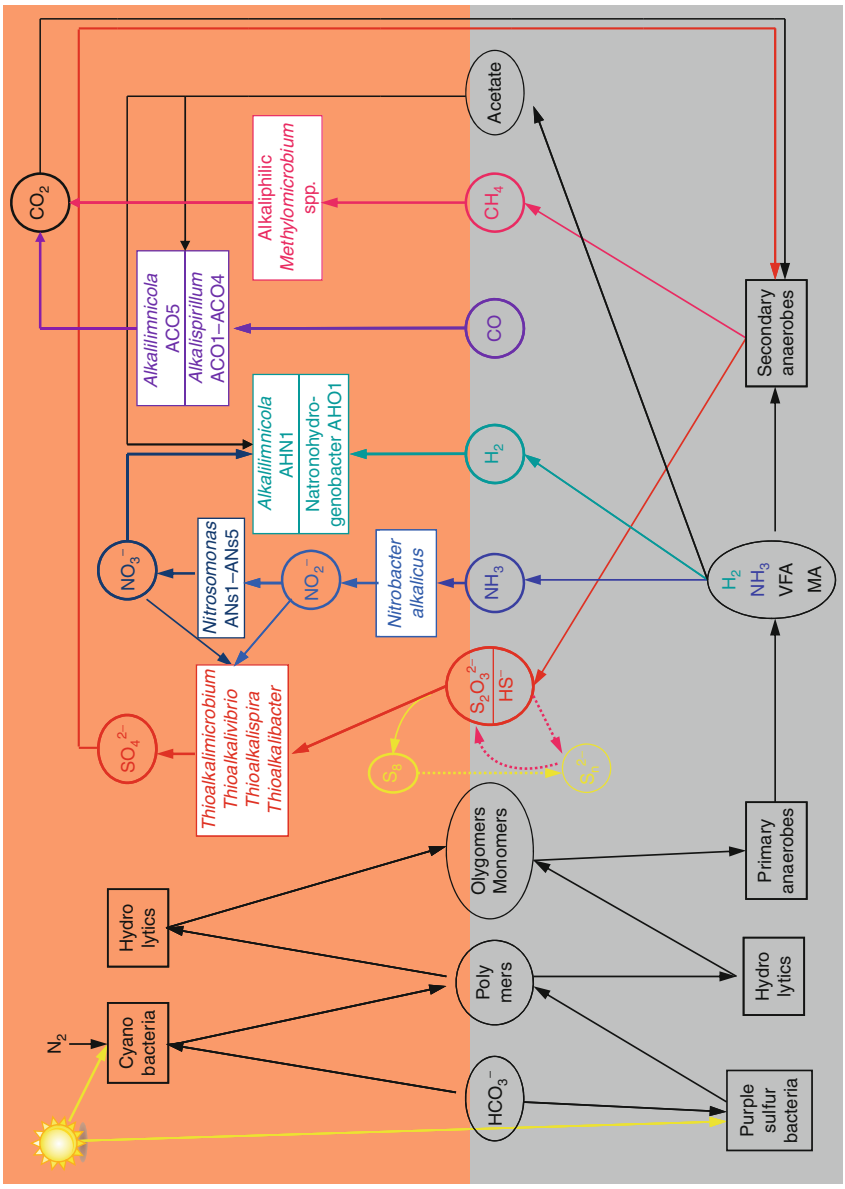

Fig. 2.2.6
Generalized scheme of the soda lake microbial system showing the place of chemolithoautotrophic natronophiles

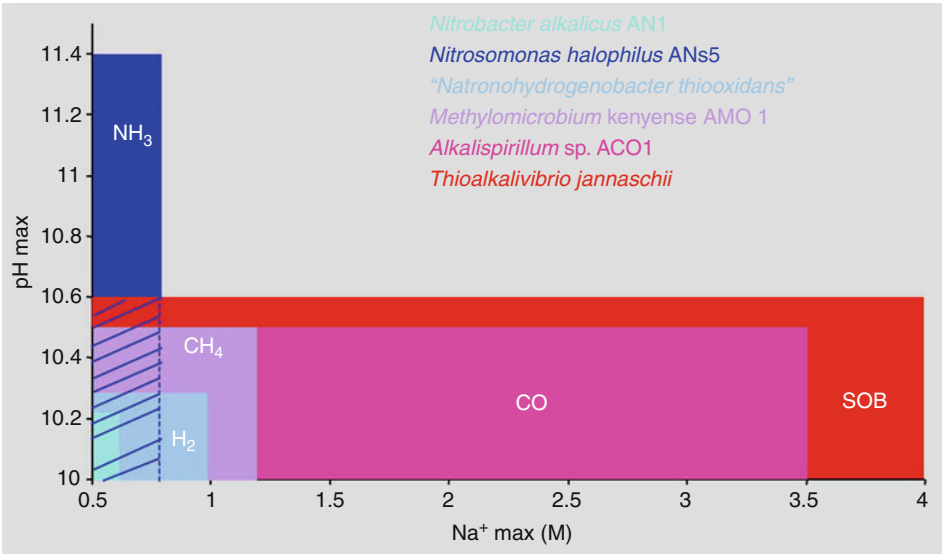

Fig. 2.2.7

Range of pH-salt values for growth of chemolithotrophic natronophiles from soda lakes

is a real domain of the soda lake natronophiles (e.g., *Nitrosomonas halophila* ANs1 and *Thioalkalimicrobium aerophilum*).

The second important characteristic is salt tolerance. From five groups of chemolithoautotrophs discovered in soda lakes, only some of the SOB and carboxydotrophs are fully capable to function in hypersaline soda lakes, while the activity of others either partially or completely restricted to moderately saline lakes (*Fig. 2.2.7*). Their presence in hypersaline lakes might be explained by either existence of less saline microniches or by the salinity fluctuation.

Cross-References

- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 2.8 Enzymes Isolated from Alkaliphiles
- 2.11 Alkaline Enzymes in Current Detergency

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2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.3,

Background

Two categories exist for discrimination of bacteria that are able to grow at a high pH (pH 9). The first group of bacteria is categorized as alkali-tolerant bacteria. The bacteria belonging to this group 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]) are able to grow at pH 8–9 but not at pH 10. Another example is alkali-tolerant *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 bacteria. This category of bacteria is the main issue in this review. Alkaliphilic bacteria can be defined as the bacteria 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 bacteria can be further divided into facultative alkaliphiles, which can grow well below or at pH 8, and obligate alkaliphiles, which cannot grow well below or at pH 8. Some genera of bacteria include neutralophilic, alkali-tolerant, and alkaliphilic (e.g., *Bacillus*). Even among the same species of bacteria, there are those that are both alkali-tolerant and alkaliphilic bacteria (e.g., *Bacillus horikoshii* [Nielsen et al. 1995]). Even among the same species of bacteria, there are those that are both obligate and facultative alkaliphiles (e.g., *Bacillus pseudofirmus* (Nielsen et al 2005) [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.

The first reported alkaliphilic bacterial species is *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) described by Chester (1889). The bacterium has the ability to convert urea to ammonium carbonate and requires ammonia in addition to an alkaline environment. Strains belonging to S. pasteurii can be isolated from soil, water, sewage, and incrustation on urinals. In 1934, Vedder reported *Bacillus alcaliphilus* as the second example of isolation of alkaliphiles. The bacterium lacks urease activity and does not require ammonia in addition to an alkaline enveronment. This species was isolated from various materials using preliminary enrichment in broth at pH 10. In 1960, Takahara and Tanabe isolated an indigo-reducing alkaliphile, which can grow at pH 12.1 from indigo fermentation liquor, and proposed it as a strain belonging to the genus *Bacillus*. They proposed a new species for the strain as *Bacillus alkaliphiles* (Takahara and Tanabe 1962). However, the species name has not been approved and the strain is not available presently.

During the 1970s–1980s, numerous alkaliphiles belonging to the genus *Bacillus* were isolated for industrial applications of their enzymes and for physiological studies on environmental adaptation mechanisms. However, most of these bacterial strains have not been appropriately identified up to the species level. Gordon and Hyde (1982) firstly classified numerous alkaliphilic *Bacillus* strains. They grouped alkaliphilic *Bacillus* spp. into *Bacillus firmus*, *Bacillus lentus* groups I–III, and the *B. firmus–B. lentus* complex on the basis of their physiological and biochemical characteristics. A reclassification of these strains, together with additional strains, was carried out by Fritzie et al. (1990). Their reclassification was based on several phenotypic properties that correlated with distinct DNA G+C mol% values. Among the strains used in the study of Spanka and Fritzie (1993), a new species, *B. cohnii*, was proposed; it is distinct from the other strains by the absence of diaminopimelic acid (DAP) in its cell wall. The phenotypic and genotypic heterogeneities of alkaliphilic *Bacillus* strains within the same group in the study by Fritz et al. were revealed by phylogenetic analysis based on 16S rRNA gene

sequence analysis (Nielsen et al. 1994). In addition to the reported alkaliphilic bacteria described above, nine new species were proposed by Nielsen et al. (1995) on the basis of results of numerical taxonomy and DNA–DNA hybridization. Strains used for physiological studies on environmental adaptation mechanisms (Goto et al. 2005; Krulwich and Guffanti 1989; Krulwich et al. 2007; Yumoto 2002, 2003) belong to the species defined by Nielsen et al. (1995). Since the study by Nielsen et al. (1995), more than 30 new species alkaliphilic *Bacillus* have been proposed. In addition, numerous strains belonging to genera other than the genus *Bacillus* and included in the phylum Firmicutes have been proposed (Yumoto 2007). Furthermore, since the finding of alkaliphiles in soda lakes in Africa, the taxonomic distribution of alkaliphiles has become wider (Duckworth et al. 1996; Jones et al. 1998). In addition to the phylum Firmicutes, alkaliphilic microorganisms so far isolated are presently taxonomically distributed to the following phyla: Cyanobacteria, Actinobacteria, Proteobacteria (including alpha, gamma, and delta subdivision), Bacteroidetes, Thermotogae, Spirochaetas, Archaea (Euryarchaeota), and yeast (Jones et al. 1998). In the 2000s, numerous alkaliphiles are continuously being isolated and the number of approved species of alkaliphiles is continuously increasing. In this review, we focus on the environmental distribution and taxonomic diversity of alkaliphiles belonging to the phylum Firmicutes.

Aims and Significance of Taxonomy of Alkaliphilic Fermicutes

Bacteria have gone through generations of changes over a long period on earth. They exhibit tremendous genetic diversities and can adapt to a wide range of environments compared with other organisms. In addition, bacteria contribute to global material circulation. Therefore, it can be said that the maintenance of environmental conditions on earth depends on the activity of bacteria. It is expected that each existing bacterial cell in the environment has a certain role in a community of bacteria. 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. In addition, understanding the relationship between taxonomic diversity and geographical distribution, the variation of strategy for adaptation in an environment, and the concomitant evolutional process of bacteria is also important. If such knowledge is available, it 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 even in a limited environment and a limited category of bacteria in a short term. Furthermore, it is not easy to consider the ecological function of such numerous species of bacteria. However, if we consider only alkaliphilic 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 bacteria are more widely distributed in the environment than other extremophiles (Horikoshi and Grant 1998). Therefore, we are able to consider a wide range of environments for a limited category of bacteria compared with neutralophilic bacteria. This approch will simplify the difficult problem in microbial ecology and will bring us a simpler model of bacterial environmental distribution and taxonomic diversity compared with an exhaustive approach.

It is considered that four environmental categories exist for bacterial habitats considering the size and naturally existing or artificial environments (*Fig. 2.3.1*). These are 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:

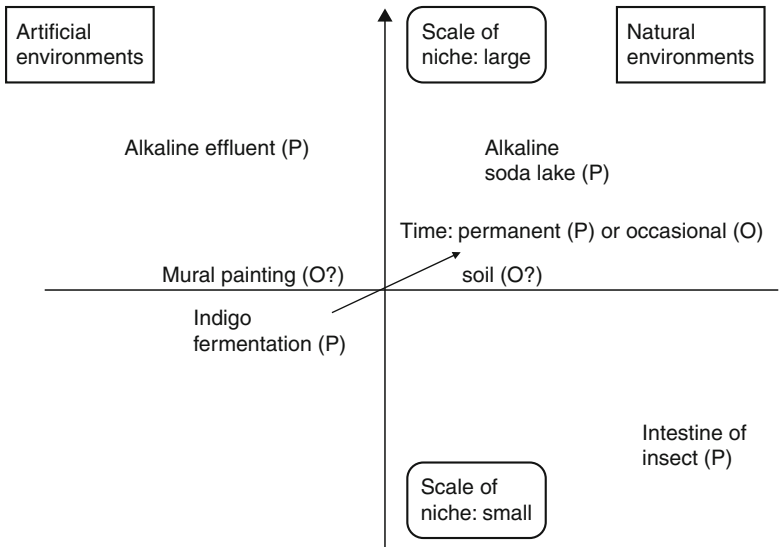

Fig. 2.3.1

Classification of habitats of alkaliphiles. The horizontal axis indicates natural or artificial environments and the vertical axis indicates the scale of the habitat. The third axis indicates time of exposure to alkaline conditions. P: permanent alkaline condition, O: occasionally alkaline condition. It is difficult to define soil and soil-like solid environments because it is difficult to monitor pH in small niches

large natural environments such as alkaline soda lakes, large artificial environments such as alkaline containing waste water treatment systems, small natural environments such as the gut of termites, and small artificial environments such as alkali-fermented food "Hongeohoe" in Korea and laboratory enrichment culture for alkaliphiles. To a greater or lesser extent, many microorganisms can be classified into these four environmental categories. It will be important to know the differences in actual status and dynamics of bacterial flora between natural and artificial environments, and global material circulation and symbiotic systems for application of microbial ecosystems to benefit the human society. However, it is obviously difficult to understand these differences by exhaustively considering all bacteria. In this case also, the study of a limited category of alkaliphilic bacteria will simplify the analysis. If we present a more concrete proposal of a study of the above-mentioned problem on the four categories of bacterial habitats, clarification of 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 interesting to considerer why alkaliphilic bacteria distribute not only in alkaline environments but also in conventional garden soil. To solve such a problem, it is important to accumulate data on the characteristics of corresponding species from different habitats. Targeting alkaliphilic bacteria will be useful from the microbial ecological and physiological points of view.

Considering the different characteristics in terms of cytochrome content, cell wall composition and fatty acid composition of alkaliphiles, as well as alkaline adaptation mechanisms of alkaliphiles may differ among bacterial species or strains (Aono and Horikoshi 1983; Clejan et al. 1986; Yumoto et al. 1997). In addition, it is considered that a certain diversity of alkaline

adaptation mechanisms may exist among various bacterial species or strains. Whole-genome analysis was completed in several strains of alkaliphiles (Takami et al. 2000, 2002). It is expected that diversities and origins of specific physiological functions of alkaliphiles will be clarified by selection of strains based on phylogenetic position and estimation of gene expressions of physiologically important proteins and biological materials.

Environmental Distributions

Hyperthermophiles and psychrophiles are adapted to specific environments and cannot grow under conventional laboratory conditions used for the growth of common microorganisms such as *Escherichia coli* (i.e., atmospheric pressure of 37°C, moderate ionic strength and neutral pH). These extremophiles are distributed in their specific environments in nature. In general, although there are several exceptions, major populations of extremophiles are adapted to their specific environments and they are distributed in their specific environments. Compared with such extremophiles, alkaliphiles are unique because typical alkaliphiles (e.g., alkaliphilic *Bacillus* spp.) usually exist in conventional environments such as gardens and agricultural soil and manure (Horihoshi 2006; Nielsen et al. 1995). Alkaliphilic microorganisms are also present in high pH environments such as naturally occurring alkaline environments, including soda lakes (Borsodi et al. 2005; Jan-Roblero et al. 2004; Jones et al. 1998; Joshi et al. 2008; Rees et al. 2004), underground alkaline water (Roadcap et al. 2006; Takai et al. 2001; Tiago et al. 2004), relatively small alkaline niches such as intestines of insects (Broderick et al. 2004; Thongaram et al. 2003), and artificial alkaline environments such as indigo fermentation liquor (Nakajima et al. 2005; Takahara and Tanabe 1960; Yumoto et al. 2004b, 2008) and alkaline wastes formed as by-products of food-processing industries (Collins et al. 1983; Nakamura et al. 2004; Ntougias and Russel 2000, 2001; Yumoto et al. 2004a).

Soil Samples

Numerous strains of alkaliphilc *Bacillus* spp. have been isolated from soil samples (Horikoshi 2006; Yumoto 2007). In addition to the isolation of *Bacillus* spp., several strains of alkaliphiles, *Alkalibacillus* spp. (Fritze 1996; Jeon et al. 2003) and *Paenibacilus* spp. have also been isolated (Lee et al. 2002; Yoon et al. 1998). The genus *Paenibacillus* belongs to a family different from that of the genus *Bacillus* (*Paenibacillaceae*). Indeed, alkaliphiic *Bacillus* spp. can easily be isolated from ordinary garden soil using a conventional medium containing 1% Na_2CO_3 as reported by Horikoshi (2006). Alkaliphilic *Bacillus* spp. have been isolated not only from alkaline soils but also from soils of neutral pH. The reason why alkaliphiles exist in soils other than alkaline soils has not been clarified yet, although there are several possibilities. During nitrogen circulation, the decay of proteins and the hydrolysis of urea by microorganisms may lead to localized alkalization in the soil. Another possible explanation for the localized alkalization in the soil is the production of alkaline substances by alkaliphiles and/or other microorganisms. For example, Horikoshi (2006) reported that some alkaliphiles produce extracellular alkaline substances that provide a favorable pH to their ambient environment. Another reason for the universality of alkaliphilc *Bacillus* spp. in soil may be their ability to form spores. Alkaline adapted *Bacillus* spp. living in a vast or small alkaline environment may be scattered in the soil, for example, by dust storms. Indeed, alkaline soils are very common

around the world. The pH of alkaline soils is around 10 or even higher. In addition, some of alkaliphiles can adapt to neutral pH also; thus, facultative alkaliphilic *Bacillus* spp. are distributed in a wide range of soil types compared with obligate alkaliphiles. Generally, most of the alkaliphilic *Bacillus* spp. are facultative alkaliphiles, and the population of obligate alkaliphiles is small among isolates from an ordinary soil sample.

Felske et al. (1999) estimated the predominant bacteria in Dutch grassland soils by 16S rRNA gene targeting PCR amplification using directly extracted DNA from the soils as the template, and compared them with the most abundant culturable bacteria. Although the pH of the media used for isolation of strains was 7 or 4, a strain exhibiting the highest similarity (94.8%) in the 16S rRNA gene with the alkaliphile *B. cohnii* was isolated. In this study, comparison of the culture-dependant approach and culture-independent approach by 16S rRNA gene sequence analysis of clone library on the basis of extracted DNA from the same soil samples showed no correlation between the collection of cultured strains and the 16S rRNA gene clone library. Therefore, it is not clear whether a bacterial strain related to *B. cohnii* is the predominant population in soils.

Garbeva et al. (2003) estimated predominant *Bacillus* spp. in Dutch agricultural soil under different culture management regimes using genus *Bacillus*-targeting 16S rRNA gene clone libraries. In total, 128 clones (42–43 clones per treatment) were analyzed. Although the soil sample was slightly acidic (pHs 5.5–6.5), 3 clones that exhibited 99% similarity with alkaliphilic *Bacillus halodurans* were detected. It is considered that only major groups of *Bacillus* spp. were detected in this experiment. Therefore, it is suggested that *B. halodurans* is the major population among genus Bacillus in the soils used in this study.

To isolate halophilic bacteria from soil, samples taken from 360 places were collected and spread on agar plates containing 20% NaCl with pH adjusted to 5.0, 7.0, and 9.0 (Echigo et al. 2005). The samples were from ordinary garden soil, yards, and roadways in areas surrounding Tokyo, Japan. Twenty-seven alkaliphilic strains were isolated out of 176 isolates. These isolates were tentatively identified according to their similarity in the 16S rRNA gene sequence as follows: *Alkalibacillus haloalkaliphilus* (Jeon et al. 2005) (formerly *Bacillus haloalkaliphilus* [Fritze 1996]) *Filobacillus milosensis*, *Gracillibacillus halotolerans*, *Halobacillus trueperi*, '*Bacillus nitriophilus*,' and '*Planococcus psychrotoleratus*.' However, their similarities are not sufficiently high (87.3–97.2%) to classify them into their corresponding species except *A. haloalkaliphilus*. Therefore, most of the isolated alkaliphiles are unknown species. Among the isolated alkaliphiles, four strains of an isolate exhibit 100% similarity and six strains exhibit 98.0–99.8% similarity with *A. haloalkaliphilus* (formerly *B. haloalkaliphilus*). *A. haloalkaliphilus* is a spore-forming obligate alkaliphile and is strictly an aerobe. The genus *Alkalibacillus* belongs to the same family as the genus *Bacillus* (family: *Bacillaceae*).

There are soils containing a substantial concentration of sodium carbonate among the dominant soluble salts in their upper profile such as soda or sodic, soda solonchaks. It is considered that such places are unique extreme habitats for microorganisms characterized by high alkalinity. Such soils are distributed in dry steppe and semidesert areas such as southwestern Siberia, northeastern Mongolia, north China in Central Asia, Egypt in Africa, India, Hungary in Europe, and North American steppes. A bacterial strain was isolated by enrichment with isobutyronitrile as the sole carbon, energy, and nitrogen source at pH 10 from soda solonchak soils obtained from southwestern Siberia (Sorokin et al. 2008a). The isolate was an obligate alkaliphile (pH range of growth between 7 and 10.2 [optimum, 9]) and a moderately salt-tolerant bacterium, and was proposed as a new species called *Bacillus alkalinitrilicus*. On the other hand, another alkaliphilic bacterium was isolated from northeastern Mongolia using

a nitrogen-free alkaline medium at pH 10 (Sorokin et al. 2008b). The isolate was able to fix dinitrogen gas and grew on a nitrogen-free alkaline medium, and was proposed as the species named *Bacillus alkalidiazotrophicus*. The isolate was also an obligate alkaliphile with a pH range for growth between 7.8 and 10.6 (optimum, 9.5).

On the basis of the studies of the isolation and characterization of alkaliphilic bacteria, it seems that most of the alkaliphiles living in soils belong to the genus *Bacillus*. However, many as yet undetermined genera or species still exist in the environment, as shown by the above-mentioned study on halophilic alkaliphiles.

Soda Lakes

Microbial diversities of soda lakes in Africa, Eurasia, and North America have been investigated by DNA clone analysis by amplification of DNA and isolation of microorganisms from environments (Borsodi et al. 2005; Duckworth et al. 1996; Humayoun et al. 2003; Jan-Roblero et al. 2004; Jones et al. 1998; Joshi et al. 2008; López-García et al. 2005; Mesbah et al. 2007; Rees et al. 2004). Results of those studies indicate that soda lakes are habitats of diverse microorganisms belonging to the following: Cyanobacteria, Actinobacteria, Firmicutes, Verrucomicrobiales, Proteobacteria (including alpha, beta, gamma, and delta subdivisions), Bacteroides, Verrucomicrobia, Spirochaetes, Chloroflexi, Thermotogae, and candidate divisions of bacteria.

It is considered that East African soda lakes are the most stable and productive naturally occurring environments, with pH generally higher than 10 and occasionally reaching 12 (Jones et al. 1998). This is due to the almost unlimited supply of CO_2 combined with high ambient temperature and strong daily light. The primary production in soda lakes are mainly supported by a dense population of Cyanobacteria. The density of bacterial members is remarkably constant at about 10^5 cfu ml^{-1}. The environmental conditions of soda lakes are due to a combination of geological, geographical, and climatic factors. Soda lakes are characterized by the presence of large amounts of Na^+, Cl^-, and $HCO^{3-}/CO_3{}^{2-}$ as the major ions in solution. Eleven strains associated with the genus *Bacillus* were isolated in the study of phylogenetic diversity in east African soda lakes. Three strains belonged to rRNA group 6 (Nielsen et al. 1995; Yumoto 2007) of the genus *Bacillus* and are close but not identical to obligately alkaliphilic *Bacillus pseudofirmus* that was isolated from soil and manure (*Fig. 2.3.2*). The remaining eight strains belonged to rRNA group 7 (Nielsen et al. 1995; Yumoto 2007), among which, two strains in a distinct subgroup are related to facultatively alkaliphilic *Bacillus saliphilus* (Romano et al. 2005). The remaining 6 strains are less related to known species. Isolates in rRNA group 7 are diversified also in phenotypic characteristics. Strains related to *B. pseudofirmus* seem to predominate in shoreline mud and dry foreshore soda soil that are subjected to fluctuating conditions of alkalinity and salinity with seasonal changes in water level. On the other hand, the strain in rRNA group 7 exhibiting a high Na^+ requirement for growth appear to be more prevalent in lake water and sediments where conditions are less changeable. No alkaliphilic *Bacillus* strains that were the same species from soil samples were isolated from soda lakes. This finding suggests that the origins of alkaliphiles are different. From the analysis of anaerobic environments of soda lakes, ten strains were isolated (Jones et al. 1998). Among them, seven strains were related to *Clostridium* "cluster XI." They formed a group distinct from that of previously reported obligatory anaerobic alkaliphiles. They do not require NaCl for their growth. Other isolates are haloalkaliphiles requiring 12–16% (w/v) NaCl for their growth. Although they are closely related to *Clostridium* "cluster VI" among the

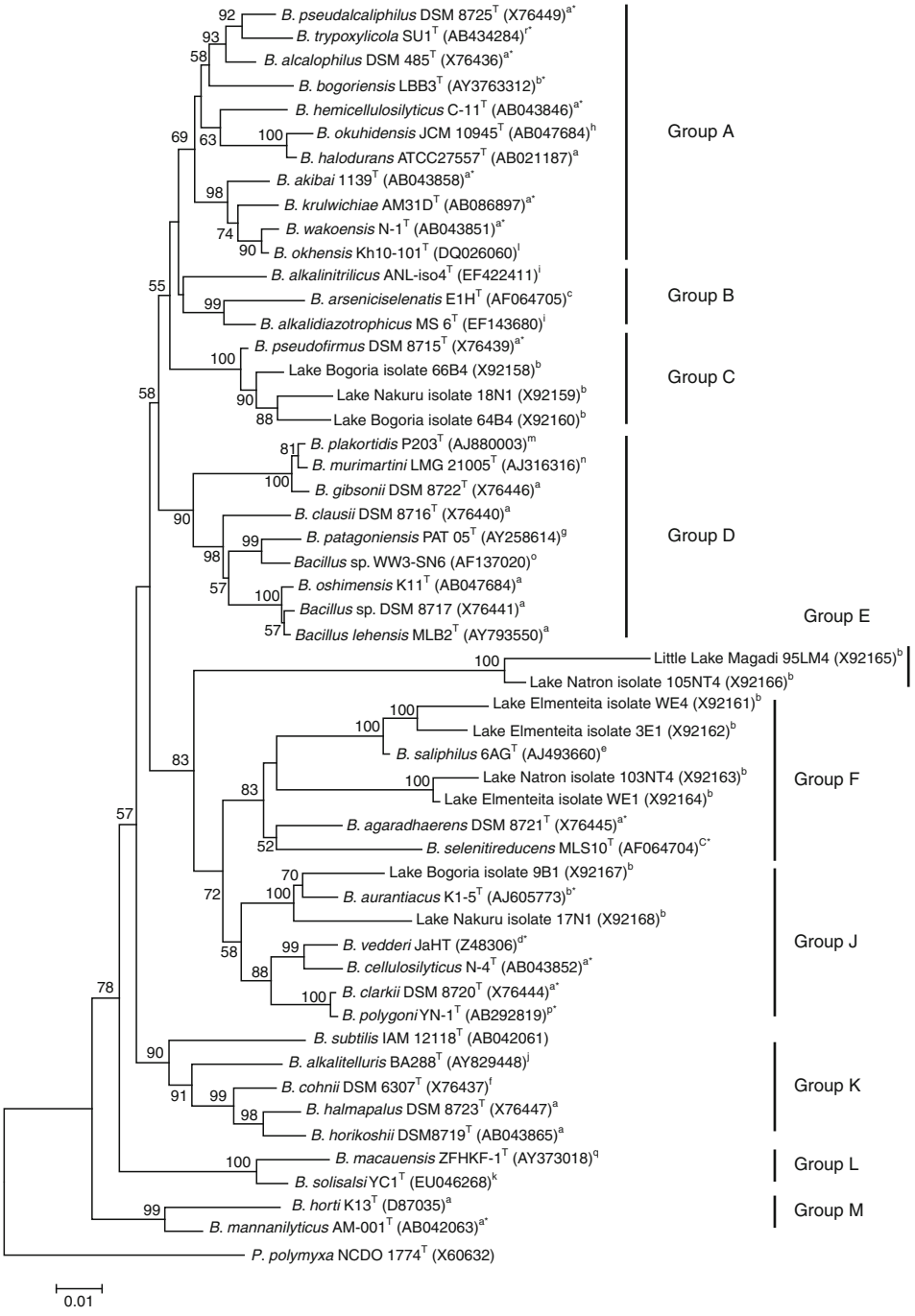

Fig. 2.3.2 (Continued)

reported species, they formed a separate position in the phylogenetic tree constructed on the basis of the 16S rRNA gene. Therefore, they represent a new genus of obligately anaerobic haloalkaliphiles. DNA was extracted from water and sediment samples taken from soda lakes of the Kenyan-Tanzanian Rift Valley and subjected to denaturing gradient gel electrophoresis (DGGE) on the basis of the 16S rRNA gene (Rees et al. 2004). Eight genes related to the phylum Firmicutes were detected. Three genes were related to *B. pseudofirmus* and four genes were related to *Clostridium* spp. One gene exhibited a low similarity with a reported sequence (81.4% identity to *B. halodurans*). These results suggest that most of the genes were reflected in the results of culture-dependent detection, but some of the strains corresponding to genes exhibiting a low similarity with reported sequences have not been isolated.

Mono Lake is an alkaline (pH 9.8), hypersaline (84–94 g liter^{-1}) soda lake located east of the Sierra Nevada Mountains, California, USA (Humayoun et al. 2003). The lake has strong geochemical gradients, a simple food web, and a simple microbial community. Therefore, it is suitable for studying how bacterial populations function to facilitate biochemical processes. Anoxic bottom water at a high pH with prolonged meromixis has high levels of toxic inorganic compounds such as sulfide and ammonia. These conditions may influence microbial activity and flora. In addition, the environment has high concentrations of dissolved organic carbon (DOC), dissolved inorganic phosphorus and standing corps of bacteria. These findings suggest that understanding the reasons for the situation of soda lakes will help clarify the consequence of the microbial loop in the ecology. This study revealed the depth-dependent distribution of microbial diversity in the lake. Bacterial sequences belonging to the phylum Firmicutes were detected in 19% of 212 detected gene sequences. None of the obtained sequences were closely related to any of the 16S rRNA gene sequences in the gene database. Apart from the data on soda lakes in east Africa, no sequence similar to those of *Bacillus* spp. was detected. Twenty-seven sequences formed an independent separate lineage. The remaining sequences exhibited following similarities: two sequences were distantly related (86%) to the obligate anaerobe *Anaeobranca bogoriae*; two sequences were distantly related (85%) to the obligate anaerobe *Natronoanaerobium salstagnum* isolated from Lake Magadi, Kenya; and two strains were distantly related (83%) to facultatively anaerobic *Enterococcus* spp. Only the last two sequences were obtained from the sample collected from the shallowest part.

The microbial diversities of the Indian soda lake Lonar Crater Lake have been studied by molecular analyses and culture-dependent isolation (Wani et al. 2006; Joshi et al. 2008). It is postulated that the lake originated as a meteorite impact crater around 50,000 years ago on the

Fig. 2.3.2

Phylogenetic tree derived from 16S rRNA gene sequences of alkaliphilic *Bacillus* spp., constructed using the neighbor-joining method. *Bacillus subtilis* IAM 12118^T and *Paenibacillus polymixa* NCDO 1774 are used as the representative strain of rRNA group 1 (Ash et al. 1991) and the outgroup of the phylogenetic tree, respectively. The two strains are not alkaliphiles. Origin of alkaliphiles: a, soil; b, soda lake; c, bottom sediment of soda lake; d, bauxite-processing red mud tailing pond; e, algal mat from mineral pool; f, soil and horse meadow; g, rhizosphere of shrub; h, hot spa; i, soda soil; j, sandy soil; k, saline soil; l, saltpan; m, sea; n, mural paint; o, alkaline effluent olive plant; p, indigo fermentation; q, influent of a water treatment plant; r, larval guts of beetle. The asterisks exhibit obligate alkaliphiles. GenBank/EMBL/DDBJ accession numbers are given in parentheses. Numbers indicate bootstrap values greater than 500. Bar: 0.01 K_{nuc}

basis of geological studies. It is the third largest crater in the world. In addition, it is the only known crater formed by a meteoric impact in basaltic rock. Water entered into the lake through rain, ground water seepage, and springs suited in the cliffs at the edge of the lake. The water in the lake is alkaline (pHs 10–10.5) and this high pH is due to the high concentration of sodium carbonate. The lake does not contain any industrial seepage. Therefore, it is intriguing to understand the microbial diversity in this soda lake. DNA was extracted from environmental samples, the 16S rRNA gene was amplified by PCR, and the 16S rRNA gene clone library was constructed (Wani et al. 2006). Five hundred clones were randomly selected for the microbial diversity analyses. Forty-four of unique phylotypes were obtained after restriction fragment length polymorphism (RFLP) analysis and subsequent sequencing. Among them, 34% (15 clones) of the clones were classified as Firmicutes as the major constituent in this analysis. The 15 clones were classified into 5 groups as follows: 2 clones were related to the genus *Alkalibacterium*; 5 clones were not affiliated with any known taxa and formed a cluster with 5 other uncultured bacteria from hypersaline and hydrothermal vent environments; 2 clones were distantly related to *Alkaliphilus crotonoxidans*, whereas 1 clone exhibited a maximum similarity with *Natronoincola histidinovorans*; 1 clone was related to *Anoxynatronum sibiricum*; and 2 clones were related to *B. pseudofirmus*. These findings indicate that this soda lake environment contains aerobic bacteria, facultative anaerobes, obligate anaerobes, and an uncultured cluster. Culture-dependent investigation for clarification of bacterial diversity of Lonar Crater Lake was also carried out. One hundred and ninety-six strains were isolated using different enrichment media (Joshi et al. 2008). Out of the 196 strains, 64 were selected at first selection considering the pH and salt tolerances of the isolates. Then, 31 strains were finally selected on the basis of their enzyme profile and were further subjected to phylogenetic analysis. The result indicated that the classification of the selected bacterial strains could be done as follows: 21 strains belonging to Firmicutes, 3 strains to Actinobacteria, 1 strain of α-Proteobacteria, 1 strain to β-Proteobacteria, and 5 strains to γ-Proteobacteria. The phylum Firmicutes contains the following genera: *Bacillus*, *Paenibacillus*, *Alkalibacillus*, *Exiguobacterium*, *Planococcus*, *Enterococcus*, and *Vagococcus*. Although several isolates were related to reported alkaliphilic *Bacillus* spp. such as *B. cohnii* (96% similarity) and *B. horikoshii* (99%), some of the strains were highly related to species reported to be not alkaliphiles such as *Bacillus cereus* (1 strain, 99% similarity; 1 strain, 100% similarity), *Bacillus benzoevorans* (99%), *B. firmus* (99%), *Enterococcus casseliflavus* (100%), and *Bacillus fusiformis* (100%). Apart from the strains from soda lakes in east Africa, only two strains were isolated and identified as obligate alkaliphiles and they do not belong to the phylum Firmicutes.

Gut of Insects

Termites harbor abundant microorganisms in their gut. The first proctodeal segment of the gut in higher termites has a high pH (i.e., pHs 10–12) and is rich in K^+. Thongaram et al. (2003) screened alkaliphilic bacteria from the first proctodeal region of higher termites by isolation of alkaliphilic bacteria and analysis of alkaliphilic *Bacillus*-targeting 16S rRNA gene clone libraries. The samples used in the study were soil-feeding, soil/wood-interface, wood-feeding, and unknown-feeding higher termites. Twenty-one alkaliphilic bacterial strains were isolated. Among the isolates, 20 strains were found to be affiliated with the genus *Bacillus* by phylogenetic analysis based on 16S rRNA gene sequences. The remaining one strain was affiliated with the genus *Paenibacillus* and the closest relative was *Paenibacillus lautus* among the approved

species. The isolates affiliated with the genus *Bacillus* was classified into 6 groups clustered with reported 16S rRNA gene sequences as follows: eight strains with *Bacillus* sp. DSM 8717 (*Bacillus oshimensis* among approved species), six strains with *Bacillus gibsonii* or *B. horikoshii*, two strains with *Bacillus clausii*, two strains with *Bacillus horti*, one strain with *Bacillus halmapalus*, and one strain with *Bacillus* sp. N−1 (*Bacillus alcalophilus* among approved species). Although the bacterial flora in the termite gut has been studied extensively by clone analysis, very few *Bacillius* clones have been detected. Therefore, a new primer was developed for detecting particularly *B. clausii*, *B. gibsonii*, *B. horikoshii*, *Bacillus patagoniensis* (formerly *Bacillus* sp. DSM 8714), and two alkaliphilic isolates from the termite gut. From the results, 31 DNA clones were found to be related to the above-mentioned targeted species, *Bacillus* sp. DSM 8717, and two isolates in this study. Although the clone analysis showed several clones not belonging to the genus *Bacillus*, the obtained sequences were related to the 16S rRNA gene sequence of the isolates from the same origin. Many isolates grew better in an alkaline medium containing K_2CO_3 than in that containing Na_2CO_3. Although the pH of the gut of higher termites is high, most of the isolates are facultative alkaliphiles.

The bacterial community in the gypsy moth larval midgut has been investigated by culturing, PCR amplification, and terminal restriction fragment length polymorphism (T-RFLP) (Broderick et al. 2004). The midgut of the gypsy moth is typically pHs 8–10, but can reach pH 12. The identification of PCR-amplified genes revealed that most of the obtained genes were identified as belonging to γ-Proteobacteria and Fermicutes and that the cultured isolates represent more than one-half of the phylotypes identified. The sequences indentified as Firmicutes include mainly the genera *Enterococcus* and *Staphylococcus*. In addition, the genera *Bacillus* and *Paenibacillus* were also included. Although several new bacterial phylotypes are identified in the gypsy moth midgut, their overall diversity is lower than that in the termite gut.

The above results indicate that life style and food strongly affect the bacterial community of the insect gut.

Groundwater

Analysis of the diversity of microbial communities in extremely alkaline (pH > 12) ground-temporary water owing to reaction with the fill from the Lake Calumet area of Chicago, Illinois, where historic dumping of steel slag has occurred in a wetland, was performed by 16S rRNA gene targeting PCR amplification by directly extracting DNA from samples (Roadcap et al. 2006). Many of the sequences are related to those from the soda lakes or cement-contaminated groundwater of high pH in gold mines. These are related to *Alkaliphilus*, *Natronoincola*, *Bacillus*, and *Anaerobranca*. However, these exhibit certain phylogentetic distances from known alkaliphiles. Therefore, there are possibilities of isolation of many new species from this environment. However, isolation of bacteria has not been attempted. Hence, characteristics of bacteria living in the groundwater are as yet unknown.

Sea and Sea-Related Samples

Generally, most of the isolates from seawater are Gram-negative bacteria. However, it has been reported that Gram-positive bacteria exist in mud or sediment at the bottom of the sea. Although it seems that soil samples do not exhibit a high pH, Ivanova et al. (1999) reported

that strains belonging to Gram-positive alkaliphilic *Bacillus* were isolated from samples of marine origin. They isolated 20 strains of aerobic endospore-forming bacilli from samples of marine origin obtained from different areas of the Pacific Ocean. A group of four alkaliphiles were tentatively identified as a facultative alkaliphile, *B. horti*, from soil in the sea. Takami et al. reported that a halotolerant and facultative alkaliphile, *Oceanobacillus iheyensis*, has been isolated from deep-sea sediment collected at a depth of 1,050 m on the Iheya Ridge of the Nansei Islands in Japan (27°44.18′N, 126°54.15′E) (Lu et al. 2001). The isolate is a strictly aerobic and spore-forming bacterium.

Marine non spore-forming lactic acid bacteria, *Marinilactibacillus psychrotolerans* and *Halolactibacillus halophilus*, have been isolated from living and decomposing marine organisms (Ishikawa et al. 2003, 2005). They are facultative anaerobes. *Marinilactibacillus psychrotolerans* and *Halolactibacillus* spp. are facultatively alkaliphilic bacteria. Halophilic and alkaliphilic lactic acid bacteria have been isolated from various cheeses produced in Europe (Ishikawa et al. 2007). These alkaliphiles are possibly of marine origin because sea salt is added to cheeses. The identification based on the 16S rRNA gene sequence of the isolates revealed that they belong to the genus *Marinilactibacillus* or *Alkalibacterium*. These microorganisms may contribute to the ripening of cheeses. Recently, ten new species of bacteria belonging to the genus *Alkalibacterium* were isolated from decaying marine algae, decaying seagrass, raw fish, salted fish, and salted and fermented shrimp paste (Ishikawa et al. 2009). Although previously reported *Alkalibacterium* spp. are all obligate alkaliphiles, these sea-related isolates are all facultative alkaliphiles. These finding indicate that alkaliphilic lactic acid bacteria are widely distributed in the sea and sea-related environments.

The above reports indicated that most of the alkaliphiles that are distributed in the sea and sea-related samples are facultative alkaliphiles.

Indigo Fermentation Liquor

The above-described habitats of alkaliphiles are natural environments. Indigo fermentation liquor is an artificial environment in which alkaliphilic microorganisms thrive. Indigo fermentation liquor has been produced from a Japanese indigo plant (*Polygonum tinctorium*) by two steps of processing by microorganisms. Harvested indigo leaves are first air-dried and appropriately wetted for the subsequent processing by microorganisms. In this process, the temperature of a pail of *Polygonum* leaves reaches about 55°C. It is very difficult to control the appropriate water content by adding of water and stirring to maintain the appropriate activity of microorganisms. In this process, the noncolored substance (indican) turns into blue indigo. This fermented *Polygonum* agglomerats into an indigo ball for convenience of transfer. The produced indigo ball is further processed by microbial reduction under alkaline conditions (pH above 10), at which insoluble indigo is converted into the soluble form "lueko indigo," by the action of microorganisms. This microbial reduction process can be substituted by chemical reduction using dithionite. The fermentation procedure for reducing indigo declined around 1960 in Japan because it takes a much longer time than chemical reduction and has difficulties in maintenance. Recently, traditional methods have been re-evaluated because they yield better products in terms of color than do chemical reduction.

First an indigo-reducing bacterium has been isolated by Takahara and Tanabe (1960) and proposed as a new species called *B. alkaliphiles* (1962). Although the strain is not available presently, it exhibited optimum growth at pHs 10–11.5 and requires a peptide consisting of

seven amino acids, which is contained in the fermentation liquor, for its propagation. The fermentation liquor inoculated with the isolate is reduced state, whereas the control, i.e., not inoculated with the isolate, took 168 h to be reduced. Since it is considered that a reducing agent is necessary for chemical reduction, Padden et al. (1999) isolated a novel thermophilic indigo-reducing bacterium, *Clostridium isatidis*, from a woad vat as an alternative. However, the bacterium is alkali tolerant according to the definition mentioned above.

Indigo-reducing obligately alkaliphilic bacteria, *Alkalibacterium psychrotolerans*, *Alkalibacterium iburiense*, and *Alkalibacterium indicireducens* have been isolated from indigo fermentation liquor (Nakajima et al. 2005; Yumoto et al. 2004, 2008). They are facultative anaerobes and produce lactic acid. They share several common characteristics, whereas they exhibit different substrate utilization and growth characteristics. It is considered that there are several types of indo reducing bacterium and probably more than three types of species and they support the reduction of indigo by interacting with each other and with non-indigo-reducing bacteria. Spontaneously occurring microorganisms reduce indigo in the actual fermentation process. Indigo-reducing microorganisms probably come from the indigo ball. Actually, the pH becomes higher in the first *Polygonum* fermentation process owing to ammonia production during this process. However, determination of further upstream origin of indigo-reducing microorganisms is difficult because they are obligate alkaliphiles. Alklaliphilic bacteria other than indio-reducing microorganisms exist in the vat for indigo fermentation. The microflora involved in indigo fermentation, and the relationship between indigo-reducing bacteria and other factors are not known. This interaction may be very important for the maintenance of the fermentation fluid.

Other Environments

Alkaliphiles are distributed not only in indigo fermentation liquor but also in other artificial environments such as effluents from an agricultural or fishery processing plants. The environments of effluents are different from those of indigo fermentation in size and existence of continuous inflow of water from the upstream of facility. The first example of an alkaliphile isolated from an effluent from agricultural plant is *Exiguobacterium aurantiacum* (Collins et al. 1983). The strain was isolated from potato-processing plant effluent. On the other hand, *Exiguobacterium oxidotolerans* T-2-2^T was isolated from fishery processing plant effluent (Yumoto et al. 2004a). The latter isolate was not isolated from alkaline effluent. Although there are not so many examples, it is suggested that strains belonging to the genus *Exiguobacterium* are distributed in effluents from agricultural or fishery processing plants. Facultatively alkaliphilic *Bacillus* sp. WW3-SN6 was isolated from alkaline effluent derived from the preparation of edible olives (Ntougias and Russel 2000). An alkaline solution containing NaOH was used to precipitate a bitter compound of the fruit. The isolate is related to alkaliphilic *B. patagoniensis* that was isolated from the rhizosphere of the perennial shrub *Atiplex lampa* (Oliver et al. 2005). In addition, *Alkalibacterium olivoapovliticus* was also isolated from alkaline effluent derived from the preparation of edible olives (Ntougias and Russel 2001). Other species belonging to the genus *Alkalibacterium* are isolated from indigo fermentation liquor as described above. These findings suggest that strains belonging to the genus *Alkalibacterium* are related to plant environments. In addition, the facultative alkaliphile *Amphibacillus xylanus* was isolated from compost of grass and rice straw (Niimura et al. 1990). These findings suggest that certain alkaliphilic bacteria are related to substances from

plants. Alkaliphilic Firmicutes have been isolated from animal manure. Strains of obligately alkaliphilic *B. pseudofirmus* have been isolated from deer and ostrich manure, and strains of facultatively alkaliphilic *B. halodurans* have been isolated from chicken, tiger, pigeon, and elephant manure (Nielsen et al. 1995). Strains of facultatively alkaliphilic *B. cohnii* were isolated from old horse feces. These findings suggest that certain alkaliphilic *Bacillus* spp. are related to animal manure or feces.

In addition to the reports described above, *Oceanobacillus oncorhynchi* was isolated from the skin of rainbow trout (*Oncoehynchus mykiss*), suggesting that certain alkaliphiles exist in the skin of fishes. The above examples suggest that alkaliphilic Firmicutes species are distributed in effluents of agricultural or fishery processing plants, rhizospheres of plants, manure of animals, and feces and skin of fish (Yumoto et al. 2005b). However, this wide distribution of alkaliphilic Firmicutes microorganisms cannot be accounted to only their spore-forming ability because some of the microorganisms described above do not have this ability.

Taxonomy of Isolated Alkaliphilic Firmicutes

The taxonomic distribution of alkaliphilic Firmicutes based on the approved species is listed in *Table 2.3.1*. The taxonomic distribution becomes wider than that of 10 years ago and it is expected that it will become wider in the next decade. This means that alkaliphiles distribute in a wide range of taxonomic positions. In this review, we mainly focus on the genus *Bacillus* and consider the relationship between characteristics and phylogenetic position, because until now, a lot of bacterial strains belonging to the genus *Bacillus*, which exhibit various phylogenetic positions determined on the basis of the 16S rRNA gene sequence, have been isolated from various of environments. These strains include both facultative and obligate alkaliphiles.

Genus Bacillus

Over 40 alkaliphilic *Bacillus* species had been approved by the end of 2009. The characteristics of 41 approved species belonging to alkaliphilic *Bacillus* are listed in *Table 2.3.2*. They are listed in order of serial number of G+C content of DNA. Alkaliphilic *Bacillus* share G+C contents of 33.8–42% and 48.4–53.8%. The most frequent source of isolation is soil. They distribute in not only common places such as soil, sea, and feces but also specific environments such as hot spa, sea anemone, and insect gut. Most grow in the mesophilic temperature range of 10–40°C. *Bacillus okuhidaensis* strains grow in the themophilic temperature range of 30–60°C. Most of them grow in the pH range of 7–10, 8–10 or 11. However, some of them grow in a particular pH range such as *Bacilllus solisalsi* (growth pH range of 5–13). The summarized characteristics of alkaliphilic *Bacillus* spp. indicate that they distribute not only in alkaline environments but also in conventional environments. The results suggest that the diversity of alkaliphilic *Bacillus* spp. is not defined only by bacterial habitats and various as yet undiscovered species distribute in various environments. On the other hand, this is quite surprising that one kind of extremophile exhibits this wide range of distribution. These characteristics are only found in alkaliphilic *Bacillus* spp. among the various genera of extremophiles. This means that alkaliphilic *Bacillus* spp. are quite common microorganisms rather than rare microorganisms in various environments on earth.

Table 2.3.1

Taxonomic distribution of alkaliphiles in phylum Firmicutes

Phyrum	Class	Order	Family	Genus
Firmicutes				
	Bacilli			
		Bacillales		
			Bacillaceae	
				Alkalibacillus *Amphibacillus* *Anoxybacillus* *Bacillus* *Caldalkalibacillus* *Cerasibacillus* *Halolactobacillus* *Natonobacillus* *Oceanobacillus* *Salsuginibacillus* *Terrabacillus*
			Paenibacillaceae	
				Paenibacillus
			Planococcaceae	
				Sporosarcina *Planococcus*
			Staphylococcaceae	
				Salinicoccus
			Bacillus Family XII Incertae Sedis	
				Exiguobacterium
			Unclassified Bacillales	
				Alkalilactobacillus
			Unclassified Bacilli	
				Desulfurispirillum
		Lactobacillales		
			Carnobacteriaceae	
				Alkalibacterium *Marinilactibacillus*
			Enterococcaceae	
				Enterococcus *Vagococcus*

Table 2.3.1 (Continued)

Phyrum	Class	Order	Family	Genus
	Clostridia			
		Clostridiales		
			Clostridiaceae	
				Alkaliphilus *Anoxynatronum* *Natronincola* *Tindallia*
		Natranaerobiales		
			Natranaerobiaceae	
				Natranaerobius
			Clostridialses Family XIV Incertae Sedis	
				Anaerobranca
			Eubacteriaceae	
				Alkalibacter

A phylogenetic tree constructed on the basis of the 16S rRNA gene sequence of 39 approved species of alkaliphilic *Bacillus* spp. and isolates from soda lakes is shown in *Fig. 2.3.2*. Most of the alkaliphilic *Bacillus* spp. are approved species and isolates from soda lakes belong to a similar phylogenetic position and form several groups. They are classified into rRNA group 1, 6 and 7 (Ash et al. 1991; Nielsen et al. 1994; Yumoto 2007). In this review, we would like 516 to propose a new classification of alkaliphilic Bacillus spp., as described in *Fig. 2.3.2*. Group A contains at least 8 species belonging to obligate alkaliphiles out of 11 species. Most of them were isolated from soil. It is considered that although most of them prefer high-pH environments, they are widely distributed in soil. This is attributed to their localization in alkaline environments and the limited scale of alkaline niches may exist widely in soil environments, and/or soil environments may share localized common characteristics with localized alkaline environments such as the intestine of insects. Actually, *Bacillus trypoxylicola* belonging to this group has been isolated from larval guts of the Japanese beetle. Group B consists of only facultative alkaliphiles isolated from soda soil or bottom sediments of soda lakes. The presented results suggest that bacteria belonging to this group are distributed only in limited environments. Group C contains only one approved species, obligate alkaliphilic *B. pseudofirmus*. This species is distributed in common environments such as soil, animal manure, and fresh lake water. It is expected that this group consists of obligate alkaliphiles, because the other three strains were isolated from vast and permanently alkaline environments, namely, African soda lakes. Group D contains 9 species of facultative alkaliphiles. The origins of six species are soil or soil-related environments. *Bacillus plakortidis* has been isolated from the Norwegian Sea. *Bacillus murimartini* and *Bacillus* sp. WW3-SN6 have been isolated from mural paintings and alkaline effluent, respectively. These findings suggest that this group of bacteria is distributed not only in vast natural environments but also in artificial environments. The above-described groups A–D belong to rRNA group 6 (Nielsen et al. 1994; Yumoto 2007). Obligate alkaliphiles belong to only groups A–C and all the species in group D are facultative alkaliphiles.

Table 2.3.2

Characteristics of alkaliphilic *Bacillus* spp.

Species	Isolated location	pH range (opt.[a]) (pH)	Temp. range (opt.) (°C)	Aerobe or anaerobe	Other characteristics	G+C content of DNA (%)	References
Bacillus chonii	Garden soil, indigo ball, horse faces, horse meadow, feces, contamination in agar plate	7–10 (9)	10–47	Aerobe	Grows at 7% NaCl	33.8–35.0	(Nielsen et al. 1995; Ohta et al. 1975; Spanka and Fritze 1993; Yumoto et al. 2000)
Bacillus alkalinitrilicus	Soda solonchak soils	7–10.2 (9)	15–41 (32)	Aerobe	Utilizes aliphatic nitriles, grows at 0.1–1.5 M NaCl	35.1	(Sorokin et al. 2008a)
Bacillus akibai	Soil	8–10 (9–10)	20–45 (37)	Aerobe	Grows at 7% NaCl	34.4	(Nogi et al. 2005)
Bacillus alcalophilus	Soil, feces, soda lake	8–10 (9–10)	10–40 (ND)	Aerobe	Grows at 8% NaCL	36.2–38.4	(Nielsen et al. 1995; Nogi et al. 2005; Vedder 1934)
Bacillus agaradhaerens	Soil	8–11 (10 or above)	10–45 (ND)	Aerobe	Grows at 16% NaCl	36.5–36.8	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus hemicellulosilyticus	Soil	8–11 (10)	10–40 (37)	Aerobe	Grows at 12% NaCl	36.8	(Nogi et al. 2005)
Bacillus alklidiazotrophicus	Soda soil	7.5–10.6 (9.0–9.5)	15–43 (33–35)	Aerotolerant anerobe	0.1–1.2 M Na^+ (optimum 0.2–0.4 M)	37.1	(Sorokin et al. 2008b)
Bacillus mannanilyticus	Soil	8–10 (9)	20–45 (37)	Aerobe	Grows at 3% NaCl	37.4	(Nogi et al. 2005)
Bacillus bongoriensis	Soda lake	8–11 (10)	10–40 (37)	Aerobe	Nonmotile	37.5	(Vargas et al. 2005)
Bacillus trypoxylicola	Leval guts of the Japanease horned beetle	8–10 (9)	ND (30)	Aerobic	Nonmotile	37.4–37.7	(Aizawa et al. 2010)

Table 2.3.2 (Continued)

Species	Isolated location	pH range (opt.[a]) (pH)	Temp. range (opt.) (°C)	Aerobe or anaerobe	Other characteristics	G+C content of DNA (%)	References
Bacillus alklitelluris	Sandy soil	7.0–11.0 (9–9.5)	15–40 (30)	Facultatively anaerobe	0–4% NaCl	37.9	(Lee et al. 2008)
Bacillus wakoensis	Soil	8–11 (9–10)	10–40 (37)	Aerobe	Grows at 10% NaCl	38.1	(Nogi et al. 2005)
Bacillus pseudoalcalophilus	Soil	8–10 (about 10)	10–40 (ND)	Aerobe	Grows at 10% NaCl	38.2–39.0	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus vedderi	Bauxite-processing red mud tailing pond	Opt. temp. about 10	maximum temp. for growth 40–50	Aerobe	Grows at 7.5% NaCl	38.3	(Agnew et al. 1995)
Bacillus pseudofirmus	Soil, animal manure, Fresh lake water	8–10 (about 9)	10–45 (ND)	Aerobe	Grows at 16% NaCl	38.7–40.9	(Nielsen et al. 1995)
Bacillus halmapalus	Soil	7–10 (about 8)	10–40 (ND)	Aerobe	No growth at 5% NaCl	38.6	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus oshimensis	Soil	7–10 (ND)	13–41 (28–32)	Aerobe	Grows at 0–20% NaCl, nonmotile	39.1–40.8	(Nogi et al. 2005; Yumoto et al. 2005a)
Bacillus cellulosilyticus	Soil	8–10 (9–10)	20–40 (37)	Aerobe	Grows at 12% NaCl	39.6	(Nogi et al. 2005)
Bacillus murimartini	Mural painting from a church	7–10 (8.5)	10–30 (15–20)	Aerobe	Grows at 0–4% NaCl	39.6	(Borchert et al. 2007)
Bacillus patagoniensis	Rhizosphere of shrub	7–10 (8)	5–40 (ND)	Aerobe	Grows at 15% NaCl	39.7	(Olivera et al. 2005)
Bacillus neizhouensis	Sea anemone	6.5–10 (8.5)	4–30 (25)	Aerobic	Grows at 0.5–10% NaCl	39.8	(Chen et al. 2010)

Bacillus horti	Soil	7–10 (about 10)	15–40 (ND)	Aerobe	Grows at 10% NaCl	40.2–40.9	(Yumoto et al. 1998)
Bacillus arsenicoselenatis	Bottom sediment of soda lake	7.5–10 (ND)	ND	Obligately anaerobe	Nonmotile, respiratory growth with Se(VI), As (V), Fe(III), nitrate, and fumarate	40.0	(Switzer Blum et al. 1998)
Bacillus marmarensis	Mushroom compost	8.0–12.5	ND	Aerobic	Catalase positive, oxidase negative	40.2	(Denizci et al. 2010)
Bacillus krulwichiae	Soil	8–10 (ND)	20–45 (ND)	Facultatively anaerobe	Grows at 0–14% NaCl	40.6–41.5	(Yumoto et al. 2003)
Bacillus gibsonii	Soil	8–10 (ND)	10–37 (ND)	Aerobe	Grows at 9% NaCl	40.6–41.7	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus macauensis	Influent of a water treatment plant	6.0–10.0 (8.5)	20–40 (30)	Facultatively anaerobe	Grows at 5–10% NaCl	40.8	(Zhang et al. 2006)
Bacillus okhensis	Saltpan	7–10 (9)	25–40 (37)	Aerobe	Grows at 0–10% NaCl	40–42	(Nowlan et al. 2006)
Bacillus lehensis	Soil	7.0–11.0 (8)	10–37 (25)	Aerobe	Grows at 0–12% NaCl	41.4	(Ghosh et al. 2007)
Bacillus solisalsi	Saline soil	5–13 (7–10)	15–53 (35–42)	Facultatively anaerobe	Grows at 15% NaCl	41.8	(Liu et al. 2009)
Bacillus okuhidaensis	Hot spa	6.0–11.0 (ND)	30–60 (ND)	Aerobe	Grows at 10% NaCl	41.0–41.1	(Li et al. 2002; Nogi et al. 2005)
Bacillus plakoridis	Sea	6.5–10 (8.5)	4–30 (15–20)	Aerobe	Grows at 0–12% NaCl	41.1	(Borchert et al. 2007)
Bacillus horikoshii	Soil	7–10 (ND)	10–40 (ND)	Aerobe	Grows at 8–9% NaCl	41.1–42.0	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus polygoni	Indigo ball	8–12 (9)	5–47 (29–30)	Aerobe	Grows at 3–14% NaCl	42.9	(Aino et al. 2008)
Bacillus aurantiacus	Extremely shallow, alkaline soda lakes	8.0–12.0 (9.5–10.0)	10–45 (28)	Aerobe	3–7% NaCl	42.9	(Borsodi et al. 2008)

Table 2.3.2 (Continued)

Species	Isolated location	pH range (opt.[a]) (pH)	Temp. range (opt.) (°C)	Aerobe or anaerobe	Other characteristics	G+C content of DNA (%)	References
Bacillus clausii	Soil, broiler gastrointestinal tract, wastewater, abalone	7–10 (about 8)	15–50 (ND)	Aerobe	Grows at 10% NaCl	41.7–43.5	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus halodurans	Soil, animal manure, bird manure	7–10 (ND)	15–55 (ND)	Aerobe	Grows at 12% NaCl	42.1–43.9	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus clarkii	Soil	8–11 (ND)	15–45 (ND)	Aerobe	Grows at 16% NaCl	42.4–43.0	(Nielsen et al. 1995; Nogi et al. 2005)
Bacillus saliphilus	Algal mat from mineral pool	7–10 (9.0)	4–50 (37)	Aerobe	Grows at 1–25% NaCl	48.4	(Romano et al. 2005)
Bacillus selenitireducens	Bottom sediment of soda lake	8–11 (ND)	ND	Facultatively anaerobe	nonmotile, reducing selenite	49.0	(Switzer Blum et al. 1998)
Bacillus chagannorensis	Soda lake (Lake Chagannor)	5.8–11.0	6–40 (37)	Facultatively anaerobe	Grows at 3–20% NaCl	53.8	(Carrasco et al. 2007)

ND: no data; opt.[a] = optimum

Two strains belonging to group E have been isolated from soda lakes in Africa. Until now, no approved species in group E has been reported. Therefore, there is no information on the detailed characteristics. Group F includes five strains isolated from soda lakes or environments related to soda lakes. Although growth characteristics of isolates from soda lakes are unknown, it seems that most of the strains belonging to this group are obligate alkaliphiles. The strains belonging to this group are related to water environments except *Bacillus agaradhaerens*. In group J, three strains isolated from soda lakes are located in similar phylogentic positions. On the other hand, other strains are isolated from soil or mud and artificial fermentation product. It is considered that most of the strains belonging to this group are obligate alkaliphiles. Groups F and J belong to rRNA group 7 (Nielsen et al. 1994; Yumoto 2007). Group K contains mostly facultative alkaliphiles that were isolated from soil or soil-related environments. This group corresponds to rRNA group 1 (Ash et al. 1991).

Groups L and M are new groups. Two strains belonging to group L are facultative alkaliphiles. It is understandable that group L is in an isolated phylogenetic position because the two strains belonging to the group were isolated from specific environments. On the other hand, two strains belonging to group M are highly adapted to high pH; both strains were isolated from soil. Strains belonging to group M were isolated from common sources. It is difficult to find the reason why the group exhibits an isolated position in the phylogenetic tree.

Conclusions and Perspective

In the past decade, various alkaliphilic Fermicutes species have been isolated and many species of alkaliphiles have been approved. As described above, alkaliphiles are distributed in various phylogenetic positions in the phylum Fermicutes. Therefore, it is considered that alkaliphiles are not a very particular group of bacteria. Certain alkaliphiles can be isolated from conventional environments. In addition, various alkaliphilic bacteria exist in soil or soil-related environments. However, it seems that specific alkaliphilic bacteria live in particular environments. It is considered that there are certain relationships between phylogentic position and environmental distribution in alkaliphilic bacteria. Actually, it is very difficult to define soil environments because it is considered that several microenvironments exist in a three-dimensional structure and there is no uniformity even in relatively small environments. It can be assumed that there is a mosaic distribution for alkaline environments of a very limited size. Hence, it is considered that a variety of alkaliphiles thrive in such complicated environments that harbor a variety of bacterial niches in a certain size of space. This may be one of the reasons why alkaliphiles exist in ordinary soil.

On the other hand, several certain alkaliphilic species have been isolated from various environments. In such cases, these microorganisms have high adaptability to various environments. Therefore, a wide range of adaptability in certain microorganisms is also one of the reasons for the wide range of distribution of the alkaliphiles. Actually, inserted gene sequences of transposase were more frequently observed only in alkaliphiles than in alkaliphilic *B. halodurans*, *O. iheyensis*, and neutralophilic *Bacillus subtilis* (Takami et al. 2000, 2002). It is assumed that some facultative alkaliphiles may have evolved from neutralophiles while some facultative alkaliphiles may have evolved from obligate alkaliphiles. Actually, several neutralophiles exhibit alkaliphilic characteristics and vice versa. Furthermore, certain alkaliphilic *Bacillus* spp. contain both obligate and facultative alkaliphiles.

In the future, we should further our understanding of the environmental and taxonomic distribution of alkaliphilic bacteria not only by the isolation of new species but also by metagenomic approaches to the study of several environments. In addition, genetic analyses of numerous sites of genes using numerous bacterial strains on the basis of population genetics will contribute to the clarification of the relationship between evolutional process and environmental distribution in alkaliphilic bacteria. Through these approaches we may clarify the ecological function and environmental exchange in the world of alkaliphilic bacteria.

Cross-References

- 2.1 Introduction and History of Alkaliphiles
- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles

References

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2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles

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Introduction

Over the last 10 years, many elegant and exhaustive reviews have been written about alkaliphiles and alkalithermophiles, their environments, diversity, and physiological problems (e.g., Jones et al. 1998; Grant 1992; Zarvarzin et al. 1999; Wiegel 2002; Pikuta et al 2003; Kevbrin et al. 2004; Wiegel and Kevbrin 2004; Sorokin and Kuenen 2005a, b; Krulwich et al. 2007, 2009; Mesbah and Wiegel 2008). What is new in the field are primarily the many, recently described novel alkalitolerant and alkaliphilic microorganisms. They include psychrophiles and extreme thermophiles (Keller et al. 1995). In addition, there is an increase in the number of publications describing the biodiversity by applying noncultural methods to alkaline environments (e.g., Duckworth et al. 1996; Mono Lake: Humayoun et al. 2003; Pikuta et al. 2003; Wadi an Natrun Lakes, Egypt: Mesbah et al. 2007a; Lake Elmenteita, Kenyan Rift Valley: Mwirichia et al. 2010; Lost City site, Pacific: Brazelton et al. 2010; Soap Lake: Sorokin et al. 2007). These analyses demonstrate that the biodiversity in the highly alkaline (pH 10–11) and extreme halophilic environments is much higher than previously expected, and that they harbor a vast amount of novel taxa with low phylogenetic similarities to existing bacterial and archaeal taxa. Several of these sequences even have no close relatives among environmental phylotype from other analyses (Mesbah et al. 2007a). Furthermore, from the BLAST analysis of the environmental 16S rRNA sequences from Lake Elmenteita samples, containing 596 phylotypes (98% similarity cutoff), Mwirichia et al. (2010) observed that cyanobacteria were the most abundant, but that the second group, the *Firmicutes*, contained the highest number of genera. Based on environmental 16S rRNA data and with a 97% similarity cutoff for species differentiation, about 300 bacterial taxa and a little less archaeal taxa were obtained for each of the three studied alkaline lakes (pHs between around 9 and 11) from the Wadi An Natrun region (Mesbah et al. 2007a). Furthermore, analysis demonstrated that the communities from the different lakes were statistically different as well as that the community obtained from the aerobic water column was not a subpopulation from the populations obtained from the anaerobic sediment. The analysis also showed that the later isolated anaerobic haloalkalithermophiles species of *Natranaerobius* and *Natronovirga* (Mesbah et al. 2007) are well represented base on finding several different sequences among the obtained environmental 16S rRNA sequences. In 1998, Jones et al. stated that the anaerobic environment of the soda lakes have received very little attention (Jones et al. 1998). This has recently changed only to some degree and as shown below, the description of anaerobic alkaliphiles are still few compared to published aerobic alkalitolerant and alkaliphilic taxa. Among those recently described are the novel anaerobic halophilic alkalithermophilic *Firmicutes* from the author's laboratory, which represent a novel order, *Natranaerobiales*, within the Class *Clostridia* in the Phylum Firmicutes. Apparently, they are widely distributed in the Wadi An Natrun and the Rift Valley soda lakes based on the number of different sequences obtained from the three WadianNatrun lakes studied (Mesbah et al. 2007a). These novel bacteria are anaerobic polyextremophiles and in their tolerance to the combination of stressors moved further out the combined physic-chemical boundaries within bacterial life exist (Bowers et al. 2009; Bowers 2010). A novel archaeal species is mirroring the bacterial situation for the Archaea (Bowers 2010; Bowers et al., in preparation). Another important and intriguing group of novel anaerobic haloalkaliphiles from Russian soda lakes has been recently described by Sorokin and coworkers (Sorokin and Muyzer 2010a, b, c; Sorokin et al. 2010a, b) and Boldareva (2009) These novel taxa are involved in the sulfur cycle (sulfur reduction, sulfate reduction, sulfidogenesis, thiosulfate metabolism) and thus their description contributes much

to the increasing knowledge about the sulfur cycle in soda lakes. Interestingly no members of the sulfate reducing *Desulfotomaculum* and *Desulfosporosinus* were observed; however, a novel spore forming genus *Desulfitispora* (Peptoccocceae) was obtained. The isolates from Sorokin et al. from the Russian soda lakes differ from the *Natranaerobius* group that they are mesophilic and less halophilic (Na_{opt}^{+} below 1 M) and, in contrast to the heterotrophic *Natranaerobiales*, several are chemolithoautotrophs. Another isolate is a dissimilatory MnO_2 reducer with a pH_{opt}^{RT} of 10 (Sorokin and Muyzer 2010a). The detailed description of the majority of the novel alkaliphiles is not the focus of this review nor is it possible within the frame of this chapter. The physiological diversity is related to the environmental diversity, thus the reader is referred to the original descriptions in the International Journal of Systematic and Evolutionary Microbiology. Neither can the mechanisms to overcome the effect of stressors such as alkaline pH values and concomitant salt and/or elevated temperature be discussed, even though knowledge of these mechanisms is somewhat important in order to understand the distribution and any ecological role these taxa may be playing in the environment. Much research is still needed in order to achieve the full understanding of how these microorganisms can survive and thrive in these – from the human point of view – harsh environments. The reader is referred to recent articles (e.g., Krulwich et al. 2007, 2009; Krulwich 1995; Mesbah et al. 2009 and literature cited therein) for the current understanding. Methods for the isolation and cultivation of anaerobic alkalithermophiles are described in a recently published chapter in Methods in Microbiology (Mesbah and Wiegel 2006). The reader is also referred to the separate Chap. 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles on the predominant aerobic environments and the diversity of aerobic alkaliphiles.

Definitions of Alkaliphiles and Their Ramifications

In order to discuss diversity and ecology of alkaliphiles, one has first to discuss what qualifies a microorganism to be classified as an alkaliphile and what classifies an alkaline environment. In the recent literature, one finds a variety of definitions of what constitutes an alkaliphile. Many microorganisms can grow at pH values up of 8.5–9, without being classified as alkalitolerant or alkaliphiles. There is little disagreement about the existence of the general grouping into alkalitolerant, facultative alkaliphiles, and obligate alkaliphiles, although some would like to add the fourth group, the hyperalkaliphiles, for microorganisms that can grow and multiply at and above pH values of 11. In the narrow meaning, the term "grow" describes only that an increase in biomass occurs, but not necessarily that an increase in cell number occurred; a lack of increase in cell number can occur due to disturbance of the cell division machinery. Subsequently, the term multiply refers to an increase in cell numbers, although it is possible – when cells are transferred from less alkaline media into more alkaline media – that the cell number increases without significant increase in biomass through division of cells into smaller cells as a stress response. For brevity in this chapter, the terms "grow" and "growth" refer to the concomitant increase in biomass and cell number. The author uses the marginal growth/multiplication data of microorganisms for the classification into the above categories (*Table 2.4.1*) with pH_{min} = as the more acidic pH. Probably the most useful values for categorization are the pH_{opt} and pH_{max} data. The numerical marginal data for alkaliphiles vary in the literature; for example, for pH_{min} between 8 and 9.5 depending on the author and time of publication. Krulwich (2000) proposed to use the term alkaliphiles for taxa that grow optimally at

Table 2.4.1

Definition of alkaliphiles according to their marginal data

Category	pH_{min}^{xC}	pH_{opt}^{xC}	pH_{max}^{xC}
Alkalitolerant	–	≤7	≥9.5
Facultative alkaliphile	≤7.5	≥8.5 (9–9.5)	≥10[a]
Obligate alkaliphile	≥7.5	≥8.5 (9–9.5)	*≥10*

[a] A few "alkaliphiles" have a very narrow pH range with a pH-optimum well into the alkaliphilic range, but an upper pH just short of pH 10.

pH^{xC} = the temperature at which the pH was determined and the pH meter calibrated using temperature corrected standard values.

pH above 9.6. This is based on that, to date the observed upper cytoplasmic pH value of all studied alkaliphiles does not significantly exceed an pH value of 9.6 regardless of the external pH value (see discussion about pH max below).

Obviously, there always will be isolates where one of the marginal data will fit the definition data given in *Table 2.4.1* but one of the other two will not. This is to be expected because nature is a continuum, and the more microorganisms are isolated the more exceptions to this artificial categorization will occur, and thus a new definition may be required in the future. Another problem with the definition is that the pH optimum can depend on a particular substrate (Kevbrin et al. 1997) depending on the pathway used. For example, *Morella thermoautotrophica,* when grown on glycerate or autotrophically using the Ljungdahl-Wood pathway or part of it, has a pH optimum around $pH^{25\ C}$ 5.5. However, when grown on glucose and then using the Emden-Meyerhof-Parnass pathway, the optimum for growth is around 7 (Wiegel et al. 1981; unpublished results). Because the author primarily works with (halophilic) alkalithermophiles, the pH_{opt} values given in *Table 2.4.1* might be a little more to the neutrophilic side than some other authors like to use. Thus, alternative values used by others for mesophiles are given in parentheses.

Comparisons of pH values for various types of alkaliphiles and alkaline environmental samples have to be done cautiously. This is due to the strong effect of temperature on the pK-values of components in the media and environment which result in different pH values when measured at different temperatures. In order to obtain more meaningful pH data for alkaliphiles at elevated temperatures or thermophiles at alkaline (or acidic) pH, the pH value should be determined at the corresponding growth temperature with pH meters calibrated at that temperature and the use of temperature adapted (20–30 min) electrodes and values for the calibration standards corrected for the experimental temperature. The difference between using this procedure, for example, for 60°C, and measuring the pH at room temperature with a pH meters calibrated at room temperature can yield differences more than a full pH unit (e.g., see Fig. 1 of Wiegel 1998; Mesbah and Wiegel 2006). It should be noted that this procedure is also only a first approximated correction. Absolute correct determinations require more elaborate physicochemical determinations and mathematical corrections. This is especially important for media and environments with increased ionic strength such as media for halophiles and high ionic strength environments. The situation becomes more complicated if the influence of higher NaCl concentration (>0.5 M) and temperature must be accounted for, for example, when one deals with haloalkalithermophiles or (alkaline) salt lakes heated up by

solar radiation or geothermal activity. The use of electrodes with a liquid junction present in most commercial combined pH electrodes, for measuring pH values in solutions containing high sodium concentrations is physico-chemically incorrect. Knauss and coworkers proposed to avoid liquid junctions and introduced a junctionless measurement of the pH value (Knauss et al. 1990, 1991; Mesmer 1991). The use of such electrodes is not supported by most scientists as such methods would require a revision of the current pH scale. Even modern solid state electrodes do not solve the whole problem.

The addition of sodium chloride to buffers effects the pH measurement similar at elevated temperature. Raising the concentration of NaCl from 0.5 to 5 M, the difference in the measured pH values reached more than a full pH unit (unpublished) even though a linear response of the electrode was observed, in the author's case, up to pH 10. Although it is possible to approximate the true pH value for any conditions, in praxis, these measurements are too time consuming and elaborate and thus not suitable for routine determinations in the laboratory or field. The author, as well as other investigators, have argued that exact pH values are really only required for certain cases such as bioenergetic studies, but that for describing novel taxa and comparative studies and reviews, exact pH values are not that critical. However, it is important to know how the pH values had been determined to avoid misleading conclusions and comparisons. In order to avoid uncertainties, the author introduced (Wiegel 1998) the use of superscripts to indicate the temperature at which the pH meter was calibrated and the measurement done, that is, pH^{xxC}. This notation should prevent that incorrect comparisons are made with noncomparable pH-related data. The preparation of meaningful comparisons is also frequently hampered by missing or incomplete reports of the marginal pH data for novel microorganisms, including alkalitolerant and alkaliphiles. Thus, the author likes to see much more careful analyses and descriptions of the pH-related data for novel taxa.

This problem of incomplete and neglected pH data becomes evident when one goes through the literature, in particlar in the description of novel taxa published in the International Journal of Systematic and Evolutionary Microbiology. A strong plea to pay attention to pH data for alkalipiles was published by Sorokin (2005) in the International Journal of Systematic and Evolutionary Microbiology as a letter to the editor. Frequently encountered problems include (1) measurements done only in 1 pH unit increments; (2) omission of either the range, especially the pH_{max}, or the optimum; (3) data are reporting very broadly and unprecise as "good growth obtained the range of... " without any marginal data figures; (4) length of the incubation times of the cultures are missing, a problem for the slow growth at the range of pH_{min}; the pH_{min} data are from ecological point of view extremely important, whereas the pH max data are of interest for industrial applications and physiological investigations. For a meaningful pH_{min} determination, the cultures should be incubated for about 200-times the shortest observed doubling time, i.e., doubling time at the optimal conditions. In addition, pH dependence is frequently done in batch cultures and without determining doubling times, and without checking the final pH. Sorokin (2005) pointed out some of these pitfalls in determining upper growth values for an alkaliphile but especially for values above pH 10.5. Every person working with true alkaliphiles should read Sorokin's warning before starting to work (Sorokin 2005)! The complete and correct determination of the marginal data involves general, straight forward microbial methods. Unfortunately, these methods are neglected due to the overemphasis on biochemical and molecular properties for the description of novel taxa, despite the request for a truly balanced polyphasic analysis as requested several times over the years by Stackebrandt. Another problem at the higher pH values, frequently the initial pH, is

only present for short time of the growth curve depending on the buffer capacity of the medium. Horikoshi (year) described how a *Bacillus* strain was changing its medium to the optimum pH by alkalinization or acidification. On the other hand, anaerobes are changing the pH of the media by producing CO_2 and copious amounts of organic acids.

Another important data is whether the determination of the pH-dependent growth was done in subsequent subcultures or whether data were obtained and recorded with non-adapted inocula. The experience in the author's lab is that adapted subcultures either show a wider pH range than the first inoculations, or a lower range when a strain has apparently had problems at the higher or lower pH values to produce a growth factor, which then was used up during the first culture. The author used to do determine the doubling times and pH curves determinations in a semi-continuous culture set-up, but due to problems publishing these detailed time-consuming process (pH or T-curves) has changed the determination of the doubling times by measuring the increase in OD_{600nm} only in individual serum bottles with frequent monitoring and pH adjustment.

In summary, classifications of published taxa into the different categories has to be viewed with some reservations.

Distribution of Alkaliphiles: Alkaline Environments Versus Non-Alkaline Environments as Source for Alkali(thermo)philes

It would not be surprising if one would find alkaliphiles (nearly) exclusively in environments with alkaline pH. Surely, alkaline lakes and alkaline soil, urea containing sources, manure piles, ocean waters, and alkaline springs harbor a variety of alkaliphilic microorganisms. However, already in 1982, described by Horiskoshi and Akiba (1982 and lit. cited therein), alkaliphiles have been unexpectedly found nearly everywhere, including from soils with a pH of 4. Subsequently, many alkaliphilic *Bacillus* species have been isolated from neutral or even slightly acidic (pH between 5 and 7.5) soils. An example for the obligately anaerobic alkalithermophiles is *Anaerobranca horikoshii*, which has been isolated from different hot springs with $pH^{55–70C}$ values between 5.5 and 8.2 (Engle et al. 1995).

As shown in recent years again and again, most soil and sediment environments and particle containing water bodies are not homogenous environments, but contain various micro- or even what one could call "nano" niches. In these tiny environments, the pH and temperatures – sometimes only temporary – can be significantly different from the measured "macro" or bulk parameters of that environment. Thus, alkaline environments can harbor small neutral niches due to local microbial acid production and thus support the growth of non-alkaline taxa. Also non-alkaline environments (such as, e.g., ordinary garden soil) will contain alkaline niches that support alkaliphilic and alkalitolerant microorganisms. An exception are the anaerobic haloalkalithermophiles; to date, they have only been isolated from sun- or geothermally heated alkaline salt lakes and alkaline salt crusts as found in Nevada's Black Desert (see citations in Bowers 2010).

However, from the ecological point of view, one not so well-studied issue – although discussed in meetings – arises: If one has isolated, for example, an anaerobic alkaliphile from a non-alkaline environment and perhaps its 16S rRNA sequence is also present in the environmental sequence is then this taxa really significant member of that environment, either in numbers or as an important or even critical member for a microbial food chain? The well-known statement attributed to Beijerinck (cited by Baas Becking 1934) comes

to mind: "Alles is overall" (= everything is everywhere), which then was extended by Baas Becking with "maar het mileu selecteert" (= but the environment selects). To be able to answer whether this statement is true for all environments or not, much more quantitative examination with modern tools are required. This includes the measurement of (stable) isotope incorporations, quantitative PCR with species specific primers, and mRNA analysis to aid carefully carried out culture-dependent enumerations. Furthermore, the outcome will also depend on the definition of what constitutes a "presence" of a taxa. Is a species present in a sediment if, for example, 10 cells or a few dormant spores of a taxa exist among 10^9 cells of other microorganisms? Are non-growing anaerobes in an aerobic environment truly part of the microbial community and what is about the alive but not (yet) culturable microorganisms? Thus, the response to Beijering's/Baas Becking's statements depends on how one answers the above questions and the available detection limits for microbial taxa.

An example for this issue from the author's laboratory is the enumeration of the anaerobic alkalithermophiles *Clostridium paradoxum* and *C. thermoalkaliphilum* (Li et al. 1993, 1994) present in sewage sludge with neutral bulk pH. Dilution rows of anaerobic agars-shake-roll tubes were employed. To date, these two bacterial taxa could only be isolated from sewage sludge and sewage sludge containing compost piles obtained from various continents, but not from hot and even not from the river receiving the outflow of the Athens (Georgia, USA) sewage plant. The recorded bulk temperatures and pH values in the tested sewage plants never reached temperatures of 35°C or above and pH values above 7.8. The enumeration yielded that one g of sewage sludge contained up to 1,000 CFU of these two taxa, based on growth parameters and 16S rRNA sequence analysis of a few isolate.

Are Anaerobic Alkali(thermo)philes Less Prevalent than the Aerobic Ones?

Although the first true alkaliphilic aerobic bacterium *Urobacillus pasteurii* Miquel 1889 → *Bacillus pasteurii* Chester 1898 (Approved Lists 1980) and now*Sporosarcina pasteuri* (http://www.bacterio.cict.fr/s/sporosarcina.html) was isolated more than 100 years ago, it took about 45 years before the next aerobic alkaliphilic *Bacillus* was isolated (Vedder 1934), although several alkalitolerant taxa had been described in the 1920s (see Horiskoshi and Akiba 1982; p. 3 ff). A real interest in alkaliphilic microorganisms arose when Horikoshi and coworkers started around 1969 to isolate alkaliphilic (at that time called "alkalophilic") mesophiles from soil and their alkaline proteases became the interest in 1971 for the industrial application in laundry detergent (Horikoshi 1999 and lit. cited therein). This spurred, especially in Japan, a flood of isolations of alkaliphilic microorganisms, but again predominantly of *Bacillus* and related taxa (see Chap. 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles). An enjoyable reading of this start into "A New Microbial World" – what is now a large and important physiological group of microorganisms – which was published by Horiskoshi and Akiba (1982) and Horikoshi (1991). However, it took many more years before true alkaliphilic anaerobes were isolated and until the mid 1990s before the anaerobic alkalithermophiles were described. The anaerobic polyextremophilic haloalkalithermophiles were described only a few years ago. Presently, one or more novel alkaliphilic and many alkalitolerant taxa are described nearly every month. The recently isolated taxa originated from a wide variety of environments, many of them from alkaline lakes in Asia, various soda lakes, marine sources (water, sediment, and beach samples), alkaline soil, or

anthropogenic alkaline environments such as processed food and food processing waste material. Again, interestingly, most of the alkalitolerant taxa with reported pH_{max} of 10–11 were isolated from non-alkaline environments. However, the overwhelming majority are aerobic taxa. As an illustration, from November 2009 to June of 2010 about 350 novel species validly published in the International Journal of Systematic and Evolutionary Microbiology only 57 were alkalitolerant and 18 alkaliphilic taxa, not counting those effectively published in other journals. From these validly published taxa, only three were thermophiles, only four were obligate anaerobes, and five were facultative aerobes. From the 18 alkaliphiles 14 were facultative alkaliphiles. Interestingly, about 90% of the alkalitolerant had published pH_{max} values of 10 (with pH_{min} around pH 5). Most of them had pH_{opt} values between 6.5 and 7.5 and a few had pH_{opt} around 8–8.5. Thus, many of the novel alkalitolerant exhibit a relative wide pH range over nearly 5 pH units. For several of these taxa the pH range could be even wider if the researcher would have used smaller increments than 1 pH unit in their characterization and would have also – as the author of this chapter suggest – incubation times 100–200 times of the observed shortest doubling time for obtaining the marginal data. This would lead to ecological and physiologically more relevant data. One taxa had a pH range from 5.1 to 12.1 (using increments of 1 pH unit) with a very broad reported pH optimum of 5.1–9.1. The data were obtained from growth after 14 days but no doubling time was reported. In addition, the authors did not address whether they had eliminated the problems regarding determination of growth data at such high pH values, as addressed by Sorokin (2005). The species were isolated in medium at a neutral pH inoculated with soil of a cliff on Mara Island (Republic of Korea) (Seo and Lee 2009). Unfortunately, for the type species of this genus – isolated from the same location – no pH values were provided. Thus it is not known whether the novel isolate is an exceptional isolate or whether it is typical for the strains from this location or for this genus.

Obviously, previous statements in the literature that alkalitolerant microorganisms cannot grow above pH 10 are no longer sustainable as the above examples demonstrate. These examples highlight again the importance of using correctly and carefully determined marginal data. This is especially true for the pH_{opt} to be used for the labeling of what is an alkalitolerant and what is a facultative alkaliphile (*Table 2.4.1*).

The sources for isolation of the anaerobic and facultative aerobic alkalitolerant taxa included salt and soda lakes in China with various pH values, brackish water from a lagoon (pH 8.5) and from river sediment in India, thermophilic (60°C) anaerobic digester from China (yielding a thermophile), fermented sea food from Korea, dialysis instruments and blood samples, and guts from beetle larvae. With few exceptions among the obligate alkaliphiles, the isolation was primarily done in media at neutral pH. Among the anaerobes are an alkalitolerant phototrophic gamma proteobacterium from the Baitarani River in India (with pH values varying between 6.5 and 9), an alkaliphilic nonsulfur purple bacterium (pH range for growth 7.5–10 with an optimum at 8.5) from a soda lake in central Asia isolated at pH 8.5–9, and the obligate sulfur and dissimilatory nitrate reducing bacterium *Desulfurispirillum alkaliphilum*, gen. nov. sp. nov., enriched at pH 8 –10 (Sorokin et al. 2010a, b) growing optimal at pH 9, 0.5% NaCl and a pH range above 7.9–10.2.

The isolation of the overwhelming number of aerobes could suggest that among the alkaliphiles on Earth the aerobic taxa dominate. The author believes that this is probably more an effect of the falsely perceived notion by investigators that aerobic microorganisms are so much easier to isolate and to characterize than anaerobes. Thus, the discrepancy in the ratio of described anaerobic and aerobic alkaliphiles is an artifact of the isolation procedures. If anaerobic conditions and more alkaline pH values would be used more frequently in isolation attempts,

many more anaerobic alkaliphiles would be discovered. Furthermore, the use of elevated temperature should probably also yield much more anaerobic alkalithermophiles. With 99% of the perceived existing microorganisms being unknown, many of those probably residing in intestinal tracts of varies animals including a myriad of insect species, one can speculate that these environments will harbor many anaerobes including anaerobic alkaliphiles. The isolation of the alkalithermophiles from the author's laboratory shows that one can readily isolate anaerobic alkalithermophiles from various environments as well as the anaerobic. haloalkalithermophiles from the sun-heated or geothermally heated alkaline athalassohaline salt lakes. To date, anaerobic haloalkali thermophiles have not been isolated from or observed in thalassohaline or non-alkaline haline environments.

The Importance of Temporary "Nanoniches" for the Distribution of Alkaliphiles and Alkalithermophiles in Non-Extreme Environments

It is a common trend for the deliberate isolation of alkaliphiles to use samples from alkaline environments. In order to specifically obtain anaerobic alkalithermophiles, the selective conditions of anaerobic growth media with pH >8.5 and incubation temperatures >50°C should be applied regardless of the inoculation source. The number of alkaliphilic microorganisms in neutral "ordinary" soil samples varies from 10^2 to 10^5 per gram of soil, which corresponds to 1/10 to 1/100 of the population of the neutrophiles (Horikoshi 1991). As discussed above, it is assumed that soil has a highly heterogeneous structure with microniches ideally suitable for a coexistence of different physiological types of microorganisms. The transient alkalinity important for growth of alkaliphiles is established by means of various processes, including microbial-mediated processes like ammonification, nitrate respiration, sulfate reduction, or oxygenic photosynthesis. Another mechanism to establish transient alkaline niches is through alkaline feces, which can raise the pH locally while also distributing the microorganisms present in the feces. In the author's laboratory, an interesting observation was made regarding the importance of microniches for the biodiversity and distribution of anaerobic alkalithermophiles. Among the anaerobic alkalithermophiles, those isolated from geothermally heated sources had much longer doubling times (i.e., more than 25 min) than those isolated from the mesobiotic environments (river sediments, wet meadow soil). The later isolates generally had doubling times less than 20 min and as short as 10 min. This relationship was especially evident for many isolated strains of the widely distributed *Thermobrachium celere* (T_{opt} around 66°C and pH_{opt} around 8.6 or above). The strains from mesobiotic sources had doubling times between 10 and 15 min whereas those from thermobiotic environments had doubling times between 25 and 40 min. The fast growth rates observed for this taxa under the double extreme growth conditions are nearly twice of the fastest rate for *Escherichia coli* under normal laboratory conditions. The author's hypothesis is that in mesobiotic environments the conditions for decent growth will occur only in the temporary micro("nano")niches and thus the microorganisms have to respond extremely fast in order to propagate. In contrast, the strains growing under thermobiotic conditions do not experience such environmental selection pressure for a fast growth rate. They only need to grow fast enough not to be washed out of the hot spring pools.

Although a well-proven correlation between habitat of isolation and observed fastest growth rates under laboratory conditions requires the characterization of many more taxa, the above hypothesis from the isolated anaerobic alkalithermophiles is intriguing.

However, the data demonstrate that the temporary micro("nano")niches are very important for the diversity and distribution of anaerobic alkalithermophiles.

Correlation Between Biogeochemistry and Biogeography of Anaerobic Alkalithermophiles (A Hypothesis)

Based on the to date isolated anaerobic alkalithermophiles (excluding the haloalkalithermophiles) it appears that they can be categorized into three major groups according to their observed geographical distribution and the geochemistry of the environment from which they have been isolated. Some alkalithermophiles are found:

1. Only in one place (= restricted biogeography) but with a relaxed biogeochemistry/physiology requirement, e.g., *A. horikoshi,* (Engle et al. 1995) found only in one place within Yellowstone National Park but in both alkaline and acidic springs at various temperatures;
2. In one type of environment (= restricted biogeochemistry/physiology) but from locations on different continents (= relaxed biogeography), for example, *C. paradoxum* and *C. thermoalkaliphilum* (Li et al. 1993, 1994) found only in sewage plants or hot springs receiving grey water;
3. In many different kinds of places and locations, that is, ubiquitous species (= relaxed biogeochemistry/physiology and relaxed biogeography), for example, *Bacillus* spec. and *T. celere,* (Engle et al. 1996), which have been isolated from alkaline hot springs, mesobiotic lake and river sediments, wet meadow grounds, and manure.

In conclusion, more isolates need to be obtained and characterized. But from the presently available data about the environment from where alkalithermophiles have been isolated one can conclude that the group (but not all individual taxa) of the anaerobic alkalithermophiles per se is ubiquitous in respect to geographical distribution and various environments.

Physicochemical Boundaries for Growth of Anaerobic Alkalithermophiles

Sorokin (2005) asked whether "there is a limit for high-pH life." He pointed out that for growth in media with pH above 12 the intracellular pH would be at least about 10 or even higher. This is based on the observation that in aerobic taxa the pH difference across the cytoplasmic membrane is about 2 pH units. In anaerobic alkalithermophiles the delta pH across the membrane is less than 1.5 pH, usually around 1 pH, given optimal conditions (Cook et al. 1996; Mesbah et al. 2009). To date, an intracellular pH value of 10 has not been observed in any aerobic taxa. The upper observed values are around pH 9.6 in aerobic and mesophilic taxa (Krulwich 1995; Krulwich et al. 2009). The exceptionally high pH value of around 10 at an external pH value above 10, measured in energized and non-energized cells of *Natranaerobius thermophilus*, are contributed to inherent cytoplasmic buffering and not to the maintenance of a delta pH across the membrane through the action of electrogenic sodium/potassium-proton antiporters; Even though one of the antiporters works at a pH value higher than 9.6, that is, up to pH 10 with its optimum of activity at pH value of 9.5 (Mesbah et al. 2009). At this time it is not known whether it functions in vivo at intracellular pH above 9.6. *N. thermophilus* exhibited

an unusual constant delta pH of 1 pH unit over the whole growth pH range and even beyond. It is unclear at this time whether or not this is a general property of anaerobic haloalkalithermophiles. Similar data have also been obtained for the other tested polyextremophilic species of Natranaerobius, including the temperature tolerant and the extreme thermophilic (T_{max} 75°C) species *N.* "*grantii*" and *N.* "*jones*ii," respectively (Bowers 2010).

The optimal and maximal pH values for growth of anaerobic alkalithermophiles and polyextremophiles are lower than those of simple alkaliphiles. It is assumed that this is due to the effect of additional stressors such as elevated temperatures and/or salt concentrations. Based on the comparison of the to date isolated anaerobic thermophiles, it appears that the higher the optimal growth temperature is, the lower are the pH_{opt} and pH_{max} and vice versa (Wiegel and Kevbrin 2004). Apparently, the physico-chemical conditions for life are much lower when several extreme growth conditions are combined. The most alkaliphilic alkalithermophile is *C. paradoxum* with a pH_{opt}^{55C} of 10.3 and a Topt of 54–58°C. The anaerobic alkaliphiles with the highest T_{opt} are *Thermococcus alkaliphilus* (Keller et al. 1995) and *T. fumicolans* (Godfroy et al. 1996) with a T_{opt} of 85°C but with a $pH_{opt}^{?C}$ of only 9 and 8.5, respectively. This trend is even more pronounced for the polyextremophiles. A comparison of halophilic alkalithermophiles (Bowers et al. (2009) revealed that out of all the validly published extremely halophilic bacteria for which the marginal data were published, only 19 species have pH optima of 8.5 or greater. Of these, only 10 species combine an elevated pH optimum with a [Na+] optimum of 2 M or greater. The most prevalent combinations are pH optimum 9 with a [Na+] optimum of approximately 1.7 M. The next frequent combinations are a pH_{opt} 7 and 8 with [Na+] optimum of approximately 1.7 M. However, the most intriguing result is that the taxa with the combinations of the most alkaline pH, highest temperature, and salt tolerance are the anaerobic *Natranaerobiales* and not the aerobic taxa (Bowers 2010).

Alkaline Athalassohaline Environments

As pointed out, alkaline water and soil environments exist all over the world and thus are too numerous to be described in any detail in this chapter. Although the water bodies of alkaline and soda lakes are usually (micro) aerobic, the sediments are anaerobic. Many of the alkaline soda lakes are only a few meters deep, but in some instances the deeper water layers can also become anoxic due to microbial activity such as depletion of oxygen through respiration or due to geochemical reactions of oxygen with microbially produced sulfide. In short, they all can be sources for isolating anaerobic alkaliphiles (see below).

Alkaline environments fall into several relatively broad categories based on the nature of the processes generating the alkaline conditions. These processes have to occur continuously in order to maintain long-lasting alkaline environments. The alkalinization can be based on microbial-catalyzed or on geochemical reactions. In addition to the permanent alkaline environments, there are the temporary (micro) environments, discussed above. Jones et al. (1994) published examples of alkaline environments with their ionic compositions. Grant (2006) nicely summarized and discussed two major groups of the permanent alkaline geochemistry-based environments: the high Calcium /Magnesium ions- and the carbonate-dominated environments. The detailed geochemical processes sustaining the alkaline conditions were described by Hardie and Eugster (1970). Another interesting alkaline environments based on geochemical processes are the weathered bauxite-containing soils with pH values up to and above 11. Bauxite is formed in weathered volcanic rocks and is a mixture of various

aluminum hydroxides, oxides, and silicates. Although bauxite is an extremely important mining commodity, little information is known about the associated microbial communities in weathered bauxite soils, associated clays, and abandoned mining sites.

Briefly, *the Ca^{2+}-based environments* are primarily due to weathering of earth-alkali containing Ca-Mg silicates. Usually, most of the Mg^{2+}-ions are precipitated as hydroxides or carbonates, but enough Ca^{2+}-ions remain available in order to form Ca $(OH)_2$ containing alkaline brines. Although the soluble concentration of Ca $(OH)_2$ is usually only in the mM range, this process can lead to pH values above 12. At the place of origin, the brines are anaerobic and become only (micro) aerobic when they emerge to the aerobic surface or are mixed with aerobic meteoric (surface) water, and thus, they still contain anaerobic alkaliphiles. In respect to hot springs, there are generally much fewer alkaline (pH around 8.5–10) springs than acidic hot springs with pH of 0 to 5 where frequently the low pH is formed through oxidative processes of sulfur compounds or geothermally vented SO_2.

The carbonate-dominated environments, especially the widely and naturally occurring athalassohaline soda lakes and soda flats with pH values of 10–11.5, are regarded as the most stable high-pH environments on Earth. They are numerous but, as already pointed out, from most of them only aerobic and facultative aerobic taxa were isolated so far. These environments usually are characterized by high NaCl and Na-carbonate concentrations and occur due to evaporation of either surface waters or geothermal springs or a combination of both. The surface water can originate from rivers or from precipitations or in few instances from magmatic water high in carbonate. Examples of river water-based lakes are the Wadi An Natrun lakes in North Egypt. They are formed in depressions (29 m below sea level) with no outlets. The water of the river Nile percolates over a distance of about 100 km through the ground into the depression, where the water than evaporates. There are at least seven of those sada lakes, plus a few seasonal lakes. The depression is possibly part of an ancient riverbed. The salt concentration increases from the northeast side of the lakes to the south side of the lakes due to the fresh water flowing into the lakes primarily from the northeast side.

Examples of alkaline environments formed from precipitations are the alkaline lakes (in spring and winter) and salt flats (in the summer and fall) in the Black Desert and adjacent Grabens of Nevada and East California (USA), also without outlets to the oceans. They were formed when the North American Continental plate extended. The precipitation percolates through the rocks of the original plateau and collects at the bottom of the Grabens. Moving from west to southeast in Nevada, hot springs become more alkaline. In addition, many smaller alkali-flats are found in Oregon. The percolating waters leach minerals out of the rocks and sands, which are usually high in Na^+-ions but low in Ca^{2+}- and Mg^{2+}-ions containing silicates. Evaporation then leads to the accumulation of alkali-cations, primarily Na^+, frequently together with Cl^- and carbonate anions, sometimes with some sulfates and other anions. Thus, many alkaline soda lakes are also high haline environments. The alkaline lakes of the East African Rift (Ethiopia, Kenia, Tansania) and alkaline environments due to geothermal activity, magmatic or circulating waters depleted of the earth-alkali elements, lack especially Ca^{2+} and Mg^{2+} and frequently are high in carbonate.

Ammonium-dominated environments include various naturally and many human activity-based environments and are due to nitrogen containing material, which upon degradation releases ammonium (ammonification). Manure piles on various types of animal farms will yield alkaline environments even with elevated temperatures. Frequently, they are only temporary alkaline environments.

Athalassohaline Environments for Isolation of Anaerobic (Halo) alkalithermophiles

The large, warm soda lakes with high concentrations of sodium carbonates (up to saturation) and with pH values as high as 10–11 and temperatures up to 60°C and higher at the origins of the hot springs are good sources for isolation of anaerobic alkalithermophilic microorganisms. This is especially true for samples from the upper zones of the sediment and the water zone above the sediment – frequently the region between a predominant anaerobic zone and the microaerophilic water zone. The African soda lakes, which are heated by tropical sun and in part feed by underground warm alkaline inlets (Mwaura 1999) represent relatively stable water bodies in a geological time scale. A variety of alkaliphiles including several anaerobes were isolated and characterized from these lakes (Grant et al. 1999; Jones et al. 1998; Zarvarzin et al. 1999; Matin et al. 2001; Milford et al. 2000; Bowers 2010). The laboratory of the author isolated several anaerobic alkalithermophiles from Lake Magadi (Bowers 2010). Milford et al. (2000) isolated the purple non-sulfur bacterium *Rhodobaca bogoriensis* from lake Bogoria. Another example is the North American Mono Lake with a hot spring, although most of the lake is mesobiotic. The Asian soda lakes have more profound continental mode of climate with drastic season changes (Zarvarzin et al. 1999) and are less studied with respect to anaerobic alkalithermophiles although many aerobic and some facultative aerobic alkalitolerant and alkaliphiles were described from these lakes. Recently, however, Sorokin isolated various novel anaerobic alkaliphiles from the hypersaline soda lakes in the Kulunda Steppe (Altai, Russia) (Sorokin and Muyzer 2010a, b, c; Sorokin et al 2010a, b and lit. cited therein). Kompantseva (2010) demonstrated that *Rhodovulum steppense* sp. nov., an obligately haloalkaliphilic purple nonsulfur bacterium, is widespread in saline soda lakes of Central Asia. Several of the other *Rhodovulum* species where isolated from marine environments. Imhoff et al. (1979) and Mesbah et al. (2007a) demonstrated that the soda lakes at Wadi An Natrun contain a rich microbial flora. Mesbah et al. (2007b) and Mesbah and Wiegel (2009) isolated anaerobic haloalkalithermophiles (*Natranaerobius* sp. and *Natronovirga* sp.). Similar to other soda lakes, the Wadi An Natrun lakes contain purple non-sulfur bacteria. They are widespread in the water and sediment of soda lakes, possibly because many of them can switch between photoheterotrophic, photoautotrophic and/or chemoheterotrophic growth depending on the degree of anaerobiosis in the lake and light availability.

Other athalasso environments for isolating anaerobic alkalithermophiles are municipal sewage processing plants and animal manure piles (or also manure-derived compost) with – due to ammonification and urea degradation – alkaline conditions. Sewage plant samples were the source of the first anaerobic alakalithermophiles isolated in the author's laboratory. As discussed above, during microbial decomposition of protein-bearing pollutions, alkaline pHs can easily occur in the sludge granules due to ammonification. Anthropogenic alkaline environments include also industrial waste streams from cement producing and distributing facilities, food processing and textile manufactories (including indigo dye balls) and from tanneries.

Alkaline Thalassohaline Environment

The pH of modern ocean water is around 8.3. The ocean, a rich reservoir for microorganisms (Whitman et al. 1998), is usually not seen as an alkaline reservoir for alkaliphilic

microorganisms. In fact, many marine bacteria, especially anaerobes, have a pH optimum below pH 8.3. Marine salterns harbor many aerobic alkalitolerant halophiles. To date, no anaerobic alkalithermophiles have been isolated from normal thalassohaline environments including from sun-heated saltern.

The Great Salt Lake, originated in geological times from an ancient ocean, is presently a hybrid between an athalassohaline and a thalassohaline environment in that it contains ancient ocean water but also much of the salt-containing runoff waters from the surrounding mountains. Interestingly *Natranaerobius* sequences, absent in thalassohaline salterns were also absent from this environment (B.Baxter, pers.comm.), and a first isolation attempt in the author's laboratory yielded no similar anaerobic haloalkalithermophiles but only mesophilic and moderately halophilic alkaliphiles including a novel species *Amphibacillus* "*cookii*" (Benoit, unpubl. data). An exciting thalassohaline environment are the deep-sea hydrothermal vents at Lost City, a serpentinite-hosted hydrothermal field with carbonate chimneys 30–60 m tall, pH values around 9 to 11, and temperatures up to 90°C. Lost City is located about 15 km west of the Mid-Atlantic Ridge. It harbors a rich hydrothermal field-associated macrofauna similar to other hydrothermal vents such as the black smoker vents (Kelley et al. 2005; Brazelton et al. 2006). However, the high biomass of chemolithoautotrophic microorganisms is apparently missing. According to Kelley et al. (2005), reactions between seawater and upper mantle peridotite produce methane- and hydrogen-rich fluids that support inside of the active venting chimneys an archaeal biofilm dominated by a single, unique phylotype of methanosarcinales (LCMS phylotype). They are apparently adapted to this site with thermal fluids high in methane and hydrogen. A "low diversity of microorganisms related to methane-cycling Archaea" (Kelley et al. 2005) has been observed. Some *Thermococcus* strains have been isolated from Lost City, but so far not many anaerobic alkalithermophilic taxa. However, a large sequence project (sequences of >200,000 amplicons of the 16S rRNA V6 region) was carried out in order to determine the diversity of this site. This led to an opportunity for a unique project (Brazelton et al. 2010). The sequence data were correlated with isotopic (^{230}Th) age determinations of the individual chimneys. Lost City has existed for at least 30,000 years and thus has chimneys of various ages side by side. A 16S rRNA sequence analysis inside the porous chimneys revealed that sequences rare in the younger chimneys are becoming more dominant in the older ones, demonstrating a development of specific microbial communities over more than 1,200 years. As Brazelton et al. (2010) stated, the "results suggest that a long history of selection over many cycles of chimney growth has resulted in numerous closely related species at Lost City, each of which is pre-adapted to a particular set of reoccurring environmental conditions." This to date unique site will yield in the near future many more interesting results on the biodiversity, selection of species, and the dynamic of microbial communities in thermoalkaline carbonate rich environments.

Concluding Remarks

In recent years, researchers have given more attention to anaerobic alkaliphiles and have started to elucidate the diversity and the geochemistry of their alkaline environments, using molecular and more sophisticated analysis techniques.

However, the author encourages strongly the microbial community, especially those working with alkaliphiles to focus much more on the anaerobic community. The knowledge

of the anaerobic component of any microbial community is important in order to understand the ecology and geochemical and microbial interactions in the alkaline environments on earth and perhaps also for the question of possible life in extraterrestrial environments. For this, it is mandatory that researchers pay much more attention to the determinations of the pH-ranges and pH-optima for growth of isolated taxa as pointed out above by Sorokin (2005). As expressed at other places (e.g., Bowers 2010), the author encourages other researchers to investigate the presence of the anaerobic alkalithermophiles and especially the polyextremophilic halo-alkalithermophiles in other places than done so far in order to investigate whether or not the properties of these novel isolates can extend the presently known life-limiting combinations of physico-chemical parameters to higher extremes. Still, other questions to be answered include: (1) what is the most alkaline pH that allows continuous growth (i.e., demonstrated in a continuous and pH-controlled culture experiment) (2) whether there is a difference in the maximal tolerated pH for growth between aerobic and anaerobic microorganisms, and (3) whether there is indeed a reverse correlation between the extend of the T_{opt}/T_{max} and pH_{opt}/pH_{max} among the alkalithermophiles. Thus, a lot of exiting basic and molecular research with taxa of the microbial group of anaerobic alkaliphilic extremophiles lies ahead.

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2.5 General Physiology of Alkaliphiles

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Generally, alkaliphiles require alkaline environments and sodium ions not only for growth but also for sporulation and germination. Sodium ion-dependent uptake of nutrients has been reported in some alkaliphiles. Many alkaliphiles require various nutrients, such as polypeptone and yeast extracts, for their growth; several alkaliphilic *Bacillus* strains (*Bacillus halodurans* C-125, A-59, C-3, and AH-101) can grow in simple minimal media containing glycerol, glutamic acid, citric acid, etc. One of the best strains for genetic analysis is alkaliphilic *B. halodurans* C-125 and its many mutants have been made by conventional mutation methods. Whole genome sequence was determined and annotated in 2000 (Takami et al. 2000).

Extracellular pH Values

Alkaliphilic microorganisms are ubiquitous: Many alkaliphilic bacteria and archaea can be isolated more commonly from the earth. Alkalinity in nature may be the result of the geology and climate of the area, of industrial processes, or promoted by biological activities. The most stable alkaline environments on earth are the soda lakes and soda deserts distributed throughout the world. These environments are about pH 10–13. Roadcap et al. (2005, 2006) reported extremely alkaline (pH >12) groundwater in slag-fill aquifers hosts diverse microbial community. They confirmed the presence and growth of a variety of alkaliphilic β-Proteobacteria, *Bacillus*, and *Clostridium* species at pH up to 13.2.

Outside the range of pH permitting growth, pH homeostasis gradually or abruptly fails. Nevertheless, many species can survive (remain viable) for extended periods at pH values outside their growth range, ready to grow again when the pH returns to the optimum.

Some alkaliphilic bacteria can change external pH value to a pH suitable for growth and create their own world. For instance, *B. clausii* 221 (Horikoshi 1971), which is a good alkaline protease producer, can grow slowly at neutral pH, changing the pH of the culture broth. It was found that once the pH reached about 9, the bacteria began to grow rapidly and produced a large amount of the alkaline protease. Ueyama and Horikoshi (unpublished data) isolated an alkaliphilic *Arthrobacter* sp., which utilizes an ε-caprolactam polymer. This microorganism was also capable of changing the broth pH to an optimum (*Fig. 2.5.1*). The author conducted another experiment: The following microorganisms were used in the experiment. (1) *Aspergillus oryzae*, (2) *B. circulans* IAM 1165 (Horikoshi and Iida 1958), which produces an enzyme that lyses *A. oryzae* at pH 7–8.3 and (3) Alkaliphilic *B. pseudofirmus* A-57, which can grow in the range of pH 8 and 10. These three strains were mix-cultured in Horikoshi-II medium of pH 5 in the absence of 1% Na_2CO_3. The cultivation was carried out for a week with continuous shaking at 30°C. *A. oryzae* grew in the medium first. After 3 days incubation, *A. oryzae* began to autolyze and the pH of the broth increased to 6–7. Then *B. circulans* started to grow and produce the lytic enzyme, which lysed *A. oryzae*. The pH of the broth gradually increased to 8. As a result alkaliphilic *B. pseudofirmus* A-57 grew well and produced an alkaline protease. This phenomenon is very interesting from the ecological point of view, because it may be the reason why alkaliphilic bacteria can create a microcosmos and live in acidic soil.

Since 1968, Horikoshi and his coworkers have systematically studied alkaliphilic microorganisms (Horikoshi 1971). And also they have paved the way to establish new microbiology, alkaliphilic microbiology, to use these microorganisms as microbial and genetic resources. These bacteria have an optimum pH value for growth of about 9–10.5 in Horikoshi-I medium. None of the alkaliphilic bacteria could grow at pH below 6.0 and all indicated optimum pH value for

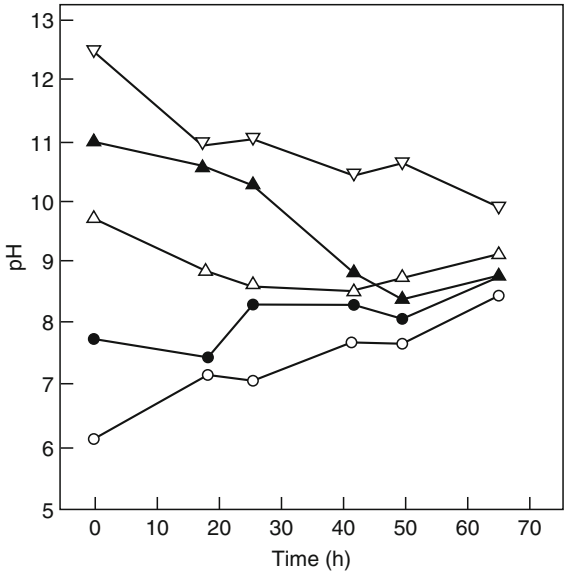


Fig. 2.5.1
Change in external pH values during cultivation of *Arthrobacter* sp

growth above 8. Therefore, bacteria with pH optima for growth in excess of pH 8, usually pH 9–12, are defined as alkaliphilic bacteria. This property is the most characteristic feature of these bacteria.

Sodium Ion

Growth

Another characteristic property of the alkaliphilic *Bacillus* strains is that for many of them sodium ions are one absolute requirement for growth and motility. The first finding of the sodium requirement was described by Horikoshi and Kurono in a patent application for extracellular production of catalase from alkaliphilic *Bacillus* sp. No. Ku-1 (FERM: No. 693). The strain isolated could not grow in nutrient broth in the absence of NaCl, but addition of NaCl (1–5%) induced good growth in the nutrient broth, although the pH value of the broth was not changed. In 1973, two scientific papers were published (Boyer et al. 1973; Kurono and Horikoshi 1973), but the sodium requirement was cited as only one of the cultural characteristics. No one was interested in sodium ions until Kitada and Horikoshi's paper was published (Kitada and Horikoshi 1977). Amino acid uptake into the cells as a function of NaCl was exhibited and they concluded that the presence of NaCl plays an important role in the active transport mechanism of amino acid into the cells. In some of them, K^+ ions can be substituted for Na^+ ions, as shown in *Table 2.5.1*.

This section discusses the effects of sodium ions on growth, sporulation, and germination. Further details of active transport by sodium are described in Chap. 2.6 Adaptive Mechanisms of Extreme Alkaliphiles by Krulwich.

Table 2.5.1

Requirement of Na^+ or K^+ for alkaliphilic *Bacillus* strains

Bacillus strains	Growth at		Na^+/K^+
	pH7	pH10	Requirement
B. pseudofirmus A-40-2	–	+	Na^+
B. halodurans A-59	+	+	Na^+
B. pseudofirmus 2b-2	–	+	Na^+
B. halodurans C-3	+	+	Na^+
B. hemicelluosilyticus C-11	–	+	Na^+
B. halodurans C-125	+	+	Na^+
Y-25	+	+	Na^+
B. clausii 221	+	+	Na^+
B. clausii M-29	+	+	K^+
57-1	–	+	K^+
C-59-2	–	+	K^+

Sporulation

Sporulation and germination are typical differentiation processes of bacteria. *B. subtilis* has been studied biochemically and genetically for many years. Several types of evidence suggest that a specific change in transcription level causes dramatic differentiation. In particular, sigma or σ like subunits are responsible for the specificity of transcription in the differentiation process.

Sodium requirement was also observed in the differentiation process: spore formation and germination. However, only one report on sporulation of an alkaliphilic *Bacillus* strain has been published. Kudo and Horikoshi (1979) isolated alkaliphilic *B. pseudofirmus* No.2b-2 from soil. This strain showed excellent spore yield in alkaline Schaeffer medium. The optimum pH for sporulation is close to that for growth, but the range is narrow. The optimum temperature for sporulation was almost the same as that for growth (34–37°C). Growth at 45°C was faster than at 34°C.

Germination

Kudo and Horikoshi (1983a, b) reported germination of alkaliphilic *B. pseudofirmus* No.2b-2. In 0.2 M NaCl solution spores of *B. pseudofirmus* No.2b-2 germinated very well in the presence of L-alanine, inosine, and NaCl but germinated poorly in the presence of L-alanine and NaCl. And no germination was observed in the absence of NaCl. The optimum temperature for germination was about 37°C, and germination occurred in the range of pH 8.5–11.1. The optimum pH for germination was around 10.0. The optimum concentration of NaCl for the germination was 0.1–0.5 M. Other cations such as K^+, NH_4^+, Rb^+, Cs^+, and Ca^{+2} did not show this stimulating effect. Only Li^+ showed weak stimulation. In the absence of Na^+, the loss of

heat resistance, acquisition of stainability, and decrease in absorbance was not observed at all even in the presence of germinants such as L-alanine and inosine. However, when Na^+ was added to the medium, the absorbance was decreased immediately. About 2 decades after Kudo's report, Krulwich's group reported Orf9 to be responsible to cation transport (Wei et al. 2003). A putative transport protein (Orf9) of alkaliphilic *B. pseudofirmus* OF4 belongs to a transporter family (CPA-2) of diverse K^+ efflux proteins. Orf9 greatly increased the concentration of K^+ required for growth of a K^+ uptake mutant of *Escherichia coli*. Nonpolar deletion mutants in the orf9 locus of the chromosome were isolated. During extensive genetic studies of the mutants, they found that endospore formation in amino acid rich medium was significantly defective and germination was modestly defective in the orf9 and orf7-orf10 deletion mutants.

The germination response of the orf9 mutant strains was less than that of the wild type with NaCl and either L-alanine or inosine. Although it is too premature to discuss the step stimulated by Na^+ and K^+, these ions stimulate the uptake of the germinants into the spores.

Temperature and Nutrition

High temperature for growth is not a characteristic property of alkaliphiles. The highest temperature for bacteria (except alkaliphilic archaea) so far reported is 57°C for *B. clausii* No. 221 (ATCC 21522), which is an alkaline protease producer. Kimura and Horikoshi (1988) isolated several psychrophilic alkaliphiles. One of them, strain 207, which is an aerobic coccus 0.8–1.2 μm in diameter, can grow at temperatures of −5°C to 39°C at pH 8.5. The optimum pH value for growth changed from 9.5 at 10°C to 9.0 at 20°C. But almost all alkaliphilic bacteria showed optimum growth temperature at the range of 25–45°C.

No precise experiment has been reported on nutrient requirements. During the development of new host-vector systems, Aono investigated more than 20 strains of alkaliphilic *Bacillus* strains and demonstrated that vitamins such as biotin, thiamine, and niacin are required for some alkaliphilic *Bacillus* strains. Kudo et al. (Kudo et al. 1990) found that some alkaliphilic *Bacillus* strains can grow on minimal media; alkaliphilic *B. halodurans* C-125 grows well on a minimal medium containing glutamate and glycerol as shown in *Table 2.5.2*.

Table 2.5.2

Horikoshi minimal medium for *B. halodurans* C-125

Glutamate	0.2%
Glycerol	0.5%
K_2HPO_4	1.4%
KH_2PO_4	0.4%
$MgSO_4\ 7H_2O$	0.02%
Na_2CO_3	1.0%
	w/v

Flagella

Flagella-based motility is a major mode of locomotion for bacteria. Flagella are typically as long as or longer than the cell body, varying in length from one to many micrometers. Several alkaliphilic bacteria demonstrate vigorous motility at alkaline pH. Flagella exposed to alkaline environments and devices for movement may operate at alkaline pH.

Flagella Formation and Flagellin

Aono et al. (1992) investigated pH-dependent flagella formation by facultative alkaliphilic *B. halodurans* C-125. This strain grown at alkaline pH had peritrichous flagella and was highly motile. However, most of the cells grown initially at pH 7 were nonmotile and possessed a few straight flagella. Sakamoto et al. (1992) cloned the flagellin protein gene (*hag* gene) of *B. halodurans* C-125 and expressed it in the *E. coli* system. Sequencing this gene revealed that it encodes a protein of 272 amino acids (Mr 29995). The alkaliphilic *B. halodurans* C-125 flagellin shares high homology with other known flagellins, such as *B. subtilis* etc. However, the sequence of the *hag* gene shows very low homology with other flagellins so far tested. This result strongly suggests that codon usage of *B. halodurans* C-125 is different from that of other bacteria.

Flagellar Motor

Flagellar movement of neutrophilic bacteria is caused by H^+-driven motors. Cells of alkaliphilic *Bacillus* sp. No. YN-1 (Koyama et al. 1976; Koyama and Nosoh 1976) in growth medium consisting of rich broth and NaCl showed vigorous motility between pH 8.5–11.5. *B. subtilis* showed motility between pH 6–8. The YN-1 cells were washed and resuspended in TG medium consisting of 25 mM Tris-HCl buffer (pH 9.0), 0.1 mM EDTA and 5 mM glucose; no translational swimming cells were observed. The addition of NaCl to the medium, however, caused quick recovery of swimming. The swimming speed increased with an increase of NaCl concentration up to 50 mM. Other cations such as Li^+ and K^+ had no effect. Hirota et al. (1981) reported on flagellar motors of alkaliphilic *B. pseudofirmus* No. 8-1 and *B. halodurans* C-125 powered by an electrochemical potential gradient of sodium ions, because the protein motive force is too low to drive flagellar motors in an alkaline environment. Such a Na^+ requirement is a unique property of alkaliphilic *Bacillus* strains. In order to clarify the role of Na^+ in motility, the effects of various ionophores, valinomycin, CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone), nigericin, and monensin were studied (Sakamoto et al. 1992). The results suggest that the membrane potential is a component of the energy source for the motility of alkaliphilic *Bacillus*. Monensin, which catalyzes Na^+/H^+ exchange, caused strong inhibition of motility, indicating that the flux of Na^+ by the chemical potential gradient of Na^+ is coupled with the motility of alkaliphilic *Bacillus* strains.

In 1999, Kojima et al. (1999) reported that the rotation of the Na^+-driven flagellar motor is specifically and strongly inhibited by phenamil, an amiloride analogue. This was the first evidence that phenamil interacts directly with the Na^+-channel components (PomA and PomB) of the motor. Sugiyama et al. (1986) analyzed the rotational characteristics of Na^+-driven flagellar motor in the presence and absence of coupling ion by the electrorotation method. The motor rotated spontaneously in the presence of Na^+, and the rotation accelerated

or decelerated following the direction of the applied external torque. The spontaneous motor rotation was inhibited by the removal of external Na^+; however, the motor could be forcibly rotated by relatively small external torque applied by the electrorotation apparatus. The observed characteristic of the motor was completely different from that of ATP-driven motor systems, which form a rigor bond when their energy source, ATP, is absent. The internal resistance of the flagellar motor increased significantly when the coupling ion could not access the inside of the motor, suggesting that the interaction between the rotor and the stator is changed by the binding of the coupling ion to the internal sites of the motor.

Then, Ito et al. (2004) identified the stator-force geerator that drives Na^+-dependent motility in alkaliphilic *B. pseudofirmus* OF4 to be MotPS, MotAB-like proteins. *B. pseudofirmus* OF4 was only motile at pH values above 8. These DNA sequences did not show homology to those of *B. halodurans* C-125, but decoded amino acids sequences exhibited very high homology to those of *B. pseudofirmus* OF4.

Further detail on cell motility is described in Chap. 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles by Ito.

Cell Wall of Alkaliphilic *Bacillus* Strains

Composition of Cell Wall of Alkaliphilic *Bacillus* Strains

The cell wall is directly exposed to high alkaline environments, but the intracellular pH of alkaliphilic bacteria is neutral (pH 7–8.5), so the pH difference must be due to cell surface components. It has been observed that protoplasts of alkaliphilic *Bacillus* strains lose their stability against alkaline environments (Aono et al. 1992). This suggests that the cell wall may play some role in protecting the cell from alkaline environments. The components of cell wall of several alkaliphilic *Bacillus* strains have been investigated by Aono and Horikoshi (1983), and Ikura and Horikoshi (1983) in comparison with those of neutrophilic *B. subtilis*. *Table 2.5.3* summarizes the composition of the cell wall of alkaliphilic *Bacillus* strains isolated in the author's laboratory.

Composition of the Peptidoglycans

The peptidoglycans appeared to be similar in composition to those of *B subtilis*. Major constituents detected commonly in hydrolyzates of the peptidoglycans were glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, meso-diaminopimelic acid and acetic acid. Essentially, the composition of peptidoglycan was not changed whether the strain was cultured at pH 7 or 10. It was therefore concluded that all of the peptidoglycans of the alkaliphilic *Bacillus* strains so far examined are of the Alγ-type of peptidoglycan, which is found in the majority of the strains of the genus *Bacillus*.

Acidic Polymers in the Cell Wall of Alkaliphilic *B. halodurans* C-125

Most strains of group 2 can grow at neutral pH and require the presence of sodium ions. The same acidic amino acids and uronic acids are found in much smaller quantities in the walls

Table 2.5.3

Composition of cell walls of alkaliphilic *Bacillus* strain

Group	Strain	Culture pH	L-Glu	DAP	Mur	GlcN	Uronic acid
1	A-40-2	10		0.42	0.36	0.67	0.90
	2b-2	10		0.35	0.34	0.65	0.81
2	C-11	10	1.05	0.33	0.22	0.26	0.60
	C-125	7	1.08	0.52	0.48	0.46	0.46
		10	1.80	0.38	0.28	0.30	0.90
	Y-25	7	0.59	0.47	0.38	0.44	0.36
		10	0.86	0.34	0.29	0.32	0.79
	A-59	7	0.52	0.56	0.39	0.42	0.44
		10	0.70	0.38	0.27	0.30	0.62
	C-3	7	0.33	0.53	0.48	0.51	0.27
		10	0.54	0.39	0.36	0.37	0.49
3	C-59-2	10		0.24	0.18	0.45	0.11
	M-29	7		0.30	0.21	0.80	0.13
		10		0.31	0.30	1,01	0.12
	57-1	7		0.29	0.19	0.22	0.08
		10		0.30	0.21	0.25	0.08

Each blank space represents not detected. The following abbreviations are used; Glu, glutamic acid; DAP, diaminopimelic acid; Mur, muramic acid; GlucN, glucosamine.

prepared from bacteria grown at neutral pH. This indicates that the acidic components in the cell walls of the group 2 bacteria play a role in supporting growth at alkaline pH. One of the alkaliphilic *Bacillus* strains isolated in our laboratory, *B. halodurans* C-125, the whole genome sequence of which was determined by Takami et al. (2000), grows well at neutral pH. The chemical composition of its nonpeptidoglycan components was relatively simple compared with other group 2 strains (Aono 1985).

Teichuronic Acid in the Nonpeptidoglycan

The nonpeptidoglycan components (TCA-soluble fraction) of alkaliphilic *B. halodurans* C-125 grown at alkaline or neutral pH contained two acidic structural polymer fractions (*Fig. 2.5.2*). The Al polymer of alkaliphilic *B. halodurans* C-125 was a teichuronic acid composed of glucuronic acid, galacturonic acid, and N-acetyl-D-fucosamine in a molar ratio of 1:1:1 (Aono and Uramot 1986). It is noteworthy that the amount of teichuronic acid is enhanced in the cell walls of *B. halodurans* C-125 grown at alkaline pH. This teichuronic acid amounted to 390 μg per mg peptidoglycan in the walls of the bacterium grown at an alkaline pH, 80μg per mg at a neutral pH. The molecular weight of the teichuronic acid from the cells grown at alkaline pH was approximately 70,000 compared to a molecular weight of 48,000 at a neutral pH as estimated by gel chromatography.

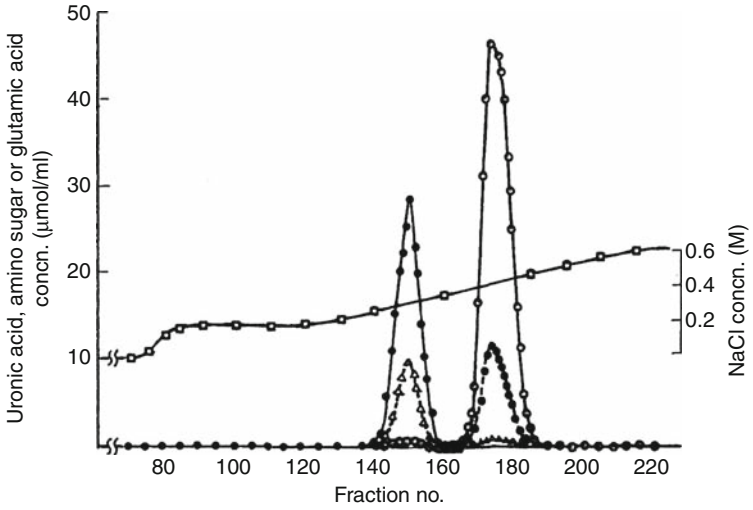


Fig. 2.5.2

DEAE-cellulose chromatography of the nondialyzable fraction of TCA extracts of alkaliphilic *Bacillus halodurans* C-152 cell walls. Uronic acids (*solid circle*), amino sugar (*triangle*), L-glutamic acid (*open circle*), and NaCl (*open square*). Fraction 142–157 (Faction A) and 168–186 (Fraction A) were analyzed

Poly-γ-L-glutamic Acid

The other fraction (A2) contained glucuronic acid and **L**-glutamic acid in a molar ratio of 1:5. This fraction was called "teichuronopeptide" by Aono (1989, 1990). In 1987, he isolated poly-γ-**L**-glutamic acid (plg; Mr about 43,000), but could not find glucuronic acid-glutamic acid copolymer (Aono 1987). Therefore, the acidic polymer found in the cell wall of *B. halodurans* was concluded to be a mixture of two kinds of polymers (poly-γ-**L**-glutamate and polyglucuronate). Then they made several mutants from wild *B. halodurans* C-125 (Aono et al. 1994; 1995). One of them, C-125-90 did not have plg in the cell walls and its growth in alkaline media was very poor. Aono et al. (1999) cloned *tupA* gene capable of restoring plg production and high alkali-tolerant growth. Their results demonstrate that the acidic polymer plays a role in pH homeostasis in *B. halodurans* C-125.

Poly-γ-**L**-glutamate has been found widely in other bacteria of the genus *Bacillus*, for example, *B. anthracis*, *B. mesentericus*, and *B. subtilis*. These are capsular structures or extracellular mucilaginous material, and do not bind to the peptidoglycan layer of the cell wall. The whole genome sequence of *B. halodurans* C-125 was determined (Takami et al. 2000) and Ashiuchi and Misono (2002) found an operon of poly-γ-**L**-glutamate synthetase genes activated by an operator in *tupA* gene fragment. These results show that the cells of *B. halodurans* C-125 are shaped by the Alγ-type of peptidoglycan, and the peptidoglycan is enclosed by at least two acidic polymers such as teichuronic acid and poly-γ-**L**-glutamatic acid with highly negative charges. Donnan equilibrium in the bacterial cell wall was calculated to estimate the pH values inside the polymer layer of the cell walls when the outer aqueous solution is alkaline in nature. The fixed charge concentration in the polymer layer was estimated to be 2–5 mol/l

from the data reported for gram-positive bacteria, particularly for an alkaliphilic bacterium *B. halodurans* C-125. According to Tsujii's calculation (Tsujii 2002) the pH values estimated to exist inside a polymer layer (cell wall) are more acidic than those of the surrounding environment by 1–1.5 U. Therefore, acidic polymers of poly-γ-L-glutamic acid and teichuronic acid are one of the important components in the cell wall of the alkaliphilic *B. halodurans* and contribute to the regulation of pH homeostasis in the cytoplasm.

Nonpeptidoglycan of Other Bacteria

Cell walls prepared from four *B. halodurans* strains (A-59, C-3, C-11, and Y-25) and were also analyzed. Although several components with different compositions were detected, the cell walls commonly contained a polypeptide of acidic amino acids such as poly-γ-L-glutamatic acid. These results suggest that the substances were similar to one another in chemical structure (Aono et al. 1993). As far as the author's group has tested, no poly-γ-L-glutamatic acid was detected in *B. pseudofirmus* group (2b-2 and A-40-2).

This is not the case with the S-layer polymer (SlpA) of *B. pseudofirmus* OF4. SlpA is not essential for growth of *B. pseudofirmus* OF4 at pH 10.5. Gilmour et al. (2000) characterized an S-layer protein with a role in alkaliphily of *B. pseudofirmus* OF4. The large majority of proteins of alkaliphilic *B. pseudofirmus* OF4 grown at pH 7.5 and 10.5 did not exhibit significant pH-dependent variation except a new surface layer protein (SlpA). A deletion mutant lacking the slpA gene grows better than the wild type at pH 7.5. Nonetheless, high levels of the major cell surface polymer are present in both pH 7.5- and 10.5-grown cells. This indicates that the organism is "hard-wired" for alkaliphily, ready to support pH homeostasis and grow optimally if there is a sudden alkaline shift even at the expense of growth at near-neutral pH

These papers strongly suggest that the outer surface of their cell walls has a part of responsibility of alkaliphily.

Internal pH Values

Measurements of Intracellular pH

Although the actual measurement of intracellular pH is difficult, several indirect measuring methods have been reported: (1) pH measurement of broken cell lysate or fluids from cells, (2) the use of a visible indicator such as bromothymol blue, (3) measurement of the external and internal distributions of weak acids or bases, (4) the fluorescence method, and (5) high-resolution ^{31}P nuclear magnetic resonance measurements.

The method most applicable to the cells of microorganisms was the weak acid or base distribution techniques. The principle of measurement is based on the ability of weak acids such as 5,5-dimethyl-2,4-oxazolidinedione (DMO) to penetrate membranes in their neutral form. DMO diffuses passively across many biological membranes and the permeability coefficient of the uncharged acid is generally much greater than that of anions. Therefore, DMO permeates cells or vesicles which are internally more alkaline than the surrounding medium. The internal pH of cells or vesicles can be calculated from the distribution by the Henderson-Hasselbalch equation. However, if the intracellular pH of the cells is lower than external pH as expected from pH optima of intracellular enzymes, most of the DMO would be outside the

cells. Actually, no DMO uptake by *B. alcalophilus* cells was observed at any external pH (Guffanti et al. 1978).

Guffanti et al. (1978) used ^{14}C-methylamine to determine intracellular pH values in *B. alcalophilus* by flow dialysis, and found that the cytoplasmic pH remained at 9.0–9.5 over a range of external pH values from 9.0 to 11.5.

Aono et al. (1997) developed a new method to measure the intracellular pH of the facultative alkaliphilic *B. halodurans* C-125. The bacterium was loaded with a pH-sensitive fluorescent probe, 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF), and cytoplasmic pH was determined from the intensity of fluorescence of the intracellular BCECF. The activity of the organism to maintain neutral cytoplasmic pH was assessed by measuring the cytoplasmic pH of the cells exposed to various pH conditions. At pH above 10.7, the organism lost its pH homeostatic activity. *B. halodurans* C-125 does not grow at pH above 11.5 as shown in *Table 2.5.4*.

Table 2.5.4

Intracellular pH values in various alkaliphiles at different external pH values

Microorganisms	External pH	Internal pH	Method
Bacillus alcaliphilus	8.0	8.0	Methylamine
B. firmus RAB	7.7	7.0	BCECF
	9.0	8.0	
	10.8	8.3	
	11.2	8.9	
	11.4	9.6	
Bacillus strain YN-2000	7.5	8.5	BCECF
	8.5	7.9	
	9.5	8.1	
	10.2	8.4	
	11.0	8.7	
B. halodurans C-125 intact cells	7.0	7.3	BCECF
	8.0	7.6	
	9.0	7.9	
	10.0	8.0	
	10.5	8.0	
	11.0	8.1	
	11.5	8.5	
	12.0	9.1	
B. halodurans C-125 protoplasts	7.0	7.5	BCECF
	8.0	7.9	
	9.0	8.8	
	9.3	8.9	
	10.0	Lyzed	

Estimation of Intracellular pH Values from Protein-Synthesizing System

Ribosomes of alkaliphilic *B. halodurans* A-59 and C-125 were of the 70S type, and no difference was observed in their thermal denaturation curves. Phenylalanyl-tRNA synthetase activity at different pH values also indicated no remarkable difference between alkaliphilic *Bacillus* and *B. subtilis* Marburg 168 (Ikura and Horikoshi 1978). Phenylalanine incorporation was tested in a series of homogeneous and heterogeneous combinations. The results are shown in *Table 2.5.5*.

The reaction mixture contained 0.2 mg ribosomal protein and 0.3 mg S-100 protein.

In the systems containing alkaliphilic *Bacillus* ribosomes or S-100, phenylalanine incorporation at pH 8.4 was higher than that at pH 7.5, although the activity of heterogeneous systems was lower than that of homogeneous systems. As a conclusion, alkaliphilic *B. halodurans* A-59 grows well under highly alkaline conditions, but the protein-synthesizing machinery is essentially the same as that of *B. subtilis*. It would be very interesting to measure the internal pH of the cells under alkaline conditions such as pH 10; however, no direct method has been established for determining the internal pH. The pH optima of the protein-synthesizing machinery strongly suggest that the internal pH value may be 8–8.5, not 10. Heterogeneous combination also supports this possibility. In 2000, Takami et al. (2000) reported the complete genome sequence of the alkaliphilic *B. halodurans* C-125 and genomic sequence comparison with *B. subtilis*. During the analysis of DNA of *B. halodurans*, Takami (Takami et al. 1999) found 41 open reading frames (ORFs) in a 32-kb DNA fragment of *B. halodurans* C-125 were very similarity to *B. subtilis* RNA polymerase subunits, elongation factor G, elongation factor Tu, and ribosomal proteins. Each ORF product showed more than 70% identity to those of *B. subtilis*.

The results clearly indicate that the differences between alkaliphilic *Bacillus* and neutrophilic *Bacillus* exist on the cell surface and not in the cells.

Table 2.5.5

Phenylalanine incorporation in homogeneous and heterogeneous systems

Ribosomes	S-100	Phenylalanine incorporated (nmol)		Ration between pH 8.4 and 7.5
		pH 7.5	pH 8.4	
B. halodurans A-59	*B. halodurans* A-59	5.82	9.37	1.61
	B. halodurans C-125	6.12	8.65	1.41
	B. subtilis 168	5.84	6.37	1.09
B. halodurans C-125	*B. halodurans* A-59	5.17	6.62	1.28
	B. halodurans C-125	5.31	8.12	1.53
	B. subtilis 168	5.63	6.46	1.15
B. subtilis 168	*B. halodurans* A-59	6.31	7.15	1.13
	B. halodurans C-125	6.71	8.34	1.24
	B. subtilis 168	9.65	6.59	0.68

Isolation and Properties of Alkali-Sensitive Mutants

Two series of experiments have been conducted by the author's colleagues. Kudo et al. (Kudo et al. 1990) focused on the cell membrane of *B. halodurans* C-125, mainly on H^+/Na^+ antiporters that regulate intracellular pH values.

They reported alkali-sensitive mutants of alkaliphilic *B. halodurans* C-125 by conventional mutagenesis. This strain was selected for the following study since it grows well in Horikoshi minimal medium over the pH range of 7–11.5 at 37°C. Alkaliphilic *B. halodurans* C-125 (Trp^- Ura^- Cm^s) was treated with nitrosoguanidine in 25 mM glycine-NaOH-NaCl buffer of pH 8.5 and plated on Horikoshi-II medium (pH 7.5) containing 5 g/l NaCl instead of sodium carbonate. Colonies that appeared on the plates were transferred onto Horikoshi-II medium (pH 10.3). After 16 h of incubation at 37°C, seven alkali-sensitive mutants that could not grow at pH 10.5 but grew well at pH 7.5 were obtained from 7×10^4 colonies.

One of the mutants, No. 38154, was unable to sustain low internal pH in the presence of either Na_2CO_3 or K_2CO_3. The internal pH was 10.4, which was the same as that for all strains in the presence of K_2CO_3. Although the other mutant, No. 18224, cannot grow at pH >9, the internal pH of the mutant was 8.7 in the presence of Na_2CO_3. This value is close to that of the parent strain (pH 8.6). Mutant No. 18224 cannot maintain a low internal pH in the presence of K_2CO_3. It is suggested that mutant No.38154 was defective in the regulation of internal pH, whereas mutant No.18224 apparently showed normal regulation of internal pH values: Na^+ ion is also assumed to play an important role in pH homeostasis.

Molecular Cloning of DNA Fragments Conferring Alkaliphily

The DNA fragment of the parental *B. halodurans* C-125 that restores alkaliphily to the alkali-sensitive mutant strain No. 38154 was found in a 2.0-kb DNA fragment, which was cloned in a recombinant plasmid pALK2 (*Fig. 2.5.3*). Another recombinant plasmid, pALK1, also restored alkaliphily to another alkali-sensitive mutant strain, No. 18824.

Plasmid pALK1 conferred alkaliphily on mutant 18224 but not on mutant 31154. Mutant 38154, which could not regulate its internal pH value in alkaline media, recovered pH homeostasis after the introduction of pALK2 but not after the introduction of pALK1. These results indicate that there are at least two factors involved in alkaliphily located in a closed linked region of chromosomal DNA, although the precise functions of these two DNA fragments are not yet known.

To identify the mutation site in the genome of mutant 38154, the nucleotide sequence of the corresponding DNA fragment was determined. A 5.1-kb DNA fragment containing ORF-1 to ORF-4 was then cloned. Another alkali-sensitive mutant, strain 18224, was found to have a mutation resulting in an amino acid substitution in the 82nd residue in the ORF-3 product (Seto et al. 1995). Mutant 18224 still retains the ability to control the internal pH, although it shows alkali-sensitive growth (Hashimoto et al. 1994). It appears that ORF-3 is not involved in Na^+/H^+ antiport itself but it may be involved in a regulatory process or some other function associated with ion transport. This is the first report of a DNA fragment responsible for a Na^+/H^+ antiporter system in the alkaliphily of alkaliphilic microorganisms.

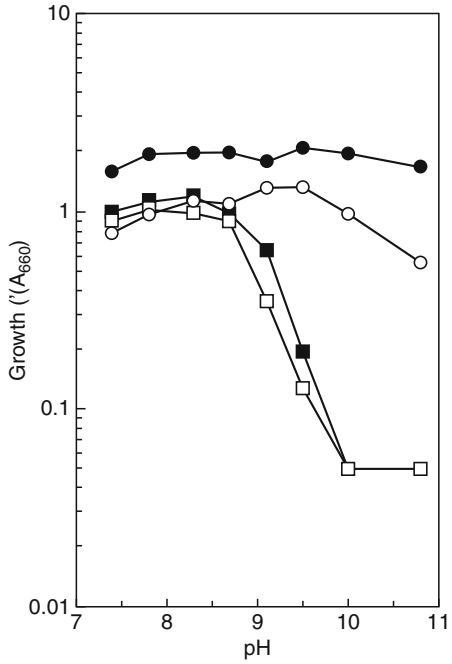


Fig. 2.5.3

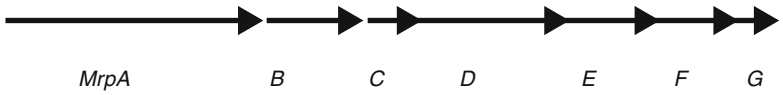
Effect of pH on growth of alkali-sensitive mutant 38154 and its derivatives. Each point represents cell growth. Parental strain of C-125 (*solid circle*), mutant 38154 (*solid square*), mutant 38154 carrying pALK2 fragment (*open circle*) and mutant 38154 carrying a vector pHW1 (*open square*) were cultivate for 7 h at 37°C

Ubiquitous Distribution of *pALK/Mrp/Sha* Operons

Almost 10 years later, *pALK* operons in four strains of alkaliphilic *Bacillus* were extensively investigated and it was clear that their distributions are very ubiquitous (*Fig. 2.5.4*).

After whole DNA sequence analysis, direct evidence for a *pALK/Mrp/Sha* operon has been shown in *B. subtilis*, *B. halodurans* C-125, *B. pseudofirmus* OF4, *Oceanobacillus iheyensis*, *B. clausii*, etc.

The functional studies conducted to date on individual pALK/Mrp/Sha systems include limited assays of cation and proton fluxes in whole cells and in everted membrane vesicles. To avoid confusion, the author uses *Mrp* system for the *pALK/Mrp/Sha* system. Information from these assays is supplemented by inferences drawn from physiological experiments on Na^+- or K^+-sensitivity, Na^+ exclusion from whole cells or the cation-dependence of Mrp-dependent pH homeostasis. Mrp-dependent antiporters in alkaliphilic *Bacillus* and in *B. subtilis* can be energized by an imposed transmembrane potential (Hashimoto et al. 1994; Ito et al. 1999, 2000) as well as by an imposed transmembrane ΔpH (Hiramatsu et al. 1998; Ito et al. 2001), that is, Mrp-dependent monovalent cation/proton antiporters behave as expected for a secondary antiporter energized by the Δp. The energization by an imposed transmembrane electrical potential is consistent with an electrogenic monovalent cation/proton exchange that involves net movement of charge during each antiporter turnover, for example, a Na^+/H^+



Bacillus halodurans						
BH 1319	BH1318	BH1317	BH1316	BH1315	BH1314	BH1313
Palk ORF1	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7
B.Pseudofirmus						
MrpA	*MrpB*	*MrpC*	*MrpD*	*MrpD*	*MrpE*	*MrpF*
Oceanobacillu iheyensis						
OB3109	OB3108	OB3107	OB3106	OB3105	OB3104	OB3103
B.clausii						
ABC1635	ABC1634	ABC1633	ABC1632	ABC1631	ABC1630	ABC1629

Fig. 2.5.4

Ubiquitous *mrp* operons. *pALK/mrp/sha* Operons in *B. halodurans, B. pseudofirmus, Oceanobacillus iheyensis*, and *B. clausii*

antiporter for which the stoichiometry of H^+:Na^+ transported per turnover is more than 1. An electrogenic Na^+/H^+ antiporter can utilize the energy of the transmembrane potential component of the Δp, the $\Delta\Psi$ (inside negative in whole cells or right-side-out membrane vesicles) that is a useful feature for antiporters supporting alkaline pH homeostasis. No detailed biochemical characterization of an Mrp antiporter has yet been conducted in membrane vesicles, for example, measuring kinetic parameters, nor are there yet any data reported for a purified, reconstituted Mrp system.

As described in the previous section, the first role established for a Mrp antiporter was in cytoplasmic pH homeostasis of alkaliphilic *B. halodurans* C-125 at highly alkaline external pH, studies that also demonstrated a Mrp role in Na^+-resistance (Hamamoto et al. 1994; Kitada et al. 2000; Krulwich et al. 2001). The Na^+/H^+ antiporter activity is attributed to MrpA since the point mutation that leads to a non-alkaliphilic phenotype was in *mrpA*. The finding that a mutant in *MrpC* is also non-alkaliphilic (Seto et al. 1995) raises the possibility that additional *mrp* genes play critical roles even if MrpA contains the cation and proton translocation pathways. A requirement for multiple *mrp* genes in the extreme alkaliphiles is consistent with our inability to recover mutants of genetically accessible *B. pseudofirmus* OF4 when attempts were made to disrupt any one of several mrp genes. The major Na^+/H^+ antiporter of alkaliphilic *Bacillus* species may be required throughout their pH range. The Na^+-specific monovalent cation/proton antiporter is necessary for the alkaliphile to maintain a cytoplasmic pH of 8.2–9.5 at external pH values from 10.5 to 11.2.

In neutrophilic *B. subtilis*, the Mrp system has a role in Na^+-resistance and in both Na^+-and K^+-dependent alkaline pH homeostasis (Seto et al. 1995; Ito et al. 1999). Recently, Kosono et al. (2004) reported that a *mrpA/shaA* mutant of *B. subtilis* changes its use of the diverse ECF (extracytoplasmic function), σW and σ-dependent transcription in transition phase; this is consistent with complex stress reaction. In contrast to the central role of Mrp in pH

homeostasis of alkaliphilic *Bacillus* species, Mrp is not the dominant antiporter in this process in *B. subtilis*; that role belongs to the multifunctional (tetracycline-divalent metal.)$^+$ (Na^+)(K^+)/H^+ antiporter Tet^+ (Ito et al. 1999). On the other hand, the *B. subtilis* Mrp system plays a dominant role in Na^+-resistance in this organism, as indicated by the Na^+-sensitive phenotypes of *mrp/sha* mutants. This result suggests that *mrp* is the essential gene of *B. subtilis* as examined on LB medium even though a *mrp* null strain is viable (Ito et al. 2000). As Swartz et al. (2005a, b), it will be of interest to compare the activity versus pH profile of alkaliphile and *B. subtilis* Mrp in many respects.

The other transport substrate for a Mrp system has been mentioned, that is, a capacity for cholate efflux by the *B. subtilis* Mrp system (Ito et al. 1999; Ito and Nagane 2001). A *mrp* null strain of *B. subtilis*, from which the entire *mrp* operon is deleted, exhibits significantly reduced resistance to growth inhibition by the addition of cholate that is complemented by the introduction of the *mrpF* gene into the chromosomal *amyE* locus under the control of an IPTG-inducible promoter (Ito et al. 2000). Reduced cholate efflux was observed in starved whole cells of the mutant relative to the wild type. This defect is complemented significantly by the reintroduction of mrpF. Homology has been noted between MrpF and Na^+-coupled bile transporters (Ito et al. 1999) and between MrpF and a region of voltage-gated Na^+ channels. However, no crucial experimental data for MrpF-mediated coupling between Na^+ and cholate fluxes has been found.

The Mrp antiporter system may have a substrate or activity in addition to the primary process/es, although still no clear result has yet been found. Further details are contributed by Krulwich (Chap. 2.6 Adaptive Mechanisms of Extreme Alkaliphiles) and Ito (Chap. 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles).

Mechanisms of Cytoplasmic pH Homeostasis in Alkaline Environments

The cytoplasmic pH values of most microbes are maintained within a narrower range than external pH, usually closer to neutrality. For example, *B. subtilis*, during optimal growth conditions, maintains its cytoplasmic pH within a range of pH 7.2–7.87 over an external pH range of 5.0–9.0. However, the alkaliphilic *B. halodurans* C-125 shows a range of cytoplasmic pH 7.3–8.1 over the range of external pH 7.0–11.0. How can alkaliphiles maintain cytoplasmic pH values of 7–8? This is most attractive and festinating field. So far our laboratory's data presented above, intracellular metabolic pathways such as protein-synthesizing system are essentially same as those of neutrophilic *Bacillus* strains.

These results strongly suggest that cell surface (cell wall and membrane) of alkaliphiles are essential for alkaliphily. Thousands of alkaliphiles have been isolated: Obligatory alkaliphilic or facultative alkaliphilic microbes. Mostly studied are *B. halodurans* C-125 and *B. pseudofirmus* OF4. There are many differences between them. For instance, *B. pseudomonas* OF4 has the S-layer polymer, which is not found in *B. halodurans* C-125. Furthermore, recently Ito found a hybrid flagellar motor (Na^+ driven and/or H^+ driven mortar) in *B. pseudomonas* OF4 (Chap. 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles).

Therefore, the author would like to show a schematic presentation of cell surface of *B. halodurans* C-125 (*Fig. 2.5.5*).

The cells have two barriers to reduce pH values from 10.5 to 8. Cell walls containing acidic polymers (teichuronic acid and poly-γ-D-glutamic acid) function as a negatively charged

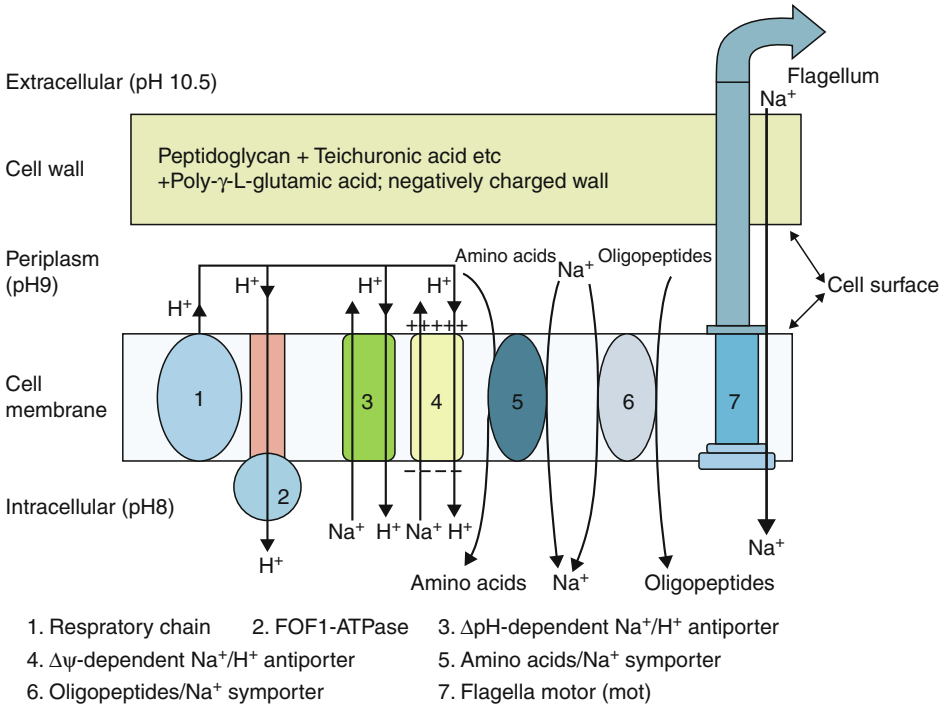


Fig. 2.5.5

Schematic presentation of cell surface of *B. halodurans* C-125

matrix and may reduce the pH value at the cell surface. The surface of the plasma membrane must presumably be kept below pH 9, because the plasma membrane is very unstable at alkaline pH values much below the pH optimum for growth.

Plasma membranes may also maintain pH homeostasis by using Na^+/H^+ antiporter system ($\Delta\Psi$-dependent and ΔpH-dependent), and ATPase-driven H^+ expulsion. The author's group isolated a non-alkaliphilic mutant strain from *B. halodurans* C-125 as the host for cloning genes related to alkaliphily. The pALK fragment from the parental strain restored the growth of the mutant at alkaline pH. The transformant was able to maintain an intracellular pH that was lower than the external pH and contained an electrogenic Na^+/H^+ antiporter driven only by $\Delta\Psi$ (membrane potential, interior negative). Beside these factors, however, it is noteworthy to write here that there are more DNA fragments conferring alkaliphily.

Phages

During the course of studies of alkaliphilic bacilli, several types of plaques were observed on the colonies. One of them was a phage A1-K-1 (Horikoshi and Yonezawa 1978). The host Bacillus sp. Y-25 was isolated from soil using Medium 11. Turbid plaques (1–2 mm in diameter) were observed after 24 h of incubation at 30°C. Growth and purification were carried out by the conventional method using CsC1. An electron micrograph of phage A1-K- I showed that the phage has an icosahedral head about 10,019 nm in diameter attached to a tail 210 nm long and 8–10 nm wide.

The phage grew well at pH 9–11, the highest titer being observed at pH 10.5. Although this was the first report on the phage of alkaliphiles, my interest focused on alkaline enzymes.

After 20 years, Jarrell et al. (1997) described the isolation and characterization of a novel bacteriophage active against the obligately alkaliphilic bacterium *B. clarkii*. The bacteriophage, designated BCJA1 is a member of the Siphoviridae family with a B1 morphology. It possesses an isometric head, which measures 65 nm between opposite apices, and a noncontractile tail of 195 nm length. It had a buoyant density of 1.518 g/ml and an estimated particle mass of 37×10^7 Da. BCJA1 was stable over the pH range of 6–11. The wild-type bacteriophage is temperate but a clear plaque mutant was isolated. Then, Kropinski et al. (2005) published details of the genome of BCJA1c. The sequence of the genome of the alkaliphilic bacteriophage has been determined. Temperate phage BCJA1 possesses a terminally redundant genome of approximately 41.6 with a mol% G + C content of 41.7 and 59 genes arranged predominantly into two divergent transcriptons. The integrase gene of this phage is unique in that it contains a ribosomal slippage site. The DNA replication, recombination, packaging, and morphogenesis proteins show their greatest sequence similarity to phages and prophages from the genus *Streptococcus*.

Although we do not have sufficient information to clarify why this phage can thrive at higher pH values, analysis of these phages may give us good solution of pH homeostasis.

Cross-References

- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles
- 2.8 Enzymes Isolated from Alkaliphiles
- 2.9 Genomics and Evolution of Alkaliphilic *Bacillus* Species
- 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic *Bacillus* for the Production of Beta-Cyclodextrin
- 2.11 Alkaline Enzymes in Current Detergency
- 9.3 Biochemistry

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2.6 Adaptive Mechanisms of Extreme Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.6,

Prologue

> "Most engineers accept the 'no free lunch' principle, which states that any mechanism that increases robustness in one setting (i.e., to one type of perturbation, or with respect to one type of output) always compromises it in another" (Lander et al. 2009).

Introduction

Extreme alkaliphiles, like extremophiles in general, possess numerous structural, metabolic, physiological, and bioenergetic adaptations that enable them to function well under their particular "extreme" condition or, in the case of poly-extremophiles, under several extreme conditions at once (see also Chaps. 2.1 Introduction and History of Alkaliphiles, 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles). If they are facultative extremophiles, many of the adaptations are present even under non-extreme growth conditions. That is, the adaptations to the extreme condition are "hard-wired" although their expression may increase further when the bacteria confront the extreme condition(s). The constitutive hard-wiring is presumed to be a mechanism that anticipates the need to survive and grow upon a sudden shift to the extreme condition(s). Here, we will summarize a number of different adaptations of alkaliphiles that support their ability to grow optimally at pH values well above 9.0. Some of these species or strains are obligate alkaliphiles that exhibit little or no growth at pH values closer to neutral. Other, facultative alkaliphiles, grow in a range from pH 7.5 to $\geq$11 (Guffanti and Hicks 1991; Yumoto 2007). The facultative alkaliphiles exhibit trade-offs of the type predicted by the "no free lunch" principle defined above. They have a remarkable capacity for growth at pH values much higher than the outer limit of pH 8.5–9.0 for growth of typical neutralophilic bacteria. Facultative alkaliphiles also transition almost seamlessly through a sudden shift from near neutral pH to extremely alkaline pH (Krulwich 1995; Krulwich et al. 2007; Padan et al. 2005; Slonczewski et al. 2009). However, these alkaliphiles exhibit a cost of these remarkable alkaliphilic properties. This deficit is reflected in lower growth rates at near neutral pH than at high pH even though there are greater energy costs for growth at alkaline pH, for example, pH homeostasis and ATP synthesis (Krulwich et al. 2007). We suggest that extreme, obligate alkaliphiles represent extreme examples of the "no free lunch" principle in having entirely lost the capacity to grow at neutral pH while excelling at highly alkaline pH values.

Each alkaliphile strain that has been examined in some detail displays multiple adaptations that address specific aspects of the challenge of growth at very high pH. For example, they have multiple types of strategies and apparently redundant transporters or enzymes to achieve alkaline pH homeostasis (Padan et al. 2005; Slonczewski et al. 2009). This is in accord with a corollary of the "no free lunch" principle which posits that such multiple strategies and their built-in redundancies confer robustness upon the system under the specific condition targeted by the strategies, that is, survival and growth at alkaline pH in our case. It further predicts that a cost will be exacted in the form of low robustness relative to other organisms under other conditions, that is, non-alkaline conditions in our case. Thus the "no free lunch" principle is also called the principle of "conservation of fragility" in the engineering literature (Lander et al. 2009). There follows a set of examples of how this conceptualization of alkaliphily explains data emerging from physiological and bioenergetic studies of several alkaliphiles. We note that a deeper

and broader understanding of the design principles underpinning alkaliphily awaits more widespread studies of different extreme alkaliphiles in which mutations can be made in the native setting. Genetically tractable strains are almost entirely unavailable for most extreme alkaliphile types, including extreme Gram-negative alkaliphiles and poly-extremophiles that are alkaliphilic as well as thermophilic and/or halophilic (Ma et al. 2004; Mesbah et al. 2007). Without many more genetically tractable and extensively characterized alkaliphile strains, we cannot test the hypotheses about models of robustness and adaptation that are developed from biochemical, genome, transcriptome, and proteome data. The "omics" studies can be complemented by studies of mutations of specific molecules in heterologous settings. However, the impact of mutational alterations in specific molecules will often be affected by systems-level adaptations in the native host in ways that cannot be anticipated by work outside the native setting.

Cytoplasmic pH Homeostasis: The Central Challenge

A small number of alkaliphilic bacteria were first described in the 1920s (Chap. 2.1 Introduction and History of Alkaliphiles). Only a few studies appeared after those first reports until the 1970s when Koki Horikoshi initiated work that spurred a sustained interest in these extremophiles, their physiology, and the potential products they might yield (Horikoshi 1991). It was immediately clear to bioenergeticists that alkaliphiles would require a capacity for robust cytoplasmic pH homeostasis. This expectation has been validated in numerous species and strains of alkaliphiles (Cook et al. 1996; Guffanti and Hicks 1991; Olsson et al. 2003; Sturr et al. 1994; Yumoto 2002). Among the most intensively studied strains is alkaliphilic *Bacillus pseudofirmus* OF4, an extreme facultative alkaliphile that is genetically tractable and grows well over a pH range from 7.5 to at least 11.4 (Krulwich et al. 2007; Sturr et al. 1994). *B. pseudofirmus* OF4 is able to maintain a cytoplasmic pH of 8.2 when the external pH is 10.5, a pH gradient of 2.3 pH units, inside acidic relative to the outside (*Fig. 2.6.1*, *upper left*); similar capacities for pH homeostasis are found in other extremely alkaliphilic *Bacillus* species, such as the facultative alkaliphile *B. halodurans* C-125 (Ito and Aono 2002), the obligate alkaliphile *B. alcalophilus* (Guffanti and Hicks 1991), as well as the alkaliphilic cyanobacterium *Spirulina platensis* (Pogoryelov et al. 2003) (*Fig. 2.6.1*, upper right). In accord with the notion that robust adaptation to one perturbation tempers the robustness in the face of other challenges, thermophilic alkaliphiles typically do not exhibit cytoplasmic pH homeostasis that is as robust as that of non-thermophilic extreme alkaliphiles. Examples of this difference are in *Fig. 2.6.1*, which shows the cytoplasmic pH at external pH values near the upper end of the optimal pH range for alkaliphilic *B. pseudofirmus* OF4, *B. halodurans* C-125, and *S. platensis* in comparison with thermoalkaliphiles *Clostridium paradoxum* (Cook et al. 1996) and *Bacillus* sp. TA2.A1 (recently proposed to be the TA2.A1 strain of *Caldalkalibacillus thermarum* (McMillan et al. 2009). It has been suggested that even more "poly-extremophilic" bacteria, such as alkaliphilic, thermophilic, and halophilic *Natranaerobius thermophilus* (Mesbah et al. 2007), may approach a physico-chemical limit of adaptations to the multiple stresses (Bowers et al. 2009) (see Chap. 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles).

The detailed pH homeostasis profile for *B. pseudofirmus* OF4, and its relationship to growth rate, was revealed by a set of assays on cells growing in continuous culture on malate-containing media at different rigorously controlled pH values from 7.5 to 11.4 (Sturr et al. 1994). These analyses showed that: (1) the growth rate was lower at pH 7.5, at which the doubling time was 54 min, than in the range of external pH from 8.5–10.5, at which the doubling time was 38 min;

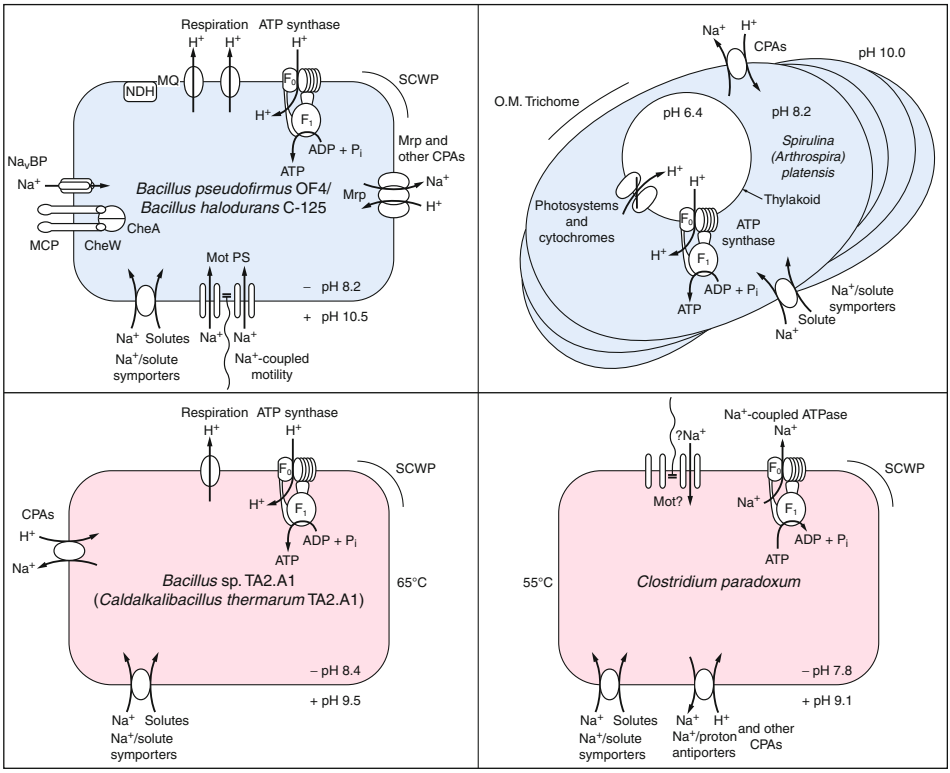


Fig. 2.6.1

Diagrammatic illustration of the pH homeostasis capacity of two alkaliphiles and two thermoalkaliphiles and elements of their membrane-associated Na^+ and H^+ translocation pathways. The cytoplasmic pH at a high external pH that supports optimum growth is shown in the top two panels for extreme alkaliphiles, *Bacillus pseudofirmus* OF4, *Bacillus halodurans* C-125 (which have comparable patterns), and *Spirulina platensis*, and in the bottom two panels for two thermoalkaliphiles, aerobic *Bacillus* sp. TA2.A1 and anaerobic *Clostridium paradoxum*. Elements of their Na^+ and H^+ pathways are shown schematically and are described in the text. The two extremely alkaliphilic *B. pseudofirmus* OF4 and *B. halodurans* C-125 have robust Mrp-dependent pH homeostasis and have voltage-gated sodium channels that play a role in Na^+ circulation in support of pH homeostasis as well as chemotaxis (Fujinami et al. 2009; Ito et al. 2004; Ren et al. 2001). For *C. paradoxum*, motility has been shown (Li et al. 1993) while Na^+-coupling of motility or of solute uptake have not yet been directly shown but are proposed (Ferguson et al. 2006). The arcs outside the membrane are shown to indicate secondary cell wall polymers (SCWP)(Schaffer and Messner 2005) or outer membrane (OM) and trichome layers (Ciferri 1983)

(2) the cytoplasmic pH was maintained at about pH 7.5 at external pH values $\leq$9.5. At more alkaline pH values the cytoplasmic pH rose, but still remained between 2.3 and 1.8 pH units lower than the outside pH values up to the highest pH tested, pH >11 (see *Fig. 2.6.2*); (3) at pH $\geq$10.5, the doubling time increased roughly in parallel with the increasing cytoplasmic pH; and (4) the cells were still capable of growth at pH 11.4, at which the cytoplasmic pH was 9.6.

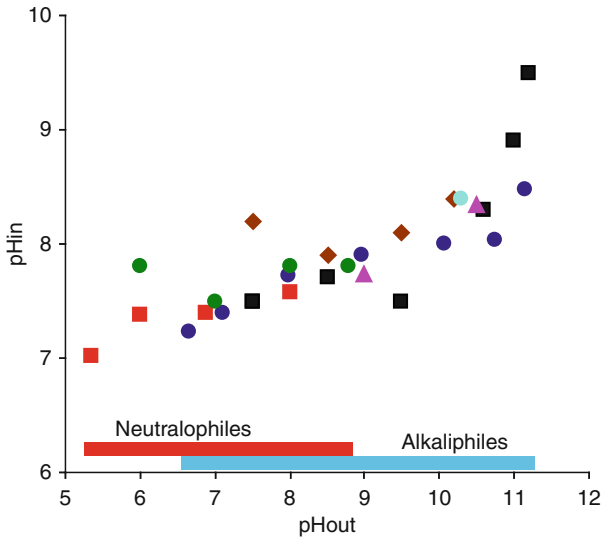


Fig. 2.6.2

Alkaline pH homeostasis by representative alkaliphilic and neutralophilic bacteria. Reported cytoplasmic pH data at the indicated external pH values are shown for two model neutralophilic bacteria, *B. subtilis* (■) (Shioi et al. 1980) and *Escherichia coli* (●) (Padan et al. 1981) and for the following alkaliphilic bacteria: *B. pseudofirmus* OF4 (■) (Sturr et al. 1994); *B. halodurans* C-125 (●) (Ito and Aono 2002); *B. cohnii* YN-2000 (◆) (Suigyama et al. 1986); *B. pseudofirmus* RAB (▲) (Kitada et al. 1982), *B. alcalophilus* ATCC 27647(●) (Hoffmann and Dimroth 1991)

The centrality of pH homeostasis for alkaliphiles is demonstrated by the direct relationship between the decrease in growth rate (monitored as increasing doubling time) and the increase in cytoplasmic pH beyond 8.2. It is also notable that, in keeping with the "no free lunch" principle, *B. pseudofirmus* OF4 grows better at pH 10.5, where the cytoplasmic pH is 8.2 than at pH of 7.5 where the cytoplasmic pH is 7.5. The optimal cytoplasmic pH for typical neutralophiles is 7.5–7.6 (see the *Escherichia coli* and *B. subtilis* patterns in *Fig. 2.6.2*) (Padan et al. 2005; Slonczewski et al. 2009). For *B. pseudofirmus* OF4, an optimal growth rate is observed together with a cytoplasmic pH of 7.5 only when the external pH is higher than 7.5, that is, in the pH 8.5–9.5 range. This suggests that at external pH values near neutral, factors on the outer surface limit growth of this alkaliphile.

Growth of Extreme Alkaliphiles at Alkaline Cytoplasmic pH Values Not Tolerated by Neutralophiles

An unanticipated finding was that optimal growth of alkaliphiles would occur at cytoplasmic pH values such as 8.2 (Sturr et al. 1994) (see *Fig. 2.6.1*). When neutralophilic bacteria such as *E. coli* and *B. subtilis* are exposed to alkaline conditions that lead to cytoplasmic pH values above 8, growth arrest results (Padan et al. 2005; Slonczewski et al. 2009). Thus it was even more surprising that slower but significant growth of *B. pseudofirmus* OF4 persists when the cytoplasmic pH is as high as 9.6 (*Fig. 2.6.2*) (Krulwich et al. 2007; Sturr et al. 1994).

We hypothesize that extreme alkaliphiles have adaptations of multiple cytoplasmic components that underpin this remarkable ability to tolerate high cytoplasmic pH values. Evidence for this hypothesis comes from the observation of apparent adaptations that foster alkaline stability and functional integrity of the cytoplasmic enzyme phosphoserine aminotransferase in both *B. alcalophilus* and *B. circulans* ssp. *alcalophilus* (Dubnovitsky et al. 2005; Kapetaniou et al. 2006) (and see Chap. 2.7 Bioenergetics: Cell motility and chemotoxis of extreme alkaliphiles). Much more investigation into the adaptations of the cytoplasmic components of the cells and their relative importance in extreme alkaliphily will be of great interest. We hypothesize that some of these adaptations of cytoplasmic components lead to poorer growth of extreme alkaliphiles at near neutral pH. For example, key processes such as translation, transcription, or function of the divisome may be optimized for a cytoplasmic pH >7.5–8.5.

Na^+/H^+ Antiporters: Key Participants in Cytoplasmic pH Homeostasis of Alkaliphiles

In spite of their tolerance of unusually high cytoplasmic pH values, alkaliphiles must maintain the cytoplasmic pH well below the external pH at the alkaline edge of their pH range for growth. Mutations that inactivate the major mechanism of alkaline pH homeostasis are either lethal to extremely alkaliphilic bacteria, as is apparently the case with *B. pseudofirmus* OF4 (Swartz et al. 2005), or render them non-alkaliphilic, as is the case for *B. halodurans* C-125 (Hamamoto et al. 1994). This major mechanism of pH homeostasis is the active uptake of H^+ mediated by Na^+/H^+ antiporters (exchangers). Na^+/H^+ antiporters are membrane transport systems that are energized by the substantial transmembrane electrical potential ($\Delta\Psi$) across the cytoplasmic membrane (indicated by the +/− in *Fig. 2.6.1*). The $\Delta\Psi$ is generated by active ion extrusion by primary ion pumps such as the respiratory chain components (see top two panels of *Fig. 2.6.1*), light-driven cation pumps, ATPases that extrude cations (as in *C. paradoxum*, see *Fig. 2.6.1*, *bottom right*), or membrane-embedded exergonic enzymes whose activity is coupled to cation extrusion (von Ballmoos et al. 2009). Thus Na^+/H^+ antiporters are secondary active transporters. The bacterial Na^+/H^+ antiporters that play a role in cytoplasmic H^+ accumulation, relative to the outside milieu, specifically extrude cytoplasmic Na^+ in exchange for H^+. A greater number of H^+ are taken up than Na^+ extruded, that is, H^+ taken up $>Na^+$ extruded, so that the overall exchange is electrogenic, with net positive charge moving inward during each turnover of the antiporter. This makes it possible for the antiporter-mediated H^+ uptake that is coupled to Na^+ efflux to be energized by the negative-inside transmembrane potential (see *Fig. 2.6.1*). In neutralophilic bacteria such as *E. coli* and *B. subtilis,* both $Na^+(Li^+)/H^+$ and K^+/H^+ antiporters participate significantly in alkaline pH homeostasis, whereas in the alkaliphilic bacteria studied to date, $Na^+(Li^+)/H^+$ antiporters have an essential, dominant, and perhaps exclusive role in this central physiological function (Hanhe et al. 2009; Padan et al. 2005; Slonczewski et al. 2009). Na^+ is therefore required for alkaliphile growth and pH homeostasis.

Although K^+/H^+ antiporter activity has been shown for members of the cation/proton antiporter complements of alkaliphiles (Fujisawa et al. 2007; Mesbah et al. 2009; Wei et al. 2007), we know of no evidence from mutants or expression data for their involvement in the specific process of alkaline pH homeostasis in alkaliphiles. The use of Na^+ as the coupling ion is advantageous because Na^+ accumulation is cytotoxic in most bacteria; that cytotoxicity is elevated at high pH (Padan et al. 2005; Wei et al. 2007). Therefore, it is advantageous to use Na^+

to support alkaliphily via pH homeostasis since it concomitantly prevents cytotoxic accumulation of Na^+. It is further advantageous because of the inwardly directed electrochemical gradient of Na^+ that is generated by the high activity of Na^+/H^+ antiporters. This "sodium motive force" (smf) is vitally important as a mode of energizing other bioenergetic work, such as ion-coupled solute uptake, toxin extrusion, and motility under conditions in which the protonmotive force (pmf, the transmembrane electrochemical gradient of protons) is low, as discussed below. We have further hypothesized that use of K^+/H^+ antiporters for alkaline pH homeostasis in extreme alkaliphiles would be problematic and there are probably mechanisms to preclude its use at very alkaline pH. Because extraordinarily high levels of antiport activity are required for alkaliphile growth at very high pH, use of K^+/H^+ antiporters would create the risk of lowering the levels of cytoplasmic K^+. Such reductions in cytoplasmic $[K^+]$ would be cytotoxic because sufficient levels of this cation are required for optimal function of many cytoplasmic proteins. In addition, the cytotoxicity of Na^+ is exacerbated when K^+ is depleted (Padan et al. 2005; Wei et al. 2007).

Redundancy in the Cation/Proton Antiporter Complements of Alkaliphiles, and the Importance of Mrp-Type Antiporters in Alkaliphily

With rare exceptions, individual bacterial strains possess multiple cation/proton antiporters (CPAs) that exchange Na^+, Li^+, K^+ , or Ca^{2+} or some combination of these cytoplasmic cations for external H^+ (Krulwich et al. 2009; Padan et al. 2005; Slonczewski et al. 2009). For non-marine bacteria, a typical range is 5–9 CPAs from several distinct antiporter families, as characterized by sequence-based analyses in the Transporter Classification system (Ren et al. 2007; Saier 2002). For example, the *B. subtilis* complement is 6–7 antiporters and the complement from *E. coli* contains a total of 8 CPAs (*Table 2.6.1*). While the total CPA antiporter complement of most of the examined alkaliphiles is within the range of neutralophiles, two alkaliphiles stand out as exceptions. *B. pseudofirmus* OF4 has an unusually high number of antiporters, with a total of 12, principally because of the large number of NhaC candidate proteins in its genome (6). The organism with the largest complement, however, is the poly-extremophilic *N. thermophilus* (*Table 2.6.1*). We have hypothesized that bacteria that are challenged by alkaline pH as their central and only extreme challenge rely heavily on a single, especially adapted antiporter, a Na^+/H^+ antiporter with the requisite properties to meet that challenge (Krulwich et al. 2009). Poly-extremophiles, on the other hand, have an overlapping set of challenges in which antiporters may play a role, necessitating deployment of many more antiporters that play roles under conditions in which the different aspects of the extremophily dominate.

CPA3 family members are widespread among both Gram-negative and Gram-positive bacteria (as well as archaea) although they are absent in largely fermentative bacteria whose ecological challenge is more commonly acid than alkali, for example, enteric bacteria, streptococci, and lactobacilli (Swartz et al. 2005). The common themes that emerge from studies of CPA3, Mrp-type antiporter roles and/or expression patterns in neutralophiles and alkaliphiles are alkali and salt resistance (Hanhe et al. 2009; Kosono et al. 2005; Krulwich et al. 2007; Swartz et al. 2005; Swartz et al. 2007). One Mrp-type antiporter system, Pha1 of *Sinorhizobium meliloti*, catalyzes both K^+/H^+ and Na^+/H^+ antiport activities and the former activity has a physiological role under nitrogen fixation conditions (Putnoky et al. 1998; Yamaguchi et al. 2009). In extremely alkaliphilic *Bacillus* species, *B. halodurans* C-125 and *B. pseudofirmus* OF4, and in the alkaliphilic cyanobacterium, *Anabaena* sp. PCC7120, mutational evidence indicates

Table 2.6.1

Cation/proton antiporter (CPA) candidates for $Na^+(K^+)(Ca^{2+})/H^+$ antiport capacity as revealed by genomic analyses of selected alkaliphilic and two model neutralophilic bacteria[a]

Organism	Alkaliphile/ neutralophile	Gram + or −	CPA1	CPA2	CPA3	NdhF-a[b]	NhaA	NhaB	NhaC	NhaD	CaCA[a]	Total
Bacillus pseudofirmus OF4	Extreme alkaliphile	+	3	2	1	0	0	0	6	0	0	12
Bacillus halodurans C-125	Extreme alkaliphile	+	1	1	1	0	0	0	2	0	0	5
Bacillus clausii KSM-K16	Moderate alkaliphile	+	1	0	2	0	0	0	4	0	0	7
Alkalilimnicola ehrlichei MLHE-1	Moderate alkaliphile	−	2	0	2	0	0	0	0	0	0	4
Natranaerobius thermophilus JW/NM-WN-LF[c]	Moderate alkaliphile; poly-extremophile	+	2	2	1	1	0	0	11	0	0	17
Synechocystis sp. PCC 6803	Moderate alkaliphile	−	3	3	1[d]	0	0	0	0	0	1	8
Bacillus subtilis subsp. subtilis str. 168	Neutralophile	+	1	2	1[d]	(1)	0	0	2	0	0	7(6)
Escherichia coli K-12 str. K-12 substr. MG1655	Neutralophile	−	2	3	0	0	1	1	0	0	1	8

Antiporters of three major antiporter categories that are in the Transporter Classification system are found in the representative bacteria shown: CPA-types, cation/proton antiporters that are single or two-gene product antiporters (CPA1 or CPA2) or hetero-oligomeric Mrp-type CPA3 antiporters that contain 6–7 hydrophobic proteins; Nha antiporters that are hydrophobic monomer or dimer products of a single gene; and a subset of the CaCA family antiporters that may have monovalent cation/proton antiport activity. The NdhF-a proteins include Nt-Nha antiporter from *N. thermophilum* and a homologue from *B. subtilis* that is unlikely to have antiport activity, as discussed in the text. Therefore, the total number of CPAs listed for *B. subtilis* indicates in parentheses that it should perhaps not be counted. Similarly, the two NhaD antiporter candidates in the draft sequence of *B. pseudofirmus* OF4 may instead be arsenite transporters and parentheses are again used to indicate this possibility that the CPA total would be 8 instead of 10 for this alkaliphile.

[a]Antiport data are taken from www.membranetransport.org (all organisms except *N. thermophilus, Synecoccus sp.* PCC 7002, and *B. pseudofirmus OF4*, the latter data being derived from a draft genome sequence) and supplemented with searches of annotated genomes in www.ncbi.nlm.nih.gov/genomes/lproks.cgi using antiporters or antiporter subunits from the different types. CaCA members shown to be Ca^{2+}-specific or that are apparent Na^+/Ca^{2+} antiporters are omitted.

[b]This transporter type is not classified in www.membranetransport.org but is described in (Krulwich et al. 2009).

[c]*N. thermophilus* is the only anaerobe in this table.

[d]Not listed in www.membranetransport.org.

that Na^+/H^+ antiport-specific members of the Mrp-type antiporters are crucial for alkaline pH homeostasis in the upper ranges of pH (Hamamoto et al. 1994; Swartz et al. 2005). There is a striking continuum with respect to the dominance of the Mrp role in the alkaline pH homeostasis capacity of these alkaliphiles and neutralophilic *B. subtilis*. In *B. pseudofirmus* OF4, the Mrp antiporter is apparently necessary for viability (Swartz et al. 2005). In *B. halodurans* C-125, its mutation leads to an inability to grow at pH values above 9 and also to increased sensitivity to inhibition by Na^+ (Hamamoto et al. 1994). In *Anabaena* sp. PCC7120 disruption of Mrp leads only to the inability to grow at pH values above 10 as well as a pronounced increase in Na^+ sensitivity (Blanco-Rivero et al. 2005). In neutralophilic *B. subtilis*, loss of Mrp function leads to a modest increase in alkali sensitivity but a very pronounced sensitivity to inhibition by Na^+ (Ito et al. 1999; Ito et al. 2000; Kosono et al. 1999; Swartz et al. 2005). Thus Mrp antiporters in extreme alkaliphiles are particularly adapted to essential roles at very high pH, at which pH homeostasis is the central challenge to viability. They also play roles in Na^+-resistance that are not essential to viability; other antiporters of the alkaliphile complement may contribute substantially to Na^+-resistance, for example, NhaC of *B. pseudofirmus* OF4 (Ito et al. 1997). It is notable that as the essentiality of a Mrp system in alkaline pH homeostasis declines in this series of organisms, its relative role in Na^+-resistance increases. The Mrp continuum across these bacterial examples seems, in this way, to illustrate the "no free lunch" principle at the molecular level. Interestingly, all eight antiporters from *N. thermophilus* that have been assayed in vitro so far (2 CPA1s, 1 CPA2, 1 Nt-Nha, and 4 NhaCs) have a capacity for K^+/H^+ antiport (Mesbah et al. 2009). By contrast, as noted above, the Mrp system of the more extremely alkaliphilic *B. pseudofirmus* OF4 as well as the homologous system called Mnh from the highly alkaline-tolerant *Staphylococcus aureus* are highly specific for $Na^+(Li^+)/H^+$ antiport and do not catalyze K^+/H^+ antiport (Swartz et al. 2007). Again, the multiple stresses that must be managed by poly-extremophilic *N. thermophilus* may limit the adaptive constraints it can put on antiporter-based pH homeostasis without decreasing its responses to salt and temperature challenges.

What is unique about Mrp systems that may make them adaptable to a special role in alkaline pH homeostasis? The most intensively studied Mrp antiporter systems to date are from either the Group 1 or Group 2 Mrp systems (Swartz et al. 2005). Group 1 systems are encoded by operons that contain seven genes, each of which has a hydrophobic membrane protein product, MrpA-G proteins. Group 2 systems have a fused MrpA and B so that the operons have six genes encoding six hydrophobic products, MrpA', C-G. Two properties of Group 1 and 2 systems led to their classification in their own category, the CPA3 family of the Transporter Classification system. First, the two largest Mrp proteins, MrpA and MrpD, share significant homology with a sub-complex of membrane-embedded subunits of the H^+-pumping respiratory chain Complex I (NADH dehydrogenase) that is thought to be involved in H^+ translocation (Mathiesen and Hagerhall 2002). They share an oxidoreductase motif with these Complex I subunits, for example, NuoL, M, and N in the *E. coli* nomenclature, that also have homologues among ion-pumping bacterial hydrogenases (Friedrich and Weiss 1997; Swartz et al. 2005). Similarity between MrpC and the NuoK of Complex I has also been cited, albeit less striking than between MrpA and D and its homologues among subunits of Complex I and hydrogenases (Mathiesen and Hagerhall 2003). *Figure 2.6.3* shows an unrooted tree based on alignments of the family of MrpA/MrpD/NuoL/NuoM/NuoN proteins (as well as Nt-Nha and homologues thereof that are discussed further below). The MrpA proteins (which are larger than MrpD proteins, i.e., close to 90 KDa versus the approximate 55 KDa size of MrpD proteins) are more closely related to NuoL of Complex I than to most

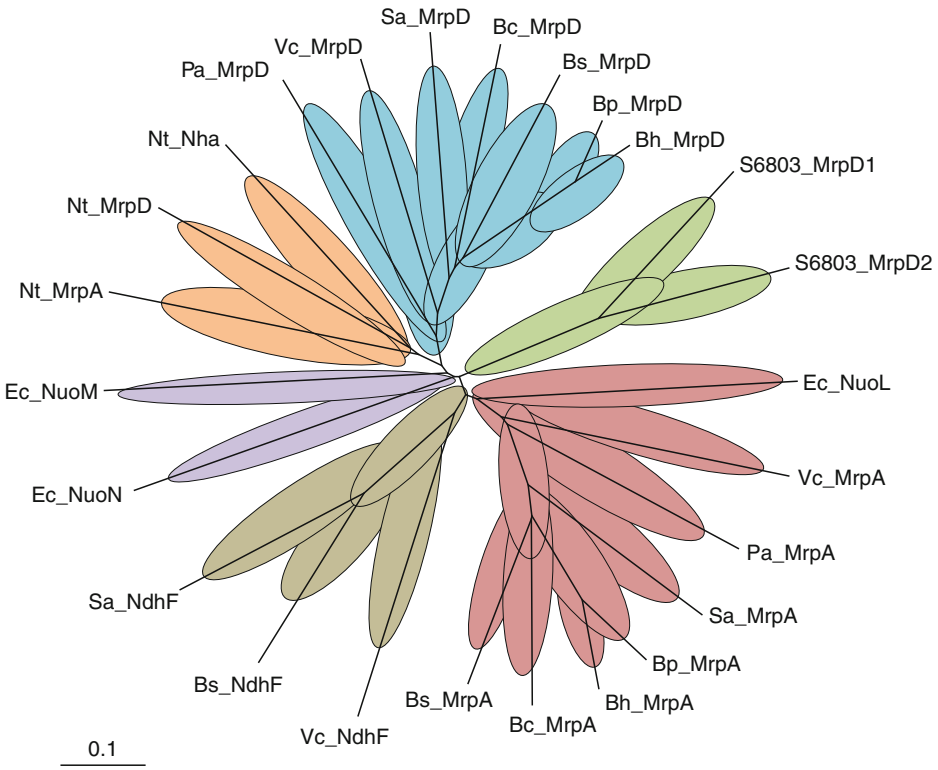


Fig. 2.6.3

The family of antiporter proteins MrpA, MrpD, and Nt-Nha; Nt-Nha homologues; and respiratory chain complex I proteins NuoL, NuoM, and NuoN. Unrooted tree (TreeView) of ClustalW (DSGene) analysis shows relationships between the two large subunits of Mrp antiporters encoded in diverse *mrp* operons, the Nt-Nha antiporter protein, three neutralophile homologues of MrpD/MrpA/Nt-Nha that, as discussed in the text, are unlikely to have cation/proton antiport activity, and the homologues NuoL, M and N subunits of the respiratory Complex I (H^+-translocating NADH dehydrogenase) from *E. coli*. The bacterial species and accession numbers for the protein sequences used are: Bc, *B. clausii* KSM-K16, MrpA (YP_174287.1), MrpD (YP_174290.1); Bh, *B. halodurans* C-125, MrpA (NP_242185.1), MrpD (NP_242182.1); Bp, *B. pseudofirmus* OF4, MrpA (AAF21812.2), MrpD (AAF21815.2); Bs, *B. subtilis* subsp. subtilis str N16961, MrpA (NP_391038.2), MrpD (NP_440574.1), NdhF (NP_388064); Ec, *E. coli* K-12 MG1655, NuoL, NuoM, NuoN (NP_416781.1, NP_416780.1 and NP_416779.2); Nt, *Natranaerobius thermophilus* JW/NM-WN-LF, MrpA (YP_001916693.1), MrpD (YP_001916692.1), Nt-Nha (YP_001916294.1); Pa, *Pseudomonas aeruginosa* PA)1, MrpA (NP_249745.1), MrpD (NP_232555.1): S6803, *Synechocystis* 6803, MrpD1 and MrpD2 (NP_440572.1, NP_440574.1); Sa, *Staphylococcus aureus* subsp. aureus COL (in which MrpA and MrpD are usually called MnhA and MnhD (Hiramatsu et al. 1998)), MrpA (YP_185821.1), Mrp D (YP_185821.1), NdhF (YP_185382.1); Vc, *Vibrio cholera* O1 biovar El Tor str. M16961, MrpA (NP_232557.1), MrpD (NP_232555.1), NdhF (NP_231221.1)

MrpD proteins and the NuoM and NuoN pair of Complex I proteins. The exception is the MrpA that is encoded in an operon in *N. thermophilus* but is significantly smaller and lacking a "MrpB-like" domain commonly found in operon-encoded MrpA proteins. The *N. thermophilus* MrpA as well as most MrpD proteins are more closely related to NuoM and NuoN; the NuoM and NuoN subunit pair from Complex I are also more closely related to each other than they are to NuoL. Only the two cyanobacterial *Synechococcus* strain 6803 MrpD proteins cluster closer to the NuoL than to NuoM and NuoN, consistent with several points of divergence of cyanobacterial Mrp operon patterns from the Group 1 and Group 2 patterns (Krulwich et al. 2007; Swartz et al. 2005).

The second property that was recognized shortly after Mrp antiporter systems were discovered is that antiport activity requires all of the 6–7 gene products of the Group 1 or 2 *mrp* operon. This was first shown for the Mnh antiporter of the CPA3 family from *S. aureus* using in vitro assays of truncated products of a full cloned *mnh* operon expressed in an antiporter-deficient *E. coli* strain (Hiramatsu et al. 1998). Subsequently, nonpolar deletions were made in each gene of the *B. subtilis* chromosomal copy of the *mrp* operon (Ito et al. 2000) and in a cloned copy of the *B. pseudofirmus* OF4 *mrp* operon expressed in an antiporter-deficient *E. coli* strain (Morino et al. 2008). Studies of both of these mutant panels confirmed the need for every Mrp protein for significant antiport activity. The only modest exception was a small residual antiport activity in the absence of MrpE (Morino et al. 2008; Yoshinaka et al. 2003). The requirement for 6–7 hydrophobic proteins and the similarity of three of the Mrp proteins to subunits of Complex I and hydrogenases that form sub-complexes led to the expectation that Mrp functions as a hetero-oligomeric complex (Hiramatsu et al. 1998). This was confirmed by demonstration of complexes containing each of the 7 Mrp proteins, first for *B. subtilis* Mrp (Kajiyama et al. 2007) and then for *B. pseudofirmus* OF4 Mrp (Morino et al. 2008). Although the analyses of the neutralophile Mrp complex and the alkaliphile Mrp complex were conducted under different conditions and with different detection strategies, both preparations contained hetero-oligomeric Mrp complexes that included all 7 Mrp proteins and were of a size close to the expected size of a complex with one copy of each Mrp protein. However, the alkaliphile samples also contained larger species, including a significant amount of a putative full Mrp dimeric hetero-oligomer (Morino et al. 2008). We hypothesize that the unusual hetero-oligomeric Mrp proteins are a consortium of transporters that may include two $Na^+(K^+)/H^+$ antiporters, or two Na^+/H^+ antiporters in extreme alkaliphiles, as well as other transporter proteins that function synergistically with the antiporters. Additional activities would account for several reports of transport capacities of Mrp systems that are not related to antiport (Dzioba-Winogrodzki et al. 2009; Kashyap et al. 2006). The large hetero-oligomeric Mrp complex is hypothesized to support antiport at high external values of pH by presenting a large protein surface on the outside surface of the membrane. Such a surface could be engineered to be an effective proton-gathering element, that could resemble, for example, a larger and more extensive version of proton-gathering funnels observed in the high resolution crystal structure of *E. coli* antiporter NhaA (Padan 2008). Larger, dimeric hetero-oligomeric complexes observed in alkaliphiles might be better adapted to serve this role than monomeric Mrp complexes.

The requirement for all 6–7 gene products of operon-encoded Mrp systems in order to observe antiport has made it difficult to test hypotheses that MrpA and MrpD are the actual antiporter proteins (Mathiesen and Hagerhall 2002) or a more recent suggestion that MrpA,

MrpD, and MrpB constitute the critical catalytic core (Kajiyama et al. 2009). Therefore, the recent study of the antiporter complement of *N. thermophilus* (Mesbah et al. 2009) that included Nt-Nha constitutes a breakthrough. Nt-Nha is a MrpD/MrpA-like protein that has antiport activity without any additional Mrp-like proteins. Nt-Nha is more closely related to MrpD than MrpA (› *Fig. 2.6.3*). It lacks the MrpB domain that is found in the MrpA but not MrpD proteins encoded in Group 1 and Group 2 operons. The MrpB domain is not present in the MrpD-like homologues of cyanobacterial and the *N. thermophilus* operon-encoded Mrp systems; these subunits are sometimes (probably erroneously) annotated as MrpA. The fact that Nt-NhaA catalyzes $Na^+(K^+)/H^+$ antiport without any other Mrp proteins supports the idea that a MrpD/MrpA-like protein is the antiporter and argues against the absolute essentiality of any other Mrp proteins to produce the antiport activity. There are other examples of bacterial genes that are not in an apparent *mrp* operon but encode MrpA/MrpD/Nt-Nha-like proteins. The genes are most commonly annotated as NdhF because they are closely related to genes found in cyanobacterial and plant gene clusters that encode ion-pumping NADH dehydrogenases (Battchikova and Aro 2007). The three examples shown in › *Fig. 2.6.3*, from *B. subtilis*, *S. aureus*, and *V. cholerae* are not themselves likely to be active $Na^+(K^+)/H^+$ antiporters since they lack one or more residues that have been shown to be essential for such activity (Kajiyama et al. 2009). These proteins may catalyze an antiport activity with different substrates and/or their activity may depend upon modulation by a conserved protein that is not a hydrophobic Mrp protein but is predicted to be co-transcribed with their genes. Currently, Mrp systems constitute a structure-function puzzle of central interest with respect to alkaliphile pH homeostasis mechanisms and the Nt-Nha antiporter may be one productive avenue to gain insights that will suggest experiments with the larger more complex Mrp systems.

Additional Structural, Enzymatic, and Metabolic Strategies for Cytoplasmic pH Homeostasis, and Their Built-In Redundancies

Extreme alkaliphiles employ additional strategies to supplement the contribution of antiporter-dependent pH homeostasis. Secondary cell wall polymers (SCWP, see › *Fig. 2.6.1*), especially teichuronopeptides, play a major role in supporting pH homeostasis in extremely alkaliphilic *B. halodurans* C-125 (Aono et al. 1999). By contrast, the slightly more robustly alkaliphilic *B. pseudofirmus* OF4 lacks comparable SCWPs. *B. pseudofirmus* OF4 expresses an acidic S-layer both at pH 7.5 and 10.5. Interestingly, deletion of the *slpA* gene that encodes the S-layer has only a modest adverse effect on the response of the alkaliphile to a sudden alkaline shift, that is, the S-layer confers a modest advantage for alkaliphily. Growth of the *slpA* mutant at pH 7.5 is greatly enhanced relative to the wild type, indicating that expression of *slpA* at near neutral pH is quite detrimental to the alkaliphile (Gilmour et al. 2000; Krulwich et al. 2007). This is a cogent example of the "no free lunch" idea that there is a palpable cost to this alkaliphile, with respect to growth at near neutral pH, for its expression of a gene that modestly optimizes its readiness for a transition to the extreme condition of alkaline pH.

Metabolic strategies are used by neutralophilic bacteria when challenged with alkali. A major one is up-regulation of deaminases that produce cytoplasmic acids (Slonczewski et al. 2009). This might be a problematic strategy for extreme alkaliphiles because the ammonia released would be protonated and could thus tend to accumulate in the cytoplasm under the prevailing condition of a higher cytoplasmic $[H^+]$ than the external medium during growth in the highly alkaline pH range. Use of deaminases to produce acids that lower cytoplasmic pH

would depend upon robust ammonium ion extrusion systems. Such systems have been suggested but their adequacy to support a robust deaminase strategy of pH homesotasis will require further investigation (Fujisawa et al. 2007; Wei et al. 2003). Fermentations that produce metabolic acids can also spare the use of energy-dependent antiport (Krulwich et al. 2007). The general physiology of alkaliphiles is reviewed elsewhere in this handbook (Chap. 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles)

A Low Bulk Protonmotive Force (pmf) Resulting from Successful pH Homeostasis, and Its Bioenergetic Consequences

The pmf energizes H^+-coupled "bioenergetic work" such as ion-coupled solute transport or toxin efflux, flagellar motility, and ATP synthesis using an F_1F_0-ATP synthase. This transmembrane electrochemical proton gradient is composed of two components. In the orientation that is productive for energization of H^+-coupled bioenergetic work, these pmf components are an electrical component ($\Delta\Psi$, inside negative relative to the outside) and a chemical component (ΔpH, inside alkaline relative to the outside) (Mitchell 1961). However, because of successful pH homeostasis, the ΔpH component is in the reverse orientation at the high end of the alkaliphiles' pH range for growth, that is, the cytoplasm is acidic relative to the outside pH. Moreover, in alkaliphiles studied to date, the $\Delta\Psi$ increases at high pH but not enough to offset the adverse effect of the reversed ΔpH on the pmf. As a result the bulk pmf is much lower in facultative alkaliphiles at the upper edge of their pH range than at near neutral pH, for example, three times lower in *B. pseudofirmus* OF4 during growth at pH 10.5 than during growth at pH 7.5 (Krulwich et al. 2007; Sturr et al. 1994). The low pmf problem is particularly acute because the cost of growing at pH 10.5 is higher because of the need for high levels of $\Delta\Psi$-driven antiport and because there is a proton involved in the catalytic reaction by which ATP is synthesized, which makes the reaction less favorable at high pH. Still *B. pseudofirmus* OF4, grows as well at pH 10.5 as at pH 7.5, with comparable molar growth yields and even faster generation times (Guffanti and Hicks 1991; Sturr et al. 1994). For solute-coupled uptake and motility (see Chap. 2.6 Adaptive mechanisms of extreme alkaliphiles), a major strategy of alkaliphiles, including many cyanobacteria, is use of Na^+-coupling instead of H^+-coupling as indicated in *Fig. 2.6.1* (Krulwich et al. 2007; Peddie et al. 2000). The sodium motive force (smf) is larger than the pmf at highly alkaline pH since the antiport that establishes the "reversed" ΔpH concomitantly generates an inwardly-directed chemical gradient of Na^+ that adds to the significant, productively oriented $\Delta\Psi$ to make a large smf. Na^+-coupled transporters of neutralophiles often work side-by-side with H^+-coupled solute transporters (see *Fig. 2.6.1*) or can couple to both cations (Zani et al. 1993). By contrast, the Na^+-coupled solute transporters of extreme alkaliphiles cannot use H^+ and are instead inhibited at the elevated $[H^+]$ at near neutral pH. This accounts for the otherwise puzzling observation that a higher $[Na^+]$ is required in the medium for growth of *B. pseudofirmus* OF4 on malate, which is transported by Na^+-dependent transport, at pH 7.5 than at pH 10.5 (Gilmour et al. 2000; Krulwich et al. 2007). Some alkaliphiles may partially bypass this suboptimal property of alkaline-adapted Na^+-coupled solute transport at low pH, by employing transporters that are not coupled to either the pmf or smf. It was noted in the *B. halodurans* C-125 genome that this alkaliphile makes much greater use of ABC type, ATP-coupled transporters than found in neutralophilic *B. subtilis* (Takami et al. 2000).

Strategies for Oxidative Phosphorylation at Low Protonmotive Force

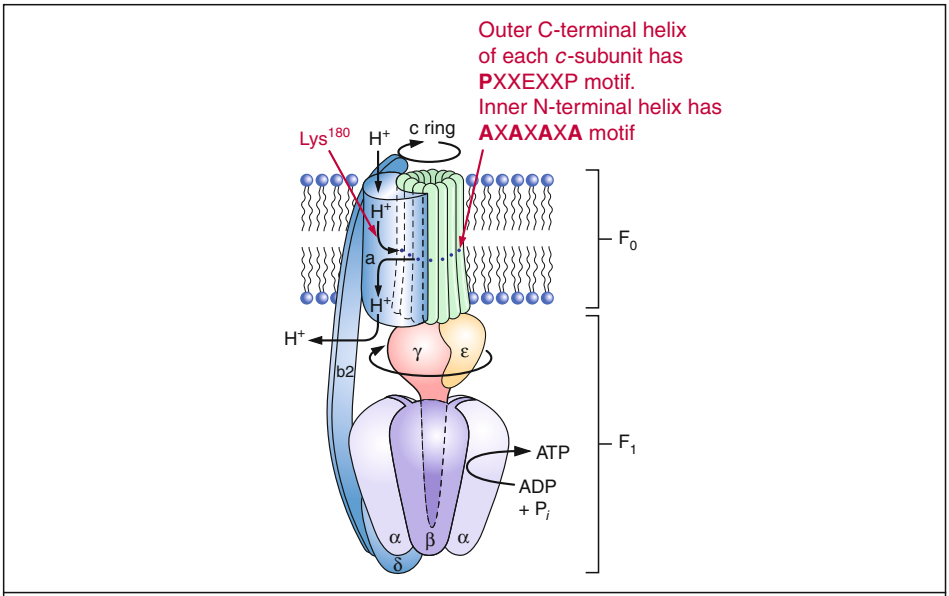
The biggest conundrum with respect to bioenergetic work is that both extreme and moderate alkaliphiles use H^+-coupled rather than Na^+-coupled ATP synthases (Krulwich et al. 2007; von Ballmoos et al. 2008). Na^+-coupled F_1F_0-ATPases are found in alkaliphilic anaerobes (see *C. paradoxum* panel in *Fig. 2.6.1*) that do not synthesize ATP with the enzyme under physiological conditions. They use it in the hydrolytic direction that generates an smf (Ferguson et al. 2006). Use of a Na^+-pumping ATPase that generates an smf fits the overall Na^+-based energetics, reduces Na^+ cytotoxicity, and also avoids cytoplasmic proton loss that would occur using a H^+-coupled ATPase at high pH. For cyanobacteria, use of a H^+-coupled ATP synthase for ATP synthesis may not be problematic because the low pmf problem applies to ATP synthases that function in the cytoplasmic membrane across which this pmf is the driving force for synthesis. Recent reports (Liberton et al. 2006; Nevo et al. 2007; Schneider et al. 2007) indicate that the thylakoid membranes in which the synthase is localized is not continuous with the cytoplasmic membrane. In that case, as shown in *Fig. 2.6.1*, the problem of low bulk pmf across the cytoplasmic membrane would not affect the energetics of ATP synthesis. On the other hand, the low pmf conundrum is a major issue for extreme alkaliphiles whose respiratory chains and ATP synthases, which constitute the machinery for oxidative phosphorylation (OXPHOS), are in the cytoplasmic membranes. The problem may be especially acute for those Gram-positive alkaliphiles that lack the outer membrane of Gram-negative bacteria or particularly protective SCWPs.

Solutions have been proposed to the conundrum of the robust H^+-coupled ATP synthesis observed in alkaliphiles at high pH and low bulk pmf and evidence for adaptations of the OXPHOS machinery has also emerged. Proposals that explain the discrepancy between the bulk pmf and ATP synthesis have focused on different ways in which the H^+ pumped by the respiratory chain may be sequestered from equilibration with the bulk medium. For example, it has been hypothesized that rapid transfers of pumped H^+ along the membrane surface allow the H^+ to reach the ATP synthase before they are equilibrated with the bulk phase outside the cell. These surface-sequestered H^+ would kinetically bypass the longer term thermodynamic inadequacy of the low $[H^+]$ concentration in the bulk phase since the effective near-membrane driving force would be higher than the low bulk pmf. Such proposals are similar to models of energization of bioenergetic work proposed originally by RJP Williams (Williams 1978). There are different recent models, including the suggestion based on computational modeling of forces near the membrane of interfacial barriers that promote retention of H^+ near the surface in a delocalized manner and the suggestion based on in vitro experimental models of H^+ microcircuits near the membrane that depend upon proximity of the pumps to the synthase and on properties of the membrane lipids (Branden et al. 2006; Mulkidjanian et al. 2005). Some form of sequestered H^+ translocation is likely to be a critical part of the alkaliphile solution to the problem of OXPHOS at low bulk pmf but the suggestions are currently hypotheses that still await more experimental data, especially in biological settings (Krulwich 1995; Krulwich et al. 2007). The alkaliphile OXPHOS capacity may, as predicted by the microcircuit hypothesis, rely upon close proximity of critical respiratory chain pumps, especially the terminal oxidases, to the ATP synthase; these partners in H^+ transfers during alkaliphile OXPHOS might be found in clusters (Goto et al. 2005; Liu et al. 2007). The high concentration of membrane cardiolipin has been suggested as a possible mediator of proximity and/or H^+ transfer itself (Haines and Dencher 2002).

A more firmly established facet of OXPHOS in alkaliphilic *Bacillus* species such as *B. pseudofirmus* OF4 is its dependence upon specific adaptations of the ATP synthase. There also appear to be parallel adaptations in the terminal oxidase of the OXPHOS respiratory chain component (Krulwich et al. 2007; Slonczewski et al. 2009). Such special adaptations of OXPHOS machinery in response to specific environmental challenges are in fact widespread (Ferguson and Ingledew 2008; von Ballmoos et al. 2008). Alkaliphile-specific sequence motifs have been identified in both the F_1F_0-ATP synthase (examples in *Fig. 2.6.4*, *top*) and the Cta caa_3-type cytochrome oxidase (examples in *Fig. 2.6.4*, *bottom*) (Ivey and Krulwich 1992; Quirk et al. 1993). In addition, unusual redox features of alkaliphile respiratory chain components have been described (Goto et al. 2005; Hicks and Krulwich 1995; Muntyan and Bloch 2008). Mutational work has not yet been conducted on the respiratory chain motifs but ATP synthase motifs have been shown to play important roles in OXPHOS at high pH and low pmf. For example, the "alkaliphile-specific" *a*-subunit Lys^{180} plays a critical role in synthesis at high pH and is proposed to be required for H^+ capture from the outside surface of the synthase and passage to the interface between the *a*-subunit and the *c*-ring rotor (see *Fig. 2.6.4*, *top*) (McMillan et al. 2007; Wang et al. 2004). There are also critical motifs on both helices of the hairpin-like *c*-subunits that constitute the oligomeric rotor; their mutational change to the consensus sequence for non-alkaliphiles severely compromises ATP synthesis at high pH (Liu et al. 2009; Wang et al. 2004). Part of the adaptive value of the ATP synthase motifs of alkaliphiles appears to be in preventing H^+ loss to the outside, since several mutations to the consensus sequence result in significant H^+ leakiness (Liu et al. 2009; Wang et al. 2004).

Another proposed adaptation that could address or partially address the alkaliphile OXPHOS conundrum relates to the number of *c*-subunits that comprise the *c*-ring rotor (*Fig. 2.6.4*, *top*). The stoichiometry of the *c*-ring varies among different organisms in which it has been determined from 10–15 (von Ballmoos et al. 2008; von Ballmoos et al. 2009). An especially high *c*-subunit stoichiometry would help address the problem of OXPHOS at low pmf (Meier et al. 2007; von Ballmoos et al. 2008). The thermoalkaliphile *Bacillus* sp.TA21.A1 has a stoichiometry of 13 subunits/ring, which is indeed toward the high end of the known range of stoichiometries, but even though the organism is a moderate alkaliphile, the stoichiometry does not account for the energetics observed (Matthies et al. 2009; Meier et al. 2007). There also does not appear to be a strong correlation between a high *c*-ring stoichiometry and OXPHOS at low pmf. The cyanobacterium *S. platensis* has the highest *c*-ring stochiometry reported to date, at 15 subunits/ring (Pogoryelov et al. 2005; Pogoryelov et al. 2009), even though the localization of its ATP synthase in intracellular thylakoids probably avoids the low pmf problem of alkaliphile OXPHOS that takes place in the cytoplasmic membrane (*Fig. 2.6.1*). The high stoichiometry in *S. platensis* may relate to other properties of the setting such as the relative contributions of the two pmf components (von Ballmoos et al. 2008; von Ballmoos et al. 2009).

Even if a somewhat high *c*-subunit stoichiometry plays, at most, a minor role in the alkaliphile OXPHOS condundrum, we note another point of interest connected with this stoichiometry. There is now a strong consensus that the stoichiometry of the *c*-ring apparently does not vary within a single organism under different conditions such as growth pH or growth substrate (Meier et al. 2005; von Ballmoos et al. 2008). If alkaliphiles follow the *Bacillus* sp. TA2. A1 trend of higher than average *c*-subunit stoichiometry, we would cite it as one more example of the "no free lunch" principle. A *c*-ring with a high stoichiometry supports a higher H^+/ATP stoichiometry for the ATP synthase and that, in turn, facilitates synthesis at low pmf. However, an ATP synthase with high H^+/ATP stoichiometry is a less economical energy converter than



	207	227	# acidic residues	# basic residues	Predicted pI
B. pseudofirmus OF4	E R D E Y D A W V E G M S A E V E E P - - T E		9	1	3.3
B. halodurans C-125	E R D D Y D A W V E G M M E A D A E P D T D D		11	1	3.0
B. clausii	E R D E Y D A W V E D M L A V E Q E A T - - A		8	1	3.3
B. amyloliquefaciens	P S K E F K Q W T K A M K N Y K H - T T D S G		2	6	10.8
B. pumilus	S Q D E F L G W T K K M A D Y K K P T S T K D		4	5	10.1
B. subtilis	S A K E F Q G W T K E M K N Y K S - T A E S D		4	4	7.5
B. thuringiensis	D E S E Y K K W L A D M K K I D G K K E V A S		6	6	7.4
G. kaustophilus	P R T E F D A W V E K M Q N A K K P V V T D P		4	4	7.5

Fig. 2.6.4

Examples of putative alkali-adaptive motifs in the ATP synthase and cytochrome oxidase involved in oxidative phosphorylation by extremely alkaliphilic *Bacillus* species. *Top*: The schematic diagram of a bacterial ATP synthase depicts an H^+-translocating synthase that is found in alkaliphiles. The membrane-embedded F_0 and cytoplasmic F_1 are indicated; they are, respectively the proton-conducting/rotor elements and the catalytic elements involved in ATP synthesis. The oligomeric ring of *c*-subunits, that is, the rotor for the rotary coupling mechanism for ATP synthesis is shown in *green*. The stator elements of the synthase are shaded in *blue*. As downward movement through the F_0 energizes synthesis, rotation of the *c*-ring in the direction shown leads to concomitant rotation of the interactive F_1 subunits γ and ε that result in a conformational change in the three catalytic sites (at the α/β interfaces). *Arrows* point to regions of three major adaptations of the F_0: *a*-subunit (*a*Lys^{180} in the putative H^+ uptake path) and *c*-subunits (the PXXEXXP and AXAXAXA motifs in the C- and N-terminal helices, respectively). *Bottom*: An alignment of a region of subunit II of *Bacillus* cytochrome *caa*$_3$ complexes that shows that this region in alkaliphiles (species in *blue*) is significantly more acidic than the same stretches found in neutralophiles. Acidic residues are shown in *red* and basic residues in *blue*. The acidic and basic residue composition of each species is shown on the right, along with the predicted isoelectric point (pI) of that region. The numbering refers to the mature form of the *B. pseudofirmus* OF4 subunit II

a synthase with a lower *c*-subunit stoichiometry (Pogoryelov et al. 2005). Therefore, the *c*-ring with 13 subunits/ring would help the alkaliphile ATP synthase function better at high pH and low pmf than if it had a stoichiometry of 10, but at near neutral pH and high pmf, the synthase would function less well than those of neutralophiles that have stiochiometries of 10 subunits/ring.

Conclusions

Adaptations of extreme alkaliphiles optimize growth and survival at pH values above pH 10 but these adaptations convey a cost at near-neutral pH. A fuller understanding of the range and utility of different adaptations will depend upon concerted "omics" efforts in a greater range of genetically tractable alkaliphiles so that mutations can be used to test hypotheses about structure-function and systems level relationships.

Cytoplasmic pH homeostasis is centrally important to alkaliphily and depends upon active H^+ accumulation by electrogenic, secondary cation/proton antiporters that in extremely alkaliphilic *Bacillus* use Na^+ as the efflux substrate coupled to H^+ uptake.

The tolerance of alkaliphiles for unusually high cytoplasmic pH values and a very limited amount of experimental data suggest that there are likely to be important adaptations of cytoplasmic components in extreme alkaliphiles. Bioinformatic approaches to the increasing number of fully sequenced genomes may lead to testable hypotheses about such adaptations and also to suggestions for components on the outside of alkaliphile cells whose poor function at near neutral pH contributes to limitations on growth in this range of pH for many alkaliphiles.

The hetero-oligomeric Mrp antiporter is the dominant cation/proton antiporter of extremely alkaliphilic *Bacillus* species examined to date. The basis of the complexity of these antiporters remains unknown. Experiments also should be designed to test the hypotheses that the large outer surface of the Mrp antiporter plays a role in H^+ capture at elevated pH. Roles for the other members of the antiporter complements still await definition.

Further experimental tests and models based on established parameters of alkaliphile energetics should also be developed to test various proposals of sequestered H^+ pathways from respiratory and other H^+ pathways to the ATP synthase both in alkaliphiles and beyond.

Progress has been made on alkaliphile-specific motifs of the ATP synthase that await correlation with structural data and parallel development of insights into apparent adaptations of the respiratory H^+ pumps. The hypothesized importance of specific membrane lipids, especially cardiolipin, also awaits experimental examination.

Acknowledgments

Work conducted in the authors' laboratories was supported by research grant GM28454 and the Systems Biology Center-NY grant P50-GM071558 from the National Institute of General Medical Sciences (T.A.K) and a grant from the 21st Century Center of Excellence program of the Ministry of Education, Culture, Sports, Science and Technology of Japan (M.I.).

Cross-References

- 2.1 Introduction and History of Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 2.5 General Physiology of Alkaliphiles
- 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles

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2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.7,

Preface

Alkaliphilic microorganisms are extremophiles that actively grow in an extremely alkaline environment and generally require sodium ions for growth (Krulwich 1995; Krulwich et al. 2007). There are many interesting and unresolved issues with respect to how alkaliphilic microorganisms adapt to their extremely alkaline environment (see also Chap. 2.5 General Physiology of Alkaliphiles). The mechanisms of this adaptation have been most extensively studied in *Bacillus* species. Data have been presented for the roles of Na^+/H^+ antiporters, which are present in the cell membrane, and of a barrier of negatively charged cell wall-associated macromolecules in the accommodation of the bacteria to the alkaline environment (Aono et al. 1995; Ito et al. 2004b; Krulwich et al. 2007; Krulwich et al. 2001b; Padan et al. 2005).

A sodium ion circuit through the cell membrane plays a critical role for adaptation of alkaliphilic *Bacillus* species grown at high pH. Na^+/H^+ antiporters can accomplish the acidification of the cytoplasm of alkaliphiles. When alkaliphiles are placed in a medium without Na^+ at pH 10, the intracellular pH quickly rises to the value of the outside pH (*Table 2.7.1*). However, when Na^+ is present in the external medium, the intracellular pH does not rise above pH 8.5 upon shifting to the more basic medium. Furthermore, mutants of alkaliphiles that cannot grow at pH values above 9 are defective in Na^+/H^+ antiporter activity. The antiporter is electrogenic ($H^+ > Na^+$) and driven by the transmembrane potential ($\Delta\Psi$) which is generated by the primary proton pumps of respiratory chains (Swartz et al. 2005). The Multi-subunit type Na^+/H^+ antiporter Mrp complex has a dominant role in alkaline pH homeostasis for alkaliphiles (Hamamoto et al. 1994; Swartz et al. 2005).

The sodium ion circuit is completed when sodium ions enter the cell via Na^+/solute symporters that are also driven by the $\Delta\Psi$. The usage of Na^+/solute symporters has the advantage that solute transport is driven by the sodium potential rather than the proton potential, the latter being low because of the inverted ΔpH ($pH_{in} - pH_{out}$). A sodium channel that opens at high pH was predicted to be an important alternative Na^+ reentry route for support of cytoplasmic pH homeostasis when solutes whose uptake is coupled to Na^+ are scarce (Krulwich 1995; Sugiyama 1995). Recent studies of flagellar-based motility and chemotaxis in these extremophiles have again identified participating components and raised questions that extend broadly (Fujinami et al. 2007a; Terahara et al. 2008). Thus, the

Table 2.7.1

Capacities of neutralophilic *Bacillus subtilis* and alkaliphilic *Bacillus pseudofirmus* OF4 for Na^+- or K^+-dependent pH homeostasis during a moderate or extreme alkaline shift in external pH

	Cytoplasmic pH, 10 min after a sudden shift				
	pH 7.5 → 8.5			pH 8.5 → 10.5	
Strain	CholineCl	NaCl	KCl	Na_2CO_3	K_2CO_3
B. subtilis 168	8.5	7.5	7.6	10.5	10.5
B. pseudofirmus OF4	8.5	7.5	8.4	8.2	10.5

For the pH 7.5 → 8.5 shift, washed logarithmic phase cells, grown on and energized with malate, were equilibrated at pH 7.5 in the presence of 100 mM cholineCl, KCl or NaCl before the external pH was rapidly raised to 8.5. For the pH 8.5 → 10.5 shift, comparable cells were equilibrated in 50 mM Na_2CO_3 or K_2CO_3 buffer at pH 8.5 before the external pH was rapidly raised to 10.5. In both sets of shift experiments, the cytoplasmic pH was determined after 10 min from a methylamine accumulation assay as described earlier (Cheng et al. 1996; Ito et al. 1999)

alkaliphile, as a "model system" of bioenergetic work, has entered the realm of swimming, a capacity of bacteria that underpins chemotaxis, the ability to move away from detrimental conditions and toward favorable ones, and underpins the ability to inhabit niches in animal hosts as well as diverse environmental settings, or exhibit complex behaviors (Miller et al. 2009a; Ottemann and Miller 1997).

Structure of Bacterial Flagellum

The bacterial flagellum is a complex rotary nanomachine that is composed of multiple copies of at least 25 different proteins. It consists of three parts: the helical filament, the basal body that is embedded in the cytoplasmic membrane, and the hook connecting the filament to the basal body (*Fig. 2.7.1*). The flagellum is made up of the protein flagellin. Flagellin is a protein that

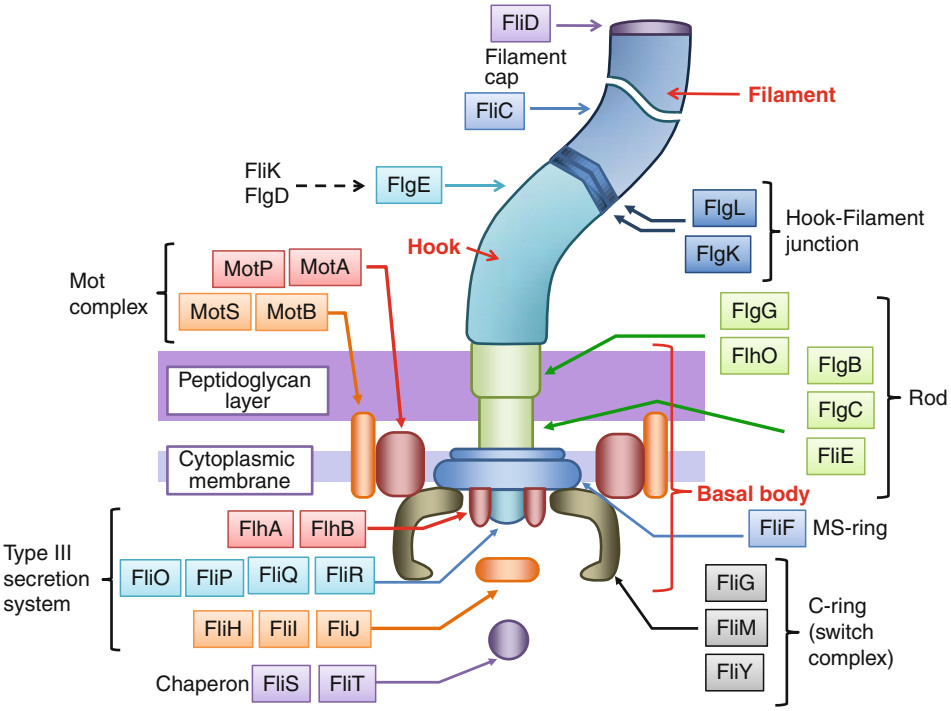

Fig. 2.7.1

The flagellum in Gram-positive bacteria that are conserved in many *Bacillus* species including alkaliphilic *Bacillus*. Gram-negative species like *Escherichia coli* have the LP-ring assembly. In *Bacillus* species, there is an FlhO protein that has been reported to substitute for FlgF (Proximal rod), FlgI (P ring), and FlgH (L ring) (Zuberi et al. 1991). An additional flagellar assembly factor, FliW, was reported in *B. subtilis* and *Campylobacter jejuni* (Titz et al. 2006). *B. subtilis* has the orthologs FliM and FliG, but instead of FliN this species has FliY, which is homologous to FliN only in its C-terminus (Bischoff and Ordal 1992). *B. subtilis* has two kinds of flagellar stator complexes, MotAB and MotPS. Some alkaliphilic *Bacillus* species have only a sodium-coupled MotPS type stator instead of proton-coupled MotAB type

arranges itself in a hollow cylinder to form the filament in the bacterial flagellum. Its shape is a 20 nm-thick hollow tube. Gram-positive bacteria have two basal body rings, one in the peptidoglycan layer and one in the cytoplasmic membrane. Gram-negative bacteria have four such rings: the L ring associates with the lipopolysaccharides, the P ring associates with peptidoglycan layer, the M ring is embedded in the cytoplasmic membrane, and the S ring is directly attached to the cytoplasmic membrane. The filament ends with a capping protein (Dioszeghy et al. 2004; Macnab 2003). The flagellar motor in the basal body consists of a rotor and stator that function comparably to those of a man-made motor (*Fig. 2.7.2*). The rotor encompasses a switch complex that is composed of the proteins FliG, FliM, and FliN, and is involved in the generation of the torque for flagellar rotation as well as in the control of the rotational direction either counterclockwise (CCW) or clockwise (CW), and in the assembly of the flagella (*Fig. 2.7.1*) (Berg 2003; Brown et al. 2002; Brown et al. 2005; Kubori et al. 1992; Lloyd and Blair 1997; Lloyd et al. 1999; Park et al. 2006b; Yamaguchi et al. 1986; Zhao et al. 1996). *B. subtilis* has the orthologs FliM and FliG, but instead of FliN this species has FliY, which is homologous to FliN only in its C-terminus (Bischoff and Ordal 1992). The membrane-embedded stator functions as an ion channel at the base of the flagella, and converts chemi-osmotic energy into mechanical energy (Blair 1995). MotA and MotB proteins were identified as the stator of a proton-driven motor of *Salmonella enterica Serovar Typhimurium* and *Escherichia coli* (Ridgway et al. 1977; Silverman et al. 1976; Stocker 1953). MotA has four transmembrane helices (TM1-TM4) and a large cytoplasmic domain between TM2 and TM3

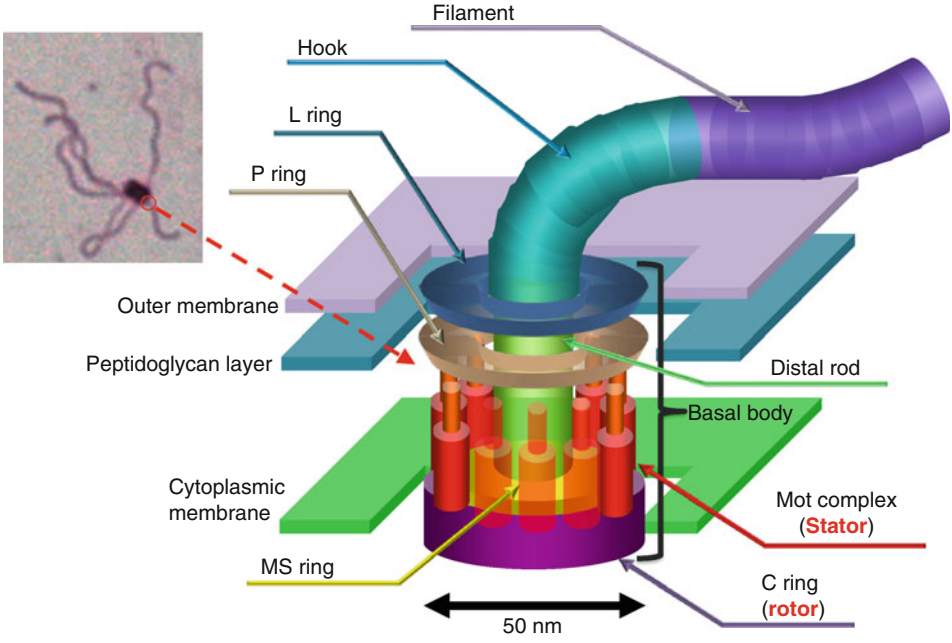

Fig. 2.7.2

Flagellum in the Gram-negative bacterium *Escherichia coli*. The diameter of the flagellar motor is about 50 nm. This bacterial rotary motor is a sophisticated nanomachine. Usually, bacterial rotary motors consist of a Stator part (in *red*) and a Rotor part (in *violet*). The Mot complex composes the stator of a flagellum and surrounds the rotor as a ring of about 8–11 particles

that is involved in generation of the torque by the electrostatic interactions with the C-terminal region of FliG (Zhou et al. 1998a) (Fig. 2.7.3). MotB has one TM and a large periplasmic domain that binds to the peptidoglycan and acts as the anchor (Chun and Parkinson 1988). The MotA/MotB complex is predicted to be a 4A:2B stoichiometry complex and to act as a proton channel (Blair and Berg 1990; Kojima and Blair 2004b). In addition, the maximum number of the MotA/MotB complexes in the flagellar motor of *E. coli* is at least 11 (Block and Berg 1984; Khan 1993; Reid et al. 2006).

Na^+-Coupled Stators of Alkaliphiles

The rotational energy of the flagellar motor is provided by the electrochemical potential of either protons or sodium ions across the cytoplasmic membrane (Berg 2003; Terashima et al. 2008). Initial studies of motility in Gram-positive bacteria showed that the rotational energy for the flagellar motor is provided by the electrochemical ion gradients across the cytoplasmic membrane, as had been established for Gram-negative bacteria. In these studies, motility of

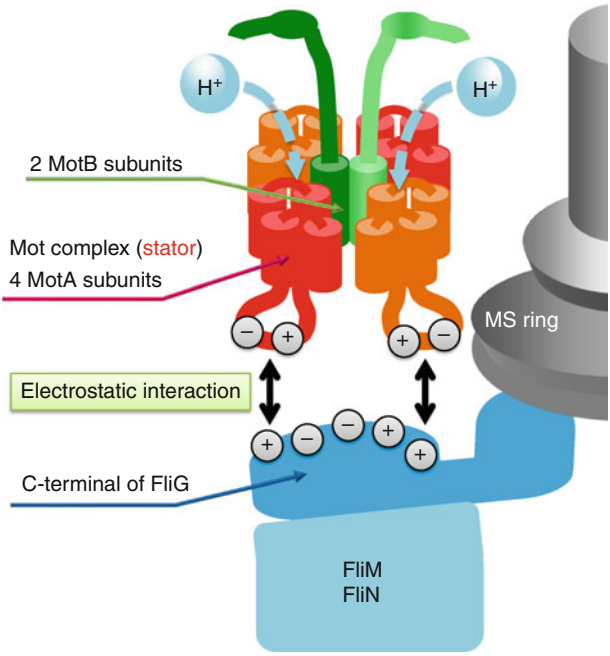

Fig. 2.7.3

The structure of stator and rotor of *Escherichia coli*. The MotA/MotB complex is predicted to be a 4A:2B stoichiometry complex and to act as a proton channel (Blair and Berg 1990; Kojima and Blair 2004b).The MotB subunit has a peptidoglycan-binding motif (protruded ball) and the MotA subunit has a large cytoplasmic domain, containing the conserved charged residues important for flagellar rotation, between the second and third transmembrane segments. The C-terminal region of FliG has a ridge containing the important charged residues, which are believed to interact with the charged residues of the cytoplasmic domain of MotA, for the flagellar rotation. FliG/FliM/FliN form a switch complex

Streptococcus sp. (Manson et al. 1977) and of *B. subtilis* (Matsuura et al. 1977; Matsuura et al. 1979) was shown to be powered by the proton motive force as had been shown for *E. coli* and *S. enterica Serovar Typhimurium*. Additional cations, such as sodium or potassium, were not required for motility.

Motility of alkaliphilic *Bacillus* species was shown in the 1980s by Imae and colleagues to be dependent upon a sodium motive force (Hirota and Imae 1983; Hirota et al. 1981). The identity of the Mot proteins have remained undocumented, however, even though rings of studs that are proposed to be the motility complexes had been visualized in membranes of alkaliphilic *B. pseudofirmus* OF4 (Khan et al. 1992). In addition to representing a new example of a sodium motive force-driven motility system to complement the extensive studies of the PomA/PomB system (Asai et al. 1997; Yorimitsu et al. 1999), the alkaliphile Mot complex is of further interest in connection with alkaliphily. In 2000, the genome sequence of alkaliphilic *Bacillus halodurans* C-125 (Takami et al. 2000) revealed only one set of genes encoding a MotA/MotB-like pair of proteins and that set of genes was downstream of a putative *ccpA* gene, which encodes a central regulator of carbon metabolism in *Bacillus* species and other Gram-positive bacteria (Fig. 2.7.4) (Henkin et al. 1991; Moreno et al. 2001). When the *B. subtilis*

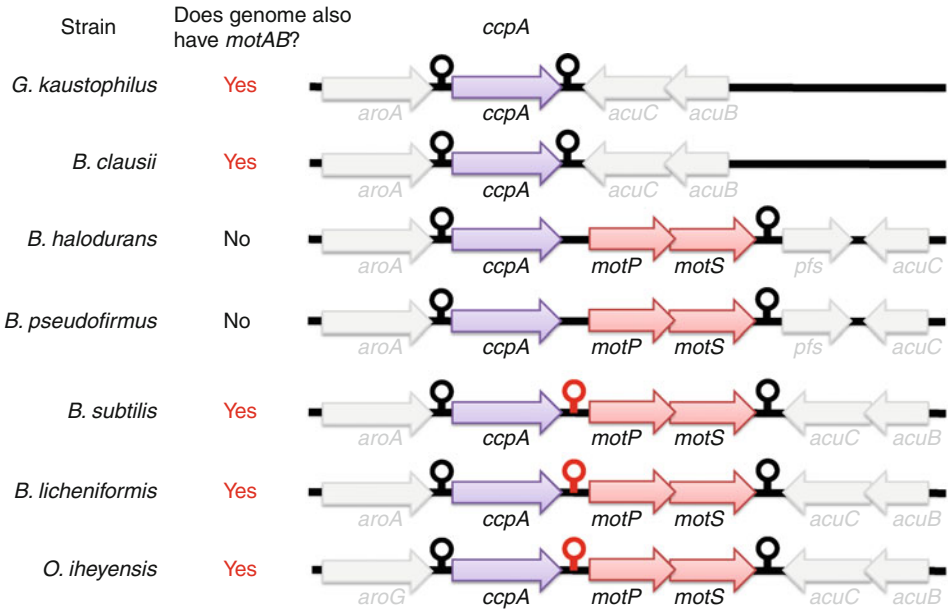

Fig. 2.7.4

The *ccpA-motPS* region in several *Bacillus* species. The two extreme alkaliphiles, *B. halodurans* C-125 and *B. pseudofirmus* OF4, have only motPS as a stator gene and it forms an operon with *ccpA*. No stem-loop is found between *ccpA* and *motPS* in these two extreme alkaliphiles, for which MotPS is the sole Mot (based, respectively, on genomic and genetic evidence [Ito et al. 2004a; Takami et al. 2000]). The stem-loop is found in moderately alkaliphile *O. iheyensis* and non-alkaliphilic *Bacillus* strains that also possess MotAB; the stem-loop reduces transcription read-through between *ccpA* and *motPS* (Terahara et al. 2006). Thermophilic *Geobacillus kaustophilus* and extreme alkaliphilic *B. clausii* have only *motAB* in their chromosome. Alkaliphilic bacteria are *underlined*

homologues of this pair, which are designated MotPS (for pH and salt), were first described, they did not appear to have a role in motility of that organism (Grundy et al. 1993). The alkaliphile genomics encouraged us to re-visit this finding along with a role for MotPS in the alkaliphile.

Disruption of *motPS* resulted in a nonmotile phenotype, and motility was restored by transformation with a multi-copy plasmid containing the *motPS* genes (Fig. 2.7.5) (Ito et al. 2004a). Purified and reconstituted MotPS from *B. pseudofirmus* OF4 catalyzed amiloride analogue-sensitive Na^+ translocation. MotPS are not required for pH homeostasis under both 100 mM and 2.5 mM Na^+ conditions, although they may play a role together with some other translocation pathway that compensated for loss of a MotPS condition (Table 2.7.2). A minor role for MotPS is suggested by the exacerbation of the cytoplasmic alkalinization in the double mutant where the sodium channel gene and MotPS are deleted (Table 2.7.2).

Both *B. halodurans* C-125 and *B. pseudofirmus* OF4 have only MotP/MotS as their stator for motility, and the flagellar motor is driven by a sodium motive force. However, there are bacteria that have two stator-force generator types. For example, moderately alkaliphilic *Oceanobacillus iheyensis* that belongs to a group of alkaliphilic marine bacteria has not only sodium-coupled MotP/MotS, but also proton-coupled MotA/MotB (Takami et al. 2000). A motility assay for this strain has not yet been reported; however, we expect that both MotA/MotB and MotP/MotS contribute to the torque which is required for rotating the flagellar filament. Moreover, some neutralophiles such as *B. subtilis* and *Bacillus licheniformis* also have both proton-coupled

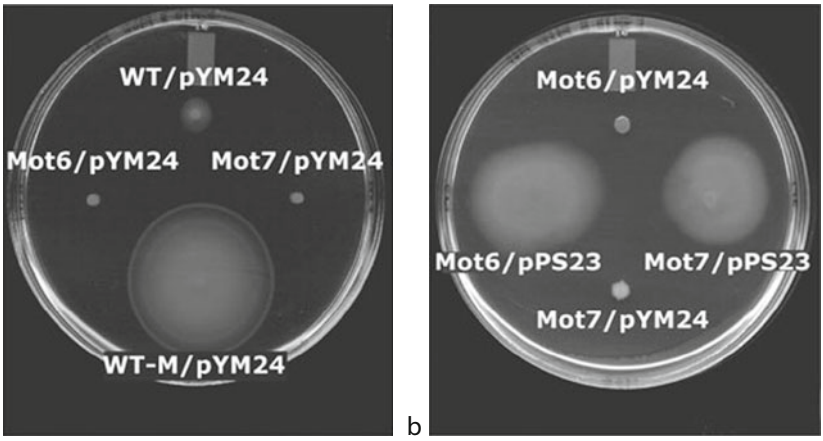

Fig. 2.7.5

Disruption of *motPS* results in a nonmotile flagellated phenotype in alkaliphilic *B. pseudofirmus* OF4. (a) Motility plates of MYE (malate-yeast extract medium [Sturr et al. 1994]), pH 10.5, showing behavior of pYM24 (empty vector) transformants of WT (wild type), Mot6 (Δ*motPS*), WT-M (up-motile variant of wild type), and Mot7 (Δ*motPS*, this mutant was constructed from the up-motile variant of wild type) after 10 h. (b) Motility plates of MYE, pH 10.5, showing behavior of Mot6/pYM24 (empty vector), Mot6/pPS23 (pYM24 + *B. pseudofirmus motPS*), Mot7/pYM24, and Mot7/pPS23 after 48 h. This figure is reproduced from Fig. 1 in Ito et al. in the Molecular Microbiology (Ito et al. 2004a)

Table 2.7.2

Mutational loss of Na_VBP and MotPS affects pH homeostasis in alkaline-shifted cells

Strain (phenotype)	Cytoplasmic pH, 10 min after a pH 8.5 → 10.5 shift	
	100 mM added Na^+, pH_{in}	2.5 mM added Na^+, pH_{in}
B. pseudofirmus OF4-811M (Wild type)	8.46	9.03
SC34 (811M Δ*ncbA* : : Spc^R)	8.66	9.43
SC34-R (SC34 *ncbA* restored)	8.48	9.02
Mot6 (811M Δ*motPS* : : Cm^R)	8.52	9.03
SC34/Mot6 (811M Δ*ncbA* : : Spc^R Δ*motPS* : : Cm^R)	8.72	9.51

ncbA encodes Na_VBP belonging to the Na_VBac Superfamily of Bacterial Voltage-Gated Na^+-Selective Ion Channels. The values are mean values from at least two independent experiments in which cells equilibrated at pH 8.5 were subjected to shift of pH_{out} to 10.5. Mutants lacking functional Na_VBP were also defective in pH homeostasis in response to a sudden alkaline shift in external pH under conditions in which cytoplasmic [Na^+] is limiting for this crucial process. The role of MotPS in pH homeostasis at high pH is apparently masked in the single *motPS* mutant by compensatory Na_VBP activity

MotA/MotB and sodium-coupled MotP/MotS. Previously, we reported that both MotA/MotB and MotP/MotS in the *B. subtilis* flagellar motor contribute to the torque required for flagellar rotation, with different relative contributions under different conditions (Terahara et al. 2006). Recently, Paulick et al. showed polar flagellar localization of both MotB and PomB in the marine bacterium *Shewanella oneidensis* MR-1 and demonstrated the coexistence of the two sets of stator units in a single flagellar motor (Paulick et al. 2009). These results suggested that MotP/MotS is widely distributed in *Bacillus* species regardless of their growth environment, and provides the sodium-dependent energy for rotating the flagellar motor. Here, we present the status of recent studies of the flagellar stator and motility of alkaliphilic *Bacillus* species.

Flagellar Stator and Motility of Alkaliphilic *Bacillus Pseudofirmus* OF4

B. pseudofirmus OF4 grows optimally above pH 10, and the minimum pH value is about 7.5 (Guffanti et al. 1986). Sturr et al. reported that the motility of *B. pseudofirmus* OF4 declined upon lowering the pH value of the medium, and no motility was observed at pH 7.5 (Sturr et al. 1994). In 2004, our group identified the MotP/MotS as the stator of the flagellar motor of *B. pseudofirmus* OF4, and showed that the flagellar motor is driven by a sodium motive force (Ito et al. 2004a). But, the details of the swimming properties and the flagellar assembly remain unknown. Aono et al. reported flagellar formation and flagellum synthesis of alkaliphilic *B. halodurans* C-125 (Aono et al. 1992). This strain showed an increased flagellar number from zero/cell up to 21/cell, when pH was elevated from pH 6.9 to pH 10. Synthesis of flagellin and the formation of flagella were shown to be suppressed during growth at pH 7, which would account for poor motility in cells grown at pH <8. Therefore, synthesis of flagellin and the formation of flagella of this bacterium were definitely regulated by external pH. On the other hand, *B. pseudofirmus* OF4 cells showed the flagella number per cell was 1–2, despite the change

of the external pH and the expression level of flagellin. These results suggested that flagellum of *B. pseudofirmus* OF4 assembles are different from that of *B. halodurans* C-125 and also independent of the external pH (Fujinami et al. 2007b).

Hirota et al. reported that the swimming speed of alkaliphilic *Bacillus sp.* YN-1 cells increased linearly with a logarithmic increase of Na^+ concentration (up to 100 mM) and the optimal pH for motility was about 10.5 (Hirota and Imae 1983). The swimming speed of *B. pseudofirmus* OF4 cells also changes with external pH and sodium concentration. Similar to a previous report (Sturr et al. 1994), no motility was observed at pH 6, and little motility was observed at pH 7. However, the swimming speed of the *B. pseudofirmus* OF4 cells increased linearly with the logarithmic increase of sodium concentration up to 230 mM. The poor motility of alkaliphilic *B. pseudofirmus* OF4 at low pH probably reflects competitive inhibition of Na^+-based motility by high $[H^+]$ rather than decreased flagella production. Similar suggestions have been made with respect to H^+ sites of the *E. coli* and *Salmonella* rotors (Minamino et al. 2003) and the Na^+ sites of the Na^+-driven rotor of *Vibrio alginolyticus* (Yoshida et al. 1990).

Flagellar Stator and Motility of Alkaliphilic *Bacillus Clausii* KSM-K16

Bacillus clausii strains belong to a group of alkaliphilic bacteria, and have been noted to produce industrially useful enzymes like alkaline protease (Kageyama et al. 2007; Kobayashi et al. 1995). Since the genome sequence of *B. clausii* KSM-K16 was finished in 2005, the various genes which related to the flagella have been identified. Interestingly, the genome sequence of this bacterium revealed a single set of genes encoding a MotA/MotB-like pair of proteins as the stator (GenBank accession no. AP006627). These stator proteins were closely related to the MotA and MotB that compose the proton-coupled stator in *B. subtilis* (identity: 52% and 45%, similarity: 73% and 64%, respectively), while these were not as related to the MotP and MotS that compose the sodium-coupled stator in *B. subtilis* (identity: 32% and 28%, similarity: 57% and 55%, respectively). Usually, as for alkaliphilic *Bacillus*, the flagella motor is driven by a sodium motive force with MotP/MotS as the stator, because a proton motive force is difficult to utilize under high alkaline pH (Ito et al. 2004a). However, proton-driven type stator genes *motA*/*motB* were only identified in this genome. All previously studied flagellar stators use either protons or sodium as coupling ions. Therefore, this fact raised the possibility that either the motility of this alkaliphile strain would be lost in the upper pH range for growth or the MotA/MotB is capable of using both proton and sodium ion coupling. This alkaliphile swims well over a range of pH from 7 to 11 and initial experiments in the native host suggested that Na^+ could be used at high pH. Since *B. clausii* is not genetically manipulatable, we characterized the properties of this MotAB stator in a stator-less (Δ*motAB,* Δ*motPS*) mutant of genetically tractable *B. subtilis*. As a result, *B. clausii* MotA/MotB (BCl-MotA/MotB) could function as stator in *B. subtilis*, as shown in ❯ *Fig. 2.7.6*. Thus, the functional properties of BCl-MotA/MotB could be evaluated by comparison with the swimming properties of *B. subtilis* cells expressing only proton-coupled MotA/MotB or sodium-coupled MotP/MotS, respectively. The motility of *B. subtilis* cells expressing BCl-MotA/MotB shows that the coupling ion was changed from proton to sodium ion with increasing external pH (❯ *Fig. 2.7.6*). These results support the hypothesis that BCl-MotA/MotB is a bifunctional stator with respect to ion-coupling capacity. This was also confirmed by using the sodium channel inhibitor EIPA, an amiloride analogue, and the protonophore CCCP which dissipates electrochemical proton gradients (Terahara et al. 2008).

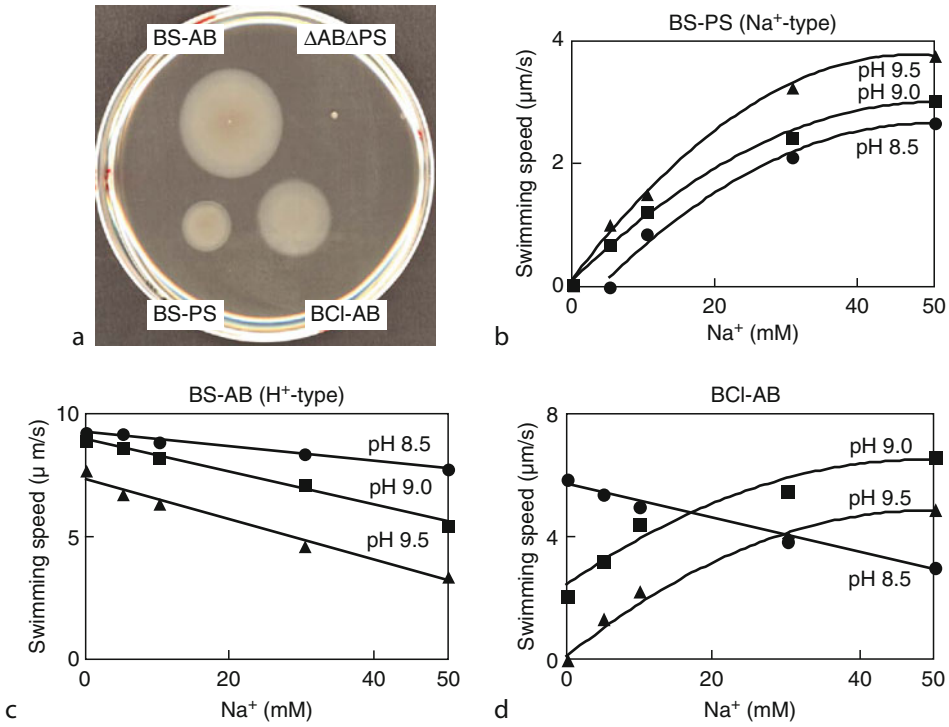

Fig. 2.7.6

The motility of a stator-less *B. subtilis* mutant (ΔABΔPS) expressing either *B. subtilis motAB* (BS-AB), *B. subtilis motPS* (BS-PS), or *B. clausii motAB* (BCl-AB). (a) Motility in a soft agar plate assay. Fresh cells were inoculated into Luria-Bertani medium (pH 7.0) soft agar and incubated at 37°C for 16 h. (B–D) Motility in liquid medium. BS-AB (b), BS-PS (c) and BCl-AB (d) cells were grown at 37°C in LB medium (pH 7.0) and the swimming speed was measured at various concentrations of sodium in 1/20 TY (Tryptone Yeast Extract) medium. 1/20 TY medium contained 0–1 mM sodium. The pH values of 1/20 TY medium used in the swimming speed assays of *B. subtilis* strains were pH 8.5 (*circle*), 9.0 (*square*), 9.5 (*triangle*). This figure is reproduced from Fig. 2 in Terahara et al. in the Proceedings of the National Academy of Sciences USA (Terahara et al. 2008)

Furthermore, we identified whether the bifunctional BCl-MotA/MotB could be changed to a stator that uses either proton or sodium ion at both neutral and alkaline pH. Our previous studies showed that a proton or sodium ion selective domain exists in MotB or MotS subunits of the Mot complex (Ito et al. 2005). On the basis of the alignment in each MotB and MotS subunit of *Bacillus* species, we identified some conserved amino acids residues (*Fig. 2.7.7*). We expected that a conserved ion selective domain would be located in the highly conserved transmembrane segment, because proteins of alkaliphiles facing outside the surface of the cytoplasmic membrane generally have a greatly reduced abundance of basic residues and an increased abundance of acidic residues compared to the same segments of neutralophile homologues (Krulwich et al. 2007). For construction of a proton-coupled stator and a sodium-coupled stator from bifunctional BCl-MotA/MotB, we initially made two distinct triple mutants (named as BCl-MotB-H and BCl-MotB-Na, respectively; shown in *Fig. 2.7.7*).

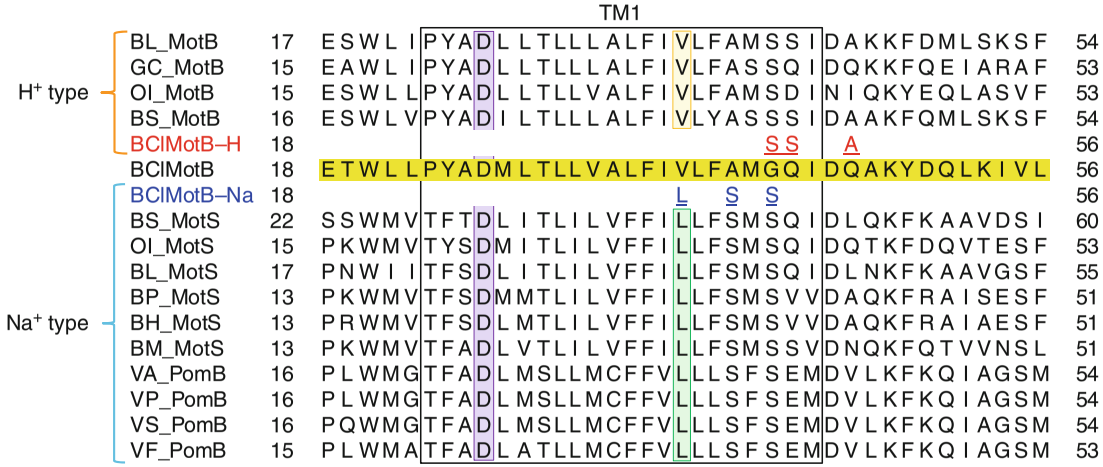

Fig. 2.7.7

Alignment of MotB, MotS, and PomB from *Bacillus* and *Vibrio* species. Alignments of the region containing the single transmembrane segment of *B. licheniformis* MotB (BL_MotB) and MotS (BL_MotS), *Geobacillus kaustophilus* MotB (GK_MotB), *O. iheyensis* MotB (OI_MotB) and MotS (OI_MotS), *B. subtilis* MotB (BS_MotB), MotS (BS_MotS), and *B. clausii* MotB (BCl-MotB), *B. pseudofirmus* MotS (BP_MotB), *B. halodurans* MotS (BH_MotB), *B. megaterium* MotB (BM_MotS), *V. alginolyticus* PomB (VA_PomB), *V. parahaemolyticus* PomB (VP_PomB), *V. splendidus* PomB (VS_PomB), and *V. fisheri* PomB (VF_PomB). The sequence of *B. clausii* MotB is highlighted with *yellow*. The *red letters* show point mutations in *B. clausii* MotB mimicking BS_MotB that yielded BCl-MotB-H. The *blue letters* show point mutations in *B. clausii* MotB mimicking BS_MotS that yielded BCl-MotB-Na. D26 is critical for rotation and is highlighted with *violet* (Kojima and Blair 2004b; Zhou et al. 1998b). V37 of *B. clausii* MotB is conserved among all the MotB-H type proteins shown and is highlighted with *light yellow*. L37 of *B. clausii* MotB-Na is conserved among the MotS and PomB proteins and is highlighted with *light green*.
This figure is reproduced from supplemental information in Terahara et al. in the Proceedings of the National Academy USA (Terahara et al. 2008)

The *B. clausii* MotB introduced mutations (BCl-MotB-H; G42S, Q43S and Q46A) was successfully selected for only protons by imitating the MotB subunit of the proton-coupled stator. In contrast, the *B. clausii* MotB mutations (BCl-MotB-Na; V37L, A40S and G42S) also successfully selected only for sodium ions by imitating the MotS subunit of the sodium-coupled stator. These results suggest that these amino acid residues are critical for ion selectivity. Additionally, by assaying each of the single and double mutants, the Q43S mutation was shown to be critical for proton-coupling, and a combination of the Q43S mutation plus either the G42S or Q46A change was required to achieve loss of sodium coupling. On the other hand, the V37L mutation was critical for sodium coupling and a combination of the V37L mutation plus either the A40S or the G42S mutation was required to achieve loss of proton-coupling at low pH. V37 is predicted to be located in the middle of the single TMS of BCl-MotB on the same face as the conserved aspartic acid residue, D26, that is critical for rotation of the flagellar (Kojima and Blair 2004a). L37 is highly conserved among the MotS and PomB components of the sodium-coupled stators of *B. subtilis* and *V. alginolyticus* (Fig. 2.7.7). Therefore, the results raise the possibility that leucine 37 may function as a sodium-selective filter for these sodium channels.

We also applied the same approach to the distinct H^+- and Na^+-coupled stators of *B. subtilis*, Bs-MotAB and Bs-MotPS, respectively, and identified mutations that conferred dual ion-coupling capacity on each of them (Terahara et al. 2008). This is the first report of a bifunctional flagellar stator which can use both Na^+ and H^+ to power motility, changing preference as a function of pH.

A Classification of Alkaliphilic *Bacillus* Flagellum Based on the Differences in the Property of the Stator

As described above, there are at least three groups of flagellar motors in alkaliphilic *Bacillus* species by the properties of each flagellar stator (Fig. 2.7.8). The group 1 organisms have only Na^+ coupled stator MotPS and a sodium motive force is used for flagellar rotation. This group belongs to *B. halodurans* C-125 and *B. pseudofirmus* OF4. The group 2 organisms have both H^+– coupled stator MotAB and Na^+– coupled stator MotPS in the same motor, and both a sodium and proton motive force is simultaneously utilized for the rotary energy. This group contains not only alkaliphiles, e.g., *Oceanobacillus iheyensis*, but also neutralophiles, e.g., *B. subtilis* and *B. megaterium*. Further analysis is required to elucidate the flagellum structure of this group. The group 3 organisms have a MotAB variant that uses protons at near neutral pH and sodium at high pH and uses both at intermediate pH values. This group belongs to *B. clausii*KSM-K16 (Terahara et al. 2008) and several other alkaliphiles (unpublished data). A MotAB type of motor with MotPS capabilities raises interesting evolutionary and physiological questions.

Chemotaxis of *Bacillus* Species

Motile bacteria can respond to stimulation by chemical and physical effectors in the external environment (Blair 1995; Miller et al. 2009b; Szurmant and Ordal 2004b; Wadhams and Armitage 2004b). A shift of temperature, pH, and oxygen concentration can be effectors (attractants or repellents) as can diverse nutrient or inhibitory chemical compounds. The most intensively studied bacterial chemotaxis system is that of *E. coli*, which is thought to

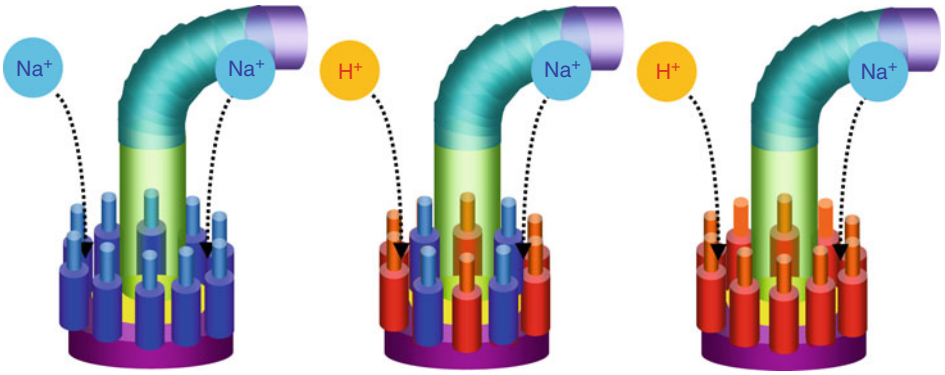

Fig. 2.7.8

A schematic diagram of a classification of alkaliphilic *Bacillus* flagella based on differences in the property of the stator. (a) A conserved pair of stator proteins, MotPS, that are homologs of MotAB mediate Na^+-coupled motility of extreme alkaliphilic *B. halodurans* C-125 and *B. pseudofirmus* OF4. (b) Moderately alkaliphilic *O. iheyensis* and neutralophilic *B. subtilis* exhibited dual ion-coupling capacity using MotPS and MotAB as stators for a single rotor. (c) Extreme alkaliphilic *B. clausii* KSM-K16 and other alkaliphiles have a novel form of dual ion coupling to motility in which a single MotAB stator uses both sodium and protons at different ranges of pH

involve a less complex set of networks than those used in *B. subtilis* chemotaxis (Garrity and Ordal 1995; Kirby 2009; Szurmant and Ordal 2004a), but yet still continues to provide novel information (Eisenbach 2007; McCarter 2005). *B. subtilis* provides one of the other chemotaxis systems for which a great amount of information is available and is by far the most studied among *Bacillus* species, revealing commonalities with *E. coli* chemotaxis but also significant differences (Garrity and Ordal 1995; Muff and Ordal 2008a; Rao et al. 2008b; Szurmant et al. 2004b; Szurmant and Ordal 2004a). In both model systems, the bacteria swim in straight runs (with CCW rotation of the flagellar bundle) with occasional tumbles (initiated by CW rotation) that promote the likelihood of change in direction (Szurmant and Ordal 2004a). Therefore, movement toward attractants is favored by smooth runs in response to an attractant and movement away from repellents is favored by increased tumbling. Chemoreceptor proteins are usually transmembrane signaling proteins that bind external signaling molecules and/or respond to physical effectors in a manner that also initiates a change that is transmitted to the cytoplasmic domain. The chemoreceptors are called MCPs (methylated chemotaxis proteins) because of an adaptation feature of chemotaxis that involves a methylation/demethylation of sites on the MCPs. Clusters of MCPs have been found at the cell poles of rod-shaped bacteria (Kirby et al. 2000b; Lamanna et al. 2005b; Maddock and Shapiro 1993b). The response of the MCPs is transmitted through the CheA histidine kinase that is complexed, together with the adaptor protein CheW, on the cytoplasmic side. The resulting state of autophosphorylation of CheA determines the phosphorylation state of the response regulator CheY which, interacting with the switch domain of the rotor, determines the direction of rotation and hence the frequency of tumbling as opposed to runs. The response of the CheA kinase activity

to binding of chemoeffectors to the MCPs is different in *E. coli* than in *B. subtilis,* and the *Bacillus* has distinct and more complex layers of adaptive capacity that correlate with the presence of chemotaxis proteins that are not found in *E. coli* (Table 2.7.3) (Muff and Ordal 2008a; Szurmant and Ordal 2004a).

B. subtilis also has redundancies of several chemotaxis proteins that are not observed in *E. coli* and is thought to increase robustness of *B. subtilis* chemotaxis (Table 2.7.3) (Miller et al. 2009a; Rao et al. 2008b; Wadhams and Armitage 2004a). For example, there are CheW and CheV as adapter proteins of the chemotaxis receptor complex (Karatan et al. 2001; Rosario et al. 1994a), CheC and FliY (and additional CheX in some species) as a phosphorylated response regulator CheY (CheY-P) phosphatase (Muff and Ordal 2008b; Park et al. 2004; Szurmant et al. 2004a; Muff et al. 2007), CheB and CheD as MCPs methylesterases (Chao et al. 2006; Rao et al. 2008a) in *Bacillus* species. On the other hand, *E. coli* has only CheW, CheZ, and CheB, respectively. The sequence homology of these functional homologues is not necessarily high (e.g., CheZ and CheC). In *Bacillus* species, it is thought that survivability has been improved by adapting it to a double track line (Miller et al. 2009b; Rao et al. 2008a; Wadhams and Armitage 2004b). These genomic features are also seen in alkaliphilic *Bacillus* species.

Chemotaxis of Alkaliphilic *Bacillus* Species

Alkaliphilic *Bacillus* species show similar chemotaxis "tool-kits" to *B. subtilis* (Table 2.7.3) and are expected to share many of the same chemoeffectors that are distinguished, as well as the general design principles of the signal transduction mechanism, since both of those show a high degree of conservation among even more distantly related bacteria (Szurmant and Ordal 2004a; Wadhams and Armitage 2004a). There is as yet no information in alkaliphilic *Bacillus* species about involvement of particular MCPs or other chemotaxis elements in responses to pH or proton motive force, as there are for *E. coli* (Krikos et al. 1985; Umemura et al. 2002; Yamamoto et al. 1990). However, evidence has emerged for a dependence of chemotaxis in alkaliphilic *B. pseudofirmus* OF4 on a voltage-gated sodium channel (see below). This incompletely understood layer of adaptation in alkaliphile chemotaxis may be distinct in these organisms, perhaps reflecting the challenge of alkaliphily and its dependence on a sodium cycle. Quite possibly, though, the involvement of a channel in alkaliphile chemotaxis is a particularly pronounced extremophile version of a role for an ion channel that will turn up in other bacteria and whose identification and study will be facilitated by the alkaliphile model.

The Existence of a Na^+ Channel, NaChBac, in Alkaliphilic *Bacillus* Species

In 2001, Ren et al. discovered a voltage-gated Na^+ channel in the alkaliphilic *Bacillus halodurans* (NaChBac) (Ren et al. 2001). NaChBac was selective for sodium, even though the sequence is similar to that of voltage-gated calcium channels. Pore-forming subunits of mammalian voltage-gated sodium and calcium channels are encoded by four repeated domains of six-transmembrane (6TM) segments. On the other hand, NaChBac is encoded by one 6TM segment. NaChBac is blocked by the dihydropyridine class (nifedipine and nimodipine) of L-type voltage-gated calcium channel blockers. NaChBac superfamily members have been identified in diverse bacteria whose common themes include marine and/or alkaline niches.

Table 2.7.3

Comparison of the chemotaxis protein homologues of alkaliphilic *Bacillus* species, *Bacillus subtilis*, and *Escherichia coli*

Protein	Functions	*Escherichia coli*	*Bacillus subtilis*	*Bacillus halodurans*	*Bacillus clausii*	*Oceanobacillus iheyensis*	References
CheW	Adaptor protein	P0A964	P39802	Q9K8N6	Q5WEU4	Q8CXH0, Q8CX96	(Rosario et al. 1994b)
CheV	Adaptor protein	–	P37599	Q9KAM5, Q9KC62	Q5WG28	–	(Rao et al. 2008b; Rosario et al. 1994b)
CheA	Histidine kinase	P07363	P29072	Q9K8N5	Q5WEU3	Q8ENE1*	(Bischoff et al. 1993)
CheY	Response regulator	P0AE67	P24072	Q9KA46, Q9K8N7	Q5WFR9	Q8EQW9	(Fuhrer and Ordal 1991)
CheR	Methyltransferase	P07364	P31105	Q9KCB8	Q5WGS9	Q8EQB5	(Burgess-Cassler and Ordal 1982; Burgess-Cassler et al. 1982)
CheB	Deamidase/ Methylesterase	P07330	Q05522	Q9KA55	Q5WFS3	Q8EQW0	(Kirby et al. 2000b; Kirsch et al. 1993)
CheC	Phosphatase	–	P40403	Q9KDX3	Q5WFS4	Q8EQV9	(Muff and Ordal 2007a, b; Rao et al. 2008b; Szurmant et al. 2004b)
CheD	Deamidase/ Methylesterase	–	P40404	Q9KA57	Q5WFS5	Q8EQV8	(Muff and Ordal 2007a; Rao et al. 2008b)
CheX	Phosphatase	–	–	Q9KDX3	–	–	(Muff et al. 2007; Muff and Ordal 2007a)
FliY	Phosphatase	–	P24073	Q9KA45	Q5WFR8	Q8EQX0	(Muff and Ordal 2007a; Szurmant et al. 2004b)
CheZ	Phosphatase	P0A9H9	–	–	–	–	(Kuo and Koshland 1987)

The GenBank accession numbers are indicated. *Asterisk* indicates the protein which was newly annotated in this research

The Physiological Functions of Na_VBP in Alkaliphilic *Bacillus Pseudofirmus* OF4

To investigate the physiological functions of the voltage-gated Na^+ channel an Na_VBP defective mutant (Δ*ncbA*) was constructed from *B. pseudofirmus* OF4 (Ito et al. 2004b). Wild-type *B. pseudofirmus* OF4, the channel mutant SC34, and the SC34-R strain to which *ncbA* was restored all exhibited doubling times that ranged from 95 to 110 min at pH 7.5 and 70–75 min at pH 10.5. The growth yield of SC34 was reduced ≦20% relative to wild type on MYE (malate-yeast extract medium) at pH 10.5 (Ito et al. 2004b).

In order to investigate the pH homeostasis ability of the Na_VBP defective mutant, pH 8.5-equilibrated cells of wild type and SC34 were suddenly shifted to non-nutrient buffer at pH 10.5 containing 100 or 2.5 mM Na^+, and the cytoplasmic pH was measured 10 min later. As shown in *Table 2.7.2*, the Na_VBP mutant SC34 could not maintain a relatively acidified intracellular pH under these conditions as well as the wild type. The double ncbA and motPS mutant exhibited even greater cytoplasmic alkalinization than SC34 (Ito et al. 2004b). These results showed that Na_VBP plays a role in Na^+ reentry in support of cytoplasmic pH homeostasis in the absence of added solutes whose uptake is coupled to Na^+.

As observed by phase-contrast microscopy, SC34 cells exhibited a "tumbly" phenotype and also poor swimming relative to wild type. A tumble refers to the transient dispersal of the helical bundle of flagella during a random change of direction that occurs when smooth *CCW* swimming is interrupted by a switch to *CW* motion (Macnab and Ornston 1977). Restoration of the *ncbA* gene to the chromosome restored wild-type motility on soft agar and reversed the tumbliness although not completely to the wild-type pattern.

The greater migration of up-motile SC34 than up-motile wild type on soft agar plates is probably an example of "pseudotaxis" (Wolfe and Berg 1989), in which greater migration in soft agar is found in bacterial mutants with an increased clockwise flagellar rotation bias relative to their wild-type parent. This greater motility was retained, and the tumbliness of up-motile SC34 was not entirely reversed by restoration of *ncbA* to the chromosome in up-motile SC34-R (*Table 2.7.4*).

The up-motile derivative is a stable derivative that was selected by repeated restreaking of cells from the motile edge of SC34 and wild-type colony on soft agar. Increased tumbliness is associated with defects in chemotaxis, the motility-based behavior whereby bacteria respond to temporal gradients of attractants and repellants (Aizawa et al. 2002; Berg 2003; Larsen et al. 1974). *As noted above,* bacteria extend smooth runs toward an attractant by reducing their tumbling frequency and increase tumbling in response to repellants, thereby increasing the chance of moving in a new direction away from the repellant. The interesting possibility of a chemotaxis defect in *ncbA* mutants was assessed by using a capillary assay of pH 10.5-grown strains of up-motile wild type, up-motile SC34, and up-motile SC34-R that swim well enough for use of this assay. The low nutrient condition compromises pH homeostasis, so a pH of 8.5 was used instead of pH 10.5. The up-motile *ncbA* mutant SC34 exhibited an inverse chemotaxis phenotype relative to the up-motile wild type in these assays (*Table 2.7.4*). Although the up-motile wild type exhibited positive chemotaxis (i.e., moved toward aspartate, proline, and glucose), up-motile SC34 exhibited negative chemotaxis. Upon *ncbA* restoration in up-motile SC34-R, positive chemotaxis toward aspartate was restored, although the response was quantitatively reduced relative to that of up-motile wild type. The *ncbA* mutant also exhibited an inverted response to high pH. The up-motile wild type exhibited negative chemotaxis to non-nutrient buffer at pH 10.5 (*Table 2.7.4*) but exhibited positive chemotaxis when malate was

Table 2.7.4

Inverse chemotaxis behavior of up-motile *ncbA* mutant

Strain	Attractants	Repellants
Wild-type SC34-R	Aspartate (pH 8.5)	pH 10.5
	Proline (pH 8.5)	
	Glucose (pH 8.5)	
	Malate (pH 10.5)	
SC34	pH 10.5	Aspartate (pH 8.5)
		Proline (pH 8.5)
		Glucose (pH 8.5)
		Malate (pH 10.5)
Wild type (with 50 mM nifedipine treatment)		Aspartate (pH 8.5)

Chemotaxis behavior was assessed by a capillary assay of pH 10.5-grown strains of up-motile wild type, SC34, and SC34-R that swim well enough for use of this assay. The low nutrient condition compromises pH homeostasis, so a pH of 8.5 was used instead of pH 10.5. Chemical effectors were added at 1 mM and the buffer pH inside the capillary was 8.5 for aspartate, proline, and glucose, and 10.5 for malate; pH 10.5 was also tried as a variable without malate. The buffer pH outside the capillary was always pH 8.5 (Ito et al. 2004b)

included in the capillary; there was no response to malate alone. The up-motile SC34 exhibited an opposite pattern to that of up-motile wild type, moving toward pH 10.5 buffer in the absence of malate and away from pH 10.5 when malate was present. Finally, if loss of Na_VBP indeed accounts for the inverse chemotaxis behavior of up-motile SC34, then a channel inhibitor should elicit inverse chemotaxis by the up-motile wild-type strain. The Na_VBP inhibitor Nifedipine (Ito et al. 2004b) caused inversion of the chemotaxis response of the up-motile wild type to aspartate (*Table 2.7.4*). Taken together, these observations raised the possibility that Na_VBP interacts with a membrane-associated component of the chemotaxis machinery of *B. pseudofirmus* OF4. While genomic data are not yet available for this alkaliphile, those from related alkaliphilic *B. halodurans* (Takami et al. 2000) and from *Oceanobacillus iheyensis* (Takami et al. 2002), both of which have Na_VBP homologues (Ito et al. 2004b), suggest that the chemotaxis proteins are similar in kind to those of the well-characterized chemotaxis system of *Bacillus subtilis* (Szurmant and Ordal 2004b). The most attractive candidate for a membrane-associated interactive partner for Na_VBP would be the methyl-accepting chemotaxis receptors (MCP). Thousands of copies of the major MCP forms are localized within bacterial cells in polar arrays (Kirby et al. 2000a) and smaller numbers of minor MCP forms also exhibit polar localization (Lybarger and Maddock 2000). The MCP has a conserved overall structure and a highly conserved domain. Na_VBP might similarly mediate its apparently global effect on chemotaxis by an interaction with MCP. We conducted an immunofluorecence microscopy (IFM) analysis of *B. pseudofirmus* OF4 cells to confirm the expectation of a polar localization for McpB and compare this localization with that of Na_VBP. Mutants lacking functional Na_VBP exhibited chemotaxis abnormality (e.g., "tumble" phenotype and "Inverse chemotaxis" (Ito et al. 2004b)). We also showed polar co-localization of Na_VBP and one of the putative MCPs, "McpX," in wild-type cells (MCPs and several chemotaxis proteins generally localize to cell poles of rod-shaped cells (Kirby et al. 2000a; Lamanna et al. 2005a; Maddock and Shapiro 1993a)) and delocalization of McpX in mutants lacking

functional Na_VBP (Fujinami et al. 2007a). In addition, polar localization of both McpX and Na_VBP was decreased in the *cheAW* mutant (Fujinami et al. 2007a). The results suggest the possibility of interactions between McpX and Na_VBP that affect their co-localization.

The finding that mutational loss of Na_VBP led to delocalization of McpX raised the possibility that the inverse chemotaxis phenotype observed in the Δ*ncbA* strain could result entirely from secondary delocalization of MCPs rather than from a channel function of Na_VBP itself. Evidence against this line of reasoning was the earlier observation that the Na_VBP channel inhibitor nifedipine caused inverse chemotaxis behavior of wild-type *B. pseudofirmus* OF4 toward the chemoattractant aspartate when the inhibitor was added to the chemotaxis assay buffer (at 50 μM) (Ito et al. 2004b). IFM experiments were conducted to test whether comparable exposure of the wild-type strain to nifedipine caused delocalization of Na_VBP or McpX. These experiments revealed no such delocalization.

The C-terminus of Na_VBP contains four aspartate residues that could serve as potential phosphoacceptors. In eukaryotic channels, the changes in C-terminal phosphorylation affect channel gating (Park et al. 2006a). The phosphorylation system of chemotaxis signal transduction may affect phosphorylation of the C-terminus of Na_VBP. The electrophysiological characteristics also indicated that the channel properties may be secondarily modulated by interaction with other proteins called "additional triggers" (Ito et al. 2004b).

The Na^+ Circuit Model of Alkaliphilic *Bacillus Pseudofirmus* OF4

A Na^+/H^+ antiporter is the major mechanism for extruding Na^+, and also functions to bring protons into the cell for pH homeostasis in alkaliphilic *Bacillus* species. The Na^+/H^+ antiporter creates the sodium potential necessary for the Na^+/solute symporters, because a primary Na^+ pump is not present. The antiporter uses the proton motive force as an energy source. Na^+/solute symporters used the sodium electrochemical potential to accumulate solutes. The flagellar rotation depends on the sodium electrochemical potential. The depiction of the respiratory chain represents a hypothesis in which protons are transferred directly from this complex to the proton-coupled F_1F_0-ATP synthase in protein–protein interaction. When Na^+ entry is limiting, e.g., at low [Na^+] or a paucity of symporter substrates, pH-activated Na^+ channels are hypothesized to provide an important Na^+ reentry path. Candidates for such channels are the Na_VBP channel and the MotPS channel. Na_VBP mutants exhibit a significant loss of pH homeostasis capacity (Ito et al. 2004b). Participation of MotPS in pH homeostasis is suggested by the enhanced deficits of the double Na_VBP and MotPS mutant. Thus, physiological studies of the channels of extremophilic *B. pseudofirmus* OF4 have led to new insight into the motility profile of a model neutralophilic *Bacillus*.

Acknowledgments

Work conducted in the authors' laboratories was supported by Grant-in-Aid for Scientific Research (C) (17613004) and (B) (21370074) from the Ministry of Education, Culture, Sports Sciences and Technology of Japan, and by a Grant for Basic Science Research Projects from the Sumitomo Foundation and from the Kurata Memorial Foundation for Promoting Science (to M.I.) and a JSPS Research Fellowships for Young Scientists (to N.T.).

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2.8 Enzymes Isolated from Alkaliphiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.8,

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. The first report concerning an alkaline enzyme published in 1971 described an alkaline protease produced by *Bacillus clausii* 221. Since that time hundreds of new enzymes have been isolated in many laboratories. Some of these have been produced on an industrial scale and commercialized.

Alkaline Protease

Isolation of Alkaline Protease

In 1971, Horikoshi (Horikoshi 1971a) reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus clausii* 221 (Fig. 2.8.1). This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5 with 75% of the activity maintained at pH 13. The enzyme was completely inhibited by diisopropylfluorophosphate or 6 M urea, but not by ethylenediamine tetraacetic acid or *p*-chloromercuribenzoate. The molecular weight of the enzyme was 30,000, which is slightly higher than those of other alkaline proteases. The addition of a 5 mM solution of calcium ions was reflected in a 70% increase in activity at the optimum temperature (60°C). Subsequently two *Bacillus* species, AB42 and PB12, were reported which also produced an alkaline protease(Aunstrup et al. 1972). These strains exhibited a broad pH range of pH 9–12, with a temperature optimum of 60°C for AB42 and 50°C for PB 12. Since these reports, many alkaline proteases have been isolated from alkaliphilic microorganisms (Tsai et al. 1983; Nomoto et al. 1984; Tsai et al. 1984, 1986). Fujiwara et al. (1993) purified a thermostable alkaline protease from a thermophilic alkaliphilic *Bacillus* sp. B18. The optimum pH and temperature for the hydrolysis of casein were pH 12–13 and 85°C, both of which are higher than those of alkaline proteases. Han and Damodaran (1998) reported the purification and characterization of an extracellular endopeptidase from a strain of *Bacillus pumilus* displaying high stability in 10% (w/v) sodium dodecyl sulfate and 8 M urea. Some of the enzymes are now commercially available as detergent additives.

Takami et al. (1989) isolated a new alkaline protease from alkaliphilic *Bacillus* sp. No. AH-101. The enzyme was most active toward casein at pH 12–13 and stable under 10 min incubation at 60°C and pH 5–13. The optimum temperature was about 80°C in the presence of 5 mM calcium ion. The alkaline protease showed a higher hydrolyzing activity against insoluble fibrous natural proteins such as elastin and keratin in comparison with subtilisins and proteinase K (Takami et al. 1990, 1992). Cheng (Cheng et al. 1995) reported a keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. This enzyme was stable from pH 5–12. The optimal reaction pHs for feather powder and casein were 8.5 and 10.5–11.5, respectively. Zaghloul et al. also reported isolation, identification, and keratinolytic activity of several feather-degrading bacteria isolated from Egyptian soil. These isolates could degrade chicken feather (Zaghloul et al. 1998).

Isolation of Detergents and H_2O_2-Resistant Alkaline Proteases

Alkaline proteases are used extensively in detergents, the food industry, and leather tanning. Enzymes produced commercially are derived only from microorganisms, and the

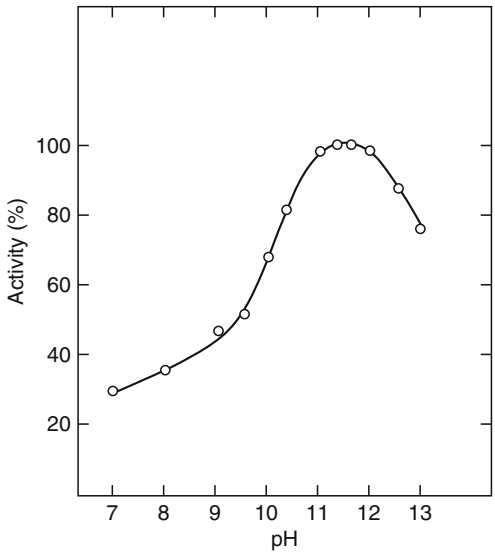

Fig. 2.8.1
Effect of pH on enzyme (*Bacillus clausii* 221 alkaline protease) activity

microorganisms must be able to produce a high enzyme yield from low-cost substrates. The success of alkaline proteases in detergents is dependent on whether the enzymes have the following properties: (1) a wide pH activity range, (2) stability under high alkaline conditions, (3) high activity and stability in the presence of surfactants, (4) high stability in the presence of builders such as chelating reagents and bleaching agents, (5) high activity over a wide temperature range, (6) long shelf-life, and (7) low production cost. Although many enzymes have been reported, the alkaline proteases described above, frankly speaking, have several weak points in their enzymatic properties, e.g., they are sensitive in the presence of oxidants and chelating agents. These disadvantages have been overcome by the isolation of new *Bacillus* strains by the author's group. Details are given below.

E-1 Enzyme of *Bacillus cohnii* D-6

In 1972, Yoshida and Horikoshi discovered a very stable alkaline protease in the presence of detergents containing high concentration of perborate in the absence of calcium ion (Japanese patent:JP 740710). *Bacillus* sp. No. D-6 (FERM No. 1592), later designated *Bacillus cohnii* D-6, produced an alkaline protease, E-1, that was more stable in the presence of detergent additives at 60°C than *Bacillus clausii* 221 protease. Several properties are presented in *Table 2.8.1*.

After our discovery, many researchers tried to industrialize it, but without success. Almost 30 years later, Saeki et al. (2000) dramatically increased the productivity to more than 10 g/l using gene technology.

Subsequently, *Bacillus* KSM-9860, *Bacillus* LP-Ya, *Bacillus* SD-521, and *Bacillus* NP-1 were isolated and the alkaline proteases of these alkaliphiles were purified by the conventional manner. Their enzymatic properties were almost the same as those of the E-1 enzyme. Furthermore, for better understanding the oxidant resistance of E-1, Saeki et al. (2002)

Table 2.8.1

Properties of an alkaline protease from *Bacillus cohnii* D-6

Property	Protease E-1 of *Bacillus cohnii* D-6
Optimum pH of enzyme action	10.5
Stable pH (60°C, 10 min)	5–15
Stable temperature, °C (at pH 9)	
$-Ca^{2+}$	75
$+Ca^{2+}$ (10 mM)	75
Molecular weight, $\times 10^4$	3
Isoelectric point	10
Active center	Ser
Stability in DBS (0.2%, 50°C, 60 min)	60

DBS: Dodecyl benenesulfonate

analyzed sequences the alkaline protease genes of KSM-9860 of LP-Ya, of SD-521, and of NP-1. The deduced amino acid sequence Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala is common to the internal sequences and coincides with the N-terminal sequence of mature E-1. The deduced amino acid sequences of KP-9860, LP-Ya, SD-521, and NP-1 showed very high homology to that of E-1 with 88.2%, 98.6%, 99.3%, and 88.2% identity, respectively.

Industrial Applications of Alkaline Proteases

Detergent Additives

The main industrial application of alkaliphilic enzymes is in the detergent industry, and detergent enzymes account for approximately 30% of total worldwide enzyme production. Not all of these are produced by alkaliphilic bacteria. However, many alkaline proteases have been produced by alkaliphilic *Bacillus* strains and are commercially available from companies.

Dehairing

Alkaline enzymes have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10. These enzymes are commercially available from several companies.

Further details are reviewed in Chap. 2.10 Beta-Cyclomaltodextrin glucanotransferase of a species of alkaliphilic Bacillus for the production of beta-cyclodextrin.

Starch-Degrading Enzymes

There are many types of starch-degrading enzymes, e.g., amylase, cyclomaltodextrin glucanotransferase, pullulanase, α-glucosidase, etc. These amylases are widely distributed in living things, where they play an important role in biochemical reactions. Its activities have been studied for industrial and pharmaceutical applications and numerous reports published. Until fairly recently all the enzymes reported showed optimum pH for enzyme action in the acid or neutral range. No report concerning an alkaline amylase with optimum activity in the alkaline pH range had been published despite extensive study by many researchers.

Horikoshi attempted to isolate alkaliphilic microorganisms producing alkaline amylases, and in 1971 an alkaline amylase was produced in Horikoshi-II medium by cultivating alkaliphilic *Bacillus pseudofirmus* No. A-40-2 (Horikoshi 1971b). Several types of alkaline starch-degrading enzymes were subsequently discovered by cultivating alkaliphilic microorganisms (Boyer and Ingle 1972; Yamamoto et al. 1972). No alkaline amylases produced by neutrophilic microorganisms have so far been reported. The above authors classified alkaline amylases into four types according to their pH activity curves, as shown in *Fig. 2.8.2*. The type-1 curve has only one peak at pH 10.5; the type-II curve has two peaks at pH 4–4.5 and 9–10; the type-III curve has three peaks at pH 4.5, 7, and 9.5–10; and the type-IV curve has one peak at pH 4 with a shoulder at pH 10. The characteristics of the four types of amylases are summarized in *Table 2.8.2*.

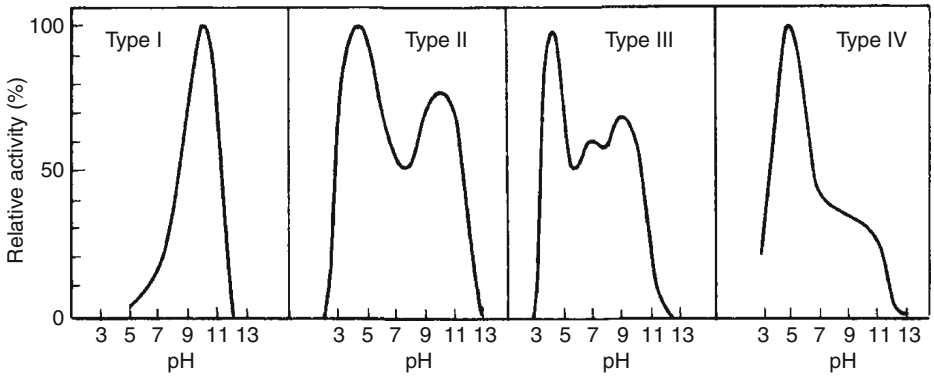

Fig. 2.8.2

Four types of pH activity curves of alkaline amylases of alkaliphilic *Bacillus* strains

Table 2.8.2

Properties of alkaline amylases of alkaliphilic *Bacillus* strains

Type	Strain No.	Optimum pH	Stable pH	Stabilized by Ca^{2+}	Production of CGTase
I	A-40-2	10.5	7–9.5	+	None
	A-59	10.5	7–9.5	+	None
	27-1	10.5	7–9	+	None
	124-1	10.5	7–9	+	None
II	135	4–4.5; 10	7–9	+	+
	169	4–4.5; 10	7–9	+	+
III	38-2	4.5; 9	5–10.5	+	+
IV	13	4.5	6.5–10	+	+
	17-1	4.5	6.5–10	+	+

Sable pH: pH range where 50% of activity remains after incubation at 50°C for 15 min.

α-Amylases

Bacillus pseudofirmus A-40-2 Amylase

Production of the alkaline amylase was first achieved in an alkaliphilic *Bacillus pseudofirmus* A-40-2 (Horikoshi 1971). The isolated strain was an aerobic, sporeforming, gram-positive, motile, rod-shaped bacterium with peritrichous flagella. Although the morphological, cultural, and biochemical characteristics of the strain resembled those of *Bacillus subtilis*, the special feature of the bacteria was that growth was very good in alkaline media, and the optimal pH for growth was about 10. No growth was detected in neutral media.

Bacillus pseudofirmus A-40-2 was grown aerobically at 37°C in Horikoshi-II medium for 3 days. The alkaline amylase in the culture fluid was purified by a DEAE-cellulose column and a hydroxyl apatite column followed by gel filtration. The molecular weight was estimated to be about 70,000 by gel filtration method. The enzyme is most active at pH 10–10.5 and retains 50% of its activity between pH 9 and 11.5. The enzyme is not inhibited by 10 mM EDTA at 30°C, and completely inactivated by 8 M urea. However, about 95% of the activity is recovered upon removal of urea by dialysis. The enzyme can hydrolyze 70% of starch to yield glucose, maltose, and maltotriose. Therefore, the enzyme is a type of saccharifyingα-α-amylase.

Bacillus Strain NRRL B-3881 Amylase

After Horikoshi's paper (Horikoshi 1971b), Boyer and Ingle (Boyer and Ingle 1972; Boyer et al. 1973) reported alkaline amylase in the strain NRRL B-3881. This was the second report of an alkaline amylase. The B-3881 amylase showed optimum pH for enzyme action at 9.2. A-40-2 amylase retains 50% of its activity between pH 9 and 11.5, and B-3881 enzyme retains the same activity between pH 7 and 10.5. Both amylases are relatively more stable against EDTA than either *Bacillus amyloliquefacienc* or *B. subtilis* amylase. The enzyme yields maltose, maltotriose, and small amounts of glucose and maltotetraose, all of which have a α-configuration.

Bacillus halodurans A-59 Amylase

Alkaliphilic *Bacillus halodurans* A-59 was isolated from soil by using Horikoshi-II medium. The properties of the strain are almost the same as those of *Bacillus subtilis*. It can grow either in neutral media or in alkaline media. This differs from *Bacillus pseudofirmus* A-40-2. The alkaline amylase was purified by conventional methods, such as DEAE-cellulose columns and gel filtration. The properties were not significantly different from the alkaline amylase of alkaliphilic *Bacillus pseudofirmus*.

Recently, Murakami (Murakami et al. 2007) isolated two alkaline, thermotolerant α-amylases from *Bacillus halodurans* 38C-2-1 and expression of the cloned gene in *Escherichia coli*. These enzymes showed maximal activities at 50–60°C and pH 10–11, and 42% and 38% relative activities at 30°C. The enzyme activity was not inhibited by a surfactant or a bleaching reagent used in detergents.

Amylases of Other Alkaliphiles

Kim and Kim (1995) isolated a maltotetraose-forming alkaline α-amylase from an alkaliphilic *Bacillus* strain, GM8901. The enzyme had an extremely high optimal pH of 11–12 and was stable in a broad pH range of 6–13. Thermostability increased in the presence of Ca^{2+} and soluble starch.

Lo et al. (2001a, b) purified TS-23 α-amylase of *Bacillus* sp. TS-23 to the homogeneous state from the culture medium of recombinant *E. coli*. The enzyme was stimulated by Mn^{2+} Co^{2+} and Fe^{2+} ions but was strongly inhibited by Hg^{2+} and Cu^{2+} and by the well-characterized inhibitors, diethylpyrocarbonate and N-bromosuccinimide. The enzyme was active in the presence of 8% sodium dodecyl sulfate (SDS). *Bacillus* sp. TS-23 α-amylase was stable when it was preincubated with 6% SDS for up to 1 h at 30°C, while inactivation was observed at 60°C. Under optimal conditions, this enzyme was able to attack the α-1.4 linkages in soluble starch, amylose, amylopectin, and glycogen to generate maltopentaose as the major end product.

Lin et al. (2003) reported replacement of methionine 208 in a truncated *Bacillus* sp TS-23 α-amylase with leucine enhanced its resistance to hydrogen peroxide. Wild-type enzyme was sensitive to chemical oxidation, but Met208Leu was stable even in the presence of 500 mM H_2O_2.

Maltohexaose-Producing Enzymes

From the beginning of the 1970s, many bacterial strains which produce amylases catalyzing the degradation of starch to malto-oligosaccharides (Gn-amylase) have been isolated and the enzymes characterized.

G6-Amylase of *Bacillus* sp. No. 707

In order to obtain hyperproducers of enzymes for industrial applications, an alkaliphilic bacterium, *Bacillus* sp. No. 707, a producer of Gn-amylase, was isolated from soil and the gene for maltohexaose-producing amylase, G6-amylase, was cloned (Kimura et al. 1988; Kimura and Horikoshi 1988). An alkaliphilic bacterium, *Bacillus* sp. No. 707, produces at least five Gn-amylase components in Horikoshi-II medium. The content of G6 in the hydrolyzate was approximately 50–60%. Recently, Kanai et al. (2004, 2006) analyzed the enzyme and reported biochemical and crystallographic properties of maltohexaose-producing amylase from alkaliphilic *Bacillus* sp 707. The face-to-face short contact between Trp140 and substrate sugars is suggested to regulate the disposition of the glucosyl residue at subsite −6 and to govern product specificity for G6 production.

G6-Amylase of *Bacillus halodurans* H-167 Enzyme

Independently, Hayashi et al. (1988a, b) isolated alkaliphilic *Bacillus halodurans* H-167 producing maltohexaose-forming enzymes in their culture broths.

The strain produced three α-amylases which yielded maltohexaose as the main product from starch. The optimum culture conditions for enzyme production were: initial medium, pH 9.4; culture temperature, 37°C; and 50–60 h cultivation under aerobic conditions. The enzymes (H-1-1, H-1-2, and H-II) were separated completely and purified to homogeneity. All enzymes were most active at pH 10.5 and stable in the range pH 7.5–12 on standing at 50°C. The maximum yield was about 25–30%.

Shirokizawa et al. (1989, 1990) cloned the gene of G6-amylases of *B. halodurans* H-167 and expressed it in the *E. coli* system. Recently, Takami et al. (2000) determined the entire DNA sequence of *B. halodurans* C-125, and its G6-formine enzyme exhibits very high homology to that of *B. halodurans* H-167, although several microbial properties of these strains are different.

Pullulan-Degrading Enzymes

In 1975, Nakamura et al. discovered an alkaline pullulanase of *Bacillus* sp. No. 202-1 (Nakamura et al. 1975). The enzyme has an optimum pH for enzyme action at 8.5–9 and is stable for 24 h at pH 6.5–11 at 4°C. The enzyme is most active at 55°C, and is stable up to 50°C for 15 min in the absence of substrate. Kelly et al. (1983) found that alkaliphilic *Bacillus halodurans* No. A-59 produced three enzymes, α-amylase, pullulanase, and α-glucosidase, in culture broth. These three enzymes were separately produced and the levels of α-glucosidase and pullulanase reached maxima after 24-h cultivation at the initial pH 9.7. Although this pullulanase was not purified, the indicated pH optimum was at 7.

Two highly alkaliphilic pullulanase-producing bacteria were isolated from Korean soils (Kim et al. 1993a, b). The two isolates were extremely alkaliphilic since bacterial growth and enzyme production occurred at pH values ranging from pH 6–12 for *Micrococcus* sp. Y-1 and pH 6–10 for *Bacillus* sp. S-1. The enzyme displayed a temperature optimum of around 60°C and a pH optimum of around pH 9. The extracellular enzymes of both bacteria were alkaliphilic and moderately thermoactive; optimal activity was detected at pH 8–10 and between 50°C and 60°C.

In screening alkaline cellulases for detergent additives, Ara et al. isolated a novel alkaline pullulanase from alkaliphilic *Bacillus* sp. KSM-1876, which was identified as a relative of *Bacillus* circulans (Ara et al. 1992; Igarashi et al. 1992). The enzyme had an optimum pH for enzyme action of around 10–10.5. This enzyme is a good candidate for use as an additive to dishwashing detergents. Then, Ara isolated new alkaliphilic *Bacillus* sp. KSM-1378 and reported two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions (Ara et al. 1995, 1996; Hatada et al. 1996). Alkaliphilic *Bacillus* sp. KSM-1378 produces an alkaline amylopullulanase that hydrolyzes both α-1,4 linkages in amylose, amylopectin, and glycogen, and α-1,6 linkages in pullulan. The enzymatic properties, improvement in thermostability and oxidative stabilization of AmyK (Hagihara et al. 2001a, b), were extensively studied and reported. And Ca^{2+}-free α-amylase, AmyK38, was discovered from an alkaliphilic *Bacillus* sp. KSM-K38 that is highly resistant to chelating reagents and chemical oxidants and requires Na^{+} for activity.

Lin et al. purified a thermostable pullulanase from thermophilic alkaliphilic *Bacillus* sp. strain TS-23. This purified enzyme had both pullulanase and amylase activities. The temperature and pH optima for both pullulanase and amylase activities were 70°C and pH 8–9, respectively. The enzyme remained more than 96% active at temperatures below 65°C, and both activities were retained at temperatures up to 90°C in the presence of 5% SDS (Lin et al. 1996).

Cyclodextrin (CD)-Forming Enzymes

The cyclodextrins, Schardinger dextrins, are a group of homologous oligosaccharides obtained from starch by the action of cyclomaltodextrin glucanotransferase (CGTase). These compounds are well known by their unique properties as follows: (1) Cyclodextrins (CD) are homogeneous cyclic molecules composed of six or more glucose units linked α-1,4 as in amylose; (2) as a consequence of cyclic arrangement they have neither a reducing end-group nor a nonreducing end-group and are not decomposed by hot aqueous alkali; (3) they are rather resistant to acid hydrolysis and the common starch-splitting amylases; (4) they crystallize very well from water and from aqueous alcohols; (5) they form an abundance of crystalline complexes called inclusion compounds with organic or inorganic substances. Mass production of these unique compounds on an industrial scale has been attempted several times in the past. However, there were serious problems in the production processes. (1) CGTase from *Bacillus macerans* is not suitable for industrial use because the enzyme is not thermostable enough. (2) Yield of CD from starch is not high, usually 20–30% on an industrial scale. (3) Toxic organic solvents such as trichloroethylene, bromobenzene, and toluene were used to precipitate CD due to the low conversion rate. The use of such harmful organic solvents is strongly prohibited in various fields, especially food processing. Therefore, the development of large-volume use was quite limited. In 1968, author's group isolated several CGTases from alkaliphilic *Bacillus* strains. One of them, a CGTase produced by alkaliphilic *Bacillus* sp. No. 38-2, overcame all these weak points and we succeeded in mass-producing crystalline α-, β-CD, and γ-CD-mixture at low cost without using any organic solvents.

Purification and Properties of *Bacillus* sp. No. 38-2 and *Bacillus oshimensis*17-1 Enzymes

Two strains, *Bacillus* sp. No. 38-2 and *Bacillus oshimensis* 17-1, were selected as the best enzyme producers from starch-degrading alkaliphilic strains (Nakamura and Horikoshi 1976a, b, c, d). The organisms were aerobically cultivated for 3 days in Horikoshi-II medium at 37°C. The crude enzymes in the supernatant fluids were purified by starch adsorption followed by conventional DEAE-cellulose column chromatography.

Since Nakamura and Horikoshi discovered and isolated the bacterium alkaliphilic *Bacillus* sp. No. 38-2, many alkaliphilic microorganisms producing CGTases have been reported. Nomoto et al. (1984, 1986) found a CGTase produced by alkaliphilic *Bacillus* sp. No. HA3-3-2 isolated from soil from Taipei, Taiwan. The enzyme showed maximum CD-forming activity in the pH range of 6.5–8 and was stable between pH 6 and 11. Abelyan et al. (1994a, b) developed an isolation method for CGTase using cyclodextrin polymers and their derivatives. CGTases were directly purified from culture broth of mesophilic, thermophilic, and alkaliphilic bacilli by affinity chromatography on a β-CD polymer.

Hamamoto and Horikoshi (1987) and Kaneko et al. (1988) have characterized a CGTase of alkaliphilic *Bacillus* sp. No. 38-2. This is a unique enzyme, especially in having wide pH optimum, pH or thermal stability and high productivity of CD from starch, compared with other microbial CGTases. Conventional purification methods showed that the crude enzyme preparation contained three enzymes: acid-, neutral-, and alkaline-CGTase. Therefore, it is very interesting to investigate the genetic information of the enzyme(s) using gene cloning methods.

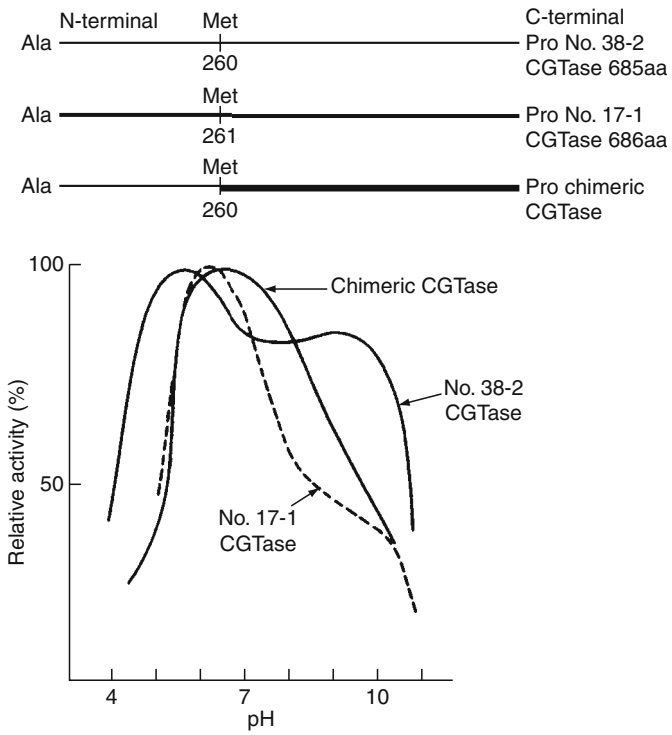

Fig. 2.8.3

pH activity curves No. 38-2, No.17-1 and chimeric CGTases

The CGTase gene of alkaliphilic *Bacillus* sp. No. 38-2 was cloned in *E. coli* and expressed. A CGTase gene of *Bacillus* sp. No. 17-1 was cloned and sequenced. As shown in *Fig. 2.8.3*, chimeric experiments between No. 17-1 CGTase and No. 38-2 CGTase revealed that the pH activity profile was affected by the C-terminal region of the protein molecule (Kaneko et al. 1989). Chimeric enzymes that contained the N -terminal and the C-terminal segments derived from CGTase 38-2 produced large amounts of CD and, in particular, a higher proportion of α-CD than other chimeric enzymes.

CGTase of *Bacillus* sp. No. 1011

Yamane's group has extensively investigated the molecular structure of the CGTase of alkaliphilic *Bacillus* sp. No. 1011. Kimura et al. (1987a, b) cloned the gene for β-CGTase from an alkaliphilic bacterium, *Bacillus* sp. No. 1011 in an *E. coli*. The extracellular β-CGTase of *Bacillus* sp. No. 1011 had Ala-Pro-Asp at the N-terminal.

Recently, Haga et al. (2003) reported the effects of essential carbohydrate/aromatic stacking interaction with Tyr100 and Phe259 on substrate binding of the CGTase. The stacking interaction between a tyrosine residue and the sugar ring at the catalytic subsite −1 is strictly conserved in the glycoside hydrolase family 13 enzymes. Replacing Tyr100 with leucine in the CGTase to prevent stacking significantly decreased all CGTase activities. The adjacent stacking

interaction with both Phe183 and Phe259 onto the sugar ring at subsite +2 is essentially conserved among CGTases. These structural and biochemical data suggest that substrate binding in the active site of CGTase is critically affected by the carbohydrate/aromatic stacking interaction with Tyr100 at the catalytic subsite −1 and that this effect is likely a result of cooperation between Tyr100 and Phe259 through stacking interaction with substrate at subsite +2. There are many CGTase papers reported besides those described above; therefore, all of them are not introduced.

γ-CD-Forming CGTase

Kato and Horikoshi (1986) demonstrated that one strain of *B. subtilis* produced a γ-CD-forming CGTase in culture broth. The isolate, *B. subtilis* No. 313, was grown aerobically for 5 days at 37°C in a cultivation medium containing 1% potato starch, 1% polypeptone, 0.1% yeast extract, 0.3% KH_2PO_4, 1% $MgSO_4$, and 0.02% $CaCO_3$. The γ-CD-forming activity in the crude broth was assayed by the BGC method (Kato and Horikoshi 1984). The crude enzyme preparation exhibited a relatively broad pH activity curve for CD formation with a pH optimum at 8. Analysis of the enzymatic digest by HPLC showed the product to be only γ-CD.

Recently, Takada et al. (2003) isolated a novel alkaliphilic bacterium, *Bacillus clarkii* 7364. This strain produced a γ-CD-forming enzyme that exhibited a higher conversion rate from potato starch. The CGTase secreted into the culture medium from this bacterium was purified by affinity chromatography on a γ-CD-immobilized column, followed by chromatography on a gel filtration column. The enzyme converted 13.7% of pre-gelatinized potato starch (10% w/w per reaction mixture) into CDs at pH 10 and 50°C, and the majority (79%) of the product CDs was of the γ-form. The addition of 20% (v/v) ethanol to the reaction mixture gave 23% yield of γ-CD from gelatinized corn starch (1% w/v). This property is unique among the known CGTases.

From the industrial point of view, γ-CD is an ideal compound for the production of pharmaceutical chemicals and food additives because of its large cavity diameter and biodegradability. Thus, enzymes having higher thermostability and higher yield of γ-CD from corn starch are desirable for industrial production.

Further properties of CGTase and industrial production of cyclodextrin are contributed the following chapter by Nakamura (2.10 Beta-Cyclomaltodextrin glucanotransferase of a species of alkaliphilic *Bacillus* for the production of beta-cyclodextrin).

Cellulases of Alkaliphilic *Bacillus* Strains

No enzyme with an alkaline optimum pH for activity (pH 10 or higher) had been reported before our report (Horikoshi et al. 1984). They found newly isolated bacteria. One of these, alkaliphilic *B. cellulosilyticus* N-4, produced at least three CMCases. Another bacterium, *B. akibai* 1139, produced one CMCase which was entirely purified, and the enzyme had an optimum pH for activity at pH 9. All alkaline cellulases so far discovered could not or only slightly hydrolyze cellulose fibers. Our finding of alkaline cellulases paved the way for the industrial application of these enzymes as a laundry detergent additive and hundreds of scientific papers and patents have been published.

Horikoshi found bacterial isolates (*Bacillus* No. N4 and No. 1139) producing extracellular alkaline carboxymethylcellulases (CMCases) (Horikoshi, Nakao et al. 1984; Fukumori et al. 1985). One of these, alkaliphilic *Bacillus cellulosilyticus* N-4 produced multiple CMCases that

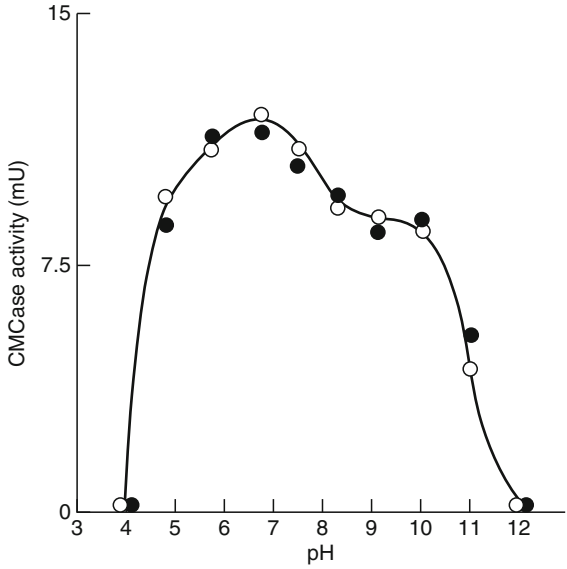

Fig. 2.8.4
Effect of pH on *Bacillus cellulosilyticus* N-4 CMCase activity

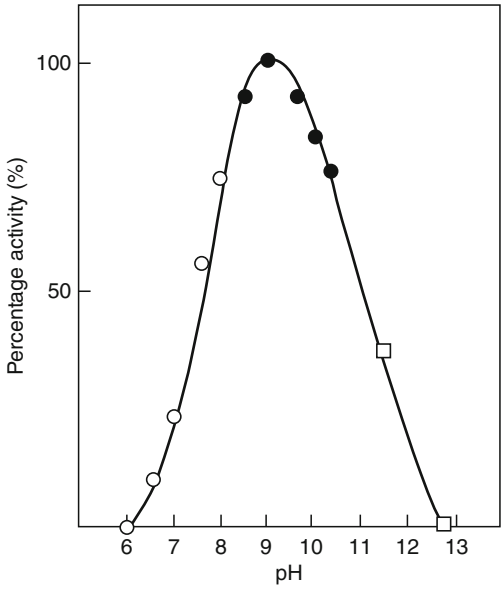

Fig. 2.8.5
Effect of pH on *Bacillus akibaii* 1139 CMCase activity

were active over a broad pH range (pH 5–10), as shown in *Fig. 2.8.4*. Sashihara cloned the cellulase genes of *Bacillus cellulosilyticus* N-4 in *Escherichia coli* HB101. Several cellulase-producing clones that have different DNA sequences were obtained (Sashihara et al. 1984). Another bacterium, *Bacillus akibai* No. 1139, produced one CMCase, which was purified and

shown to have optimum pH for activity at pH 9 (*Fig. 2.8.5*). The enzyme was stable over the range of pH 6–11 (24 h at 4°C and up to 40°C for 10 min). The CMCase gene of *Bacillus akibai* 1139 was also cloned in *E. coli* (38–42). Nakamura (Nakamura et al. 1991; Park et al. 1991) constructed many chimeric cellulases from *Bacillus subtilis* and *Bacillus cellulosilyticus* N-4 enzyme genes in order to understand the alkaliphily of N-4 enzymes. Despite the genes having high homology, the pH activity profiles of the two enzymes are quite different; *Bacillus subtilis* (BSC) has its optimum pH at 6–6.5, whereas *Bacillus cellulosilyticus* N-4 enzyme (NK1) is active over a broad pH range from 6 to 10.5. The chimeric cellulases showed various chromatographic behaviors, reflecting the origins of their C-terminal regions. The pH activity profiles of the chimeric enzymes in the alkaline range could be classified into either the BSC or NK1 type, mainly depending on the origins of the fifth C-terminal regions.

Cellulases as Laundry Detergent Additives

The discovery of alkaline cellulases created a new industrial application of cellulase as a laundry detergent additive. Ito (personal communication) mixed alkaline cellulases with laundry detergents and studied the washing effect by washing cotton underwear. The best results were obtained by one of the alkaline cellulases produced by an alkaliphilic *Bacillus* strain. However, the yield of enzyme was not sufficient for industrial purposes. Consequently, Ito and his and his coworkers (Ito et al. 1989; Yoshimatsu et al. 1990) isolated an alkaliphilic *Bacillus* sp. No. KSM-635 from the soil and succeeded in producing an alkaline cellulase as a laundry detergent additive on an industrial scale. Further details are reviewed by Ito in Chap. 2.11 Alkaline Enzymes in Current Detergency.

Xylanases

Isolation

The first paper of xylanase of alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (1973). The purified enzyme of *Bacillus* sp. No. C-59-2 exhibited a broad optimum pH ranging from 6 to 8. Then, Okazaki reported that four thermophilic alkaliphilic *Bacillus* stains (Wl, W2, W3 and W4) produced xylanases (Okazaki et al. 1984, 1985). The pH optima for enzyme action of strains Wl and W3 was 6 and for strains W2 and W4 was between 6 and 7. The enzymes were stable between pH 4.5 and 10.5 at 45°C for 1 h. The optimum temperatures of xylanases of W1 and W3 were 65°C and those of W2 and W4 were 70°C. The degree of hydrolysis of xylan was about 70% after 24 h incubation.

Consequently, two xylanases were found in the culture broth of *Bacillus halodurans* C-125 (Honda et al. 1985a). Xylanase A had molecular weight of 43,000 and that of xylanase N was 16,000. Xylanase N was most active at pH 6–7 and xylanase A was most active at a pH range of 6–10 and had some activity at pH 12. Xylanase A gene was cloned, sequenced, and expressed in *E. coli* (Honda et al. 1985a, b, c, 1986a, b).

After the demonstration that alkali-treated wood pulp could be biologically bleached by xylanases instead of by the usual environmentally damaging chemical process using chlorine, the search for thermostable alkaline xylanases has been extensive. Dey et al. (1992) isolated an alkaliphilic thermophilic *Bacillus* sp. that produced two types of cellulase-free xylanase at pH 10 and 50°C. Khasin reported alkaliphilic *Bacillus stearothermophilus* T-6 produced an

extracellular xylanase that was shown to optimally bleach pulp at pH 9 and 65°C (Khasin et al. 1993).

Nakamura et al. also reported an alkaliphilic *Bacillus* sp. strain, 41M-1, isolated from soil, produced multiple xylanases extracellularly (Nakamura et al. 1993a, b, 1995). One of the enzymes, xylanase J, was most active at pH 9. The optimum temperature for the activity at pH 9 was around 50°C. Then, an alkaliphilic and thermophilic *Bacillus* sp. strain TAR-1 was isolated from soil (Nakamura et al. 1994). The xylanase was most active over a pH range of 5–9.5 at 50°C. Optimum temperatures of the crude xylanase preparation were 75°C at pH 7 and 70°C at pH 9. These xylanases did not act on cellulose, indicating a possible application of the enzyme in biological debleaching processes.

Then, Gallardo et al. (2004) reported that the xynA gene encoding a xylanase from the recently isolated *Bacillus* sp. Strain BP-7 has been cloned and expressed in *E. coli*. Recombinant xylanase A showed high activity on xylans from hardwoods and cereals, and exhibited maximum activity at pH 6 and 60°C. The enzyme remained stable after incubation at 50°C and pH 7 for 3 h, and it was strongly inhibited by Mn^{2+}, Fe^{3+}, Pb^{2+}, and Hg^{2}.

Biobleaching

Several reports on biobleaching by xylanases were published from the industrial point of view, although the details of industrial plants have not been fully revealed. Blanco et al. reported that an enzyme from *Bacillus* sp. strain BP-23 was shown to facilitate chemical bleaching of pulp, generating savings of 38% in terms of chlorine dioxide consumption (Blanco et al. 1995). Recently, Garg reported a biobleaching effect of *Streptomyces thermoviolaceus* xylanase on birchwood kraft pulp. *S. thermoviolaceus* xylanase had the advantage of activity and stability at 65°C (Garg et al. 1996, 1998). Subsequently, many thermostable alkaline xylanases have been produced from various alkaliphiles isolated.

Mannan-Degrading Enzymes

β-Mannan is a kind of hemicellulose contained in higher plants such as konjac, guargum, locust bean, and copra, and it easily dissolves in alkaline water. Mannan-degrading enzymes of neutrophilic bacteria, actinomyces, and fungi have been studied. However, no mannan-degrading enzyme that hydrolyzes under alkaline condition has been discovered before our discovery. Akino reported isolation and properties of mannan-degrading microorganisms (Akino et al. 1987, 1988a, b).

A small amount of soil was spread on agar plates containing 1% β-mannan from larch wood, 1% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4$ $7H_20$, and 0.5% sodium carbonate. The plates were incubated at 37°C for 48–72 h. Strain AM-001 with a large clear zone around the colony was selected as the enzyme producer. The isolate grew at temperatures from 20°C to 45°C, with an optimum at 37°C in the medium described above. The pH range for growth was from pH 7.5–11.5 with the optimum at pH 8.5–9.5. The taxonomical characteristics of this alkaliphilic *Bacillus* strain were almost the same as those of *Bacillus hortii*.

The enzyme produced in culture broth contained three β-mannanases and β-mannosidase. Two of β-mannanases was most active at pH 9, and one demonstrated optimum enzyme action at pH 8.5. These properties of the enzyme are good for the production of D-mannose from β-mannan in the presence of the β-mannanases described above. Since then several mannan-degrding alkaliphiles have been published although no industrial application has been established yet.

Pectinases

The first paper on alkaline endo-polygalacturonase produced by alkaliphilic *Bacillus halodurans* P-4-N was published in 1972 (Horikoshi 1972). The optimum pH for enzyme action was 10 for pectic acid. Fogarty and Kelly (Fogarty and Ward 1977; Kelly and Fogarty 1978) then reported that *Bacillus* sp. No. RK9 produced endo-polygalacturonate lyase. The optimum pH for the enzyme activity towards acid-soluble pectic acid was 10. Subsequently, several papers on potential applications of alkaline pectinase have been published. The first application of alkaline pectinase-producing bacteria in the retting of Mitsumata bast was reported by Yoshihara and Kobayashi (1982). The pectic lyase (pH optimum 9.5) produced by an alkaliphilic *Bacillus* sp. No. GIR 277 has been used in improving the production of a type of Japanese paper. A new retting process produced a high-quality, nonwoody paper that was stronger than the paper produced by the conventional method. Tanabe et al. (1987, 1988) tried to develop a new waste treatment by using an alkaliphilic *Bacillus* sp. No. GIR 621-7. Cao et al. (1992) isolated four alkaliphilic bacteria, NT-2, NT-6, NT-33, and NT-82, producing pectinase and xylanase. One strain, NT-33, had an excellent capacity for degumming ramie fibers. Recently, author's group reidentified this strain by 16s RNA analysis, and *Bacillus* sp. P-4-N was found to be very similar or the same strain as *Bacillus halodurans* C-125.

Recently, Ito and his colleagues have isolated many pectin-degrading enzymes from alkaliphilic *Bacillus* strains and tested a possibility of industrial application of the enzymes as laundry detergent additive. However, due to difficulties to make high production in industrial scale systems, their interests were focused on other enzymes.

Cross-References

- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles
- 2.9 Genomics and Evolution of Alkaliphilic *Bacillus* Species
- 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic *Bacillus* for the Production of Beta-Cyclodextrin
- 2.11 Alkaline Enzymes in Current Detergency
- 9.3 Biochemistry

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2.9 Genomics and Evolution of Alkaliphilic *Bacillus* Species

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.9,

A great number of aerobic endospore-forming Gram-positive *Bacillus* species have been isolated on a number of occasions from a variety of terrestrial and deep-sea environments, including the Mariana Trench, which has a depth of 10,897 m (Takami et al. 1997). Some of these *Bacullus* species are known to have various capabilities for adapting to extreme environments. In fact, *Bacillus*-related species can grow in a wide range of environments – at pH 2–12, at temperatures between 5°C and 78°C, in salinity from 0% to 30% NaCl, and under pressures from 0.1 Mpa to at least 30 Mpa. We are now exploring how these adaptive capabilities, as reflected in their genomes, were acquired and what intrinsic genomic structures are present in *Bacillus*-related species that have allowed them to adapt to such a wide range of environments. To answer these questions, especially for adaptation mechanisms to alkaline environments, we initiated a genome sequencing project in early 1998 and determined the entire genomic sequences of two bacilli: alkaliphilic *Bacillus halodurans* and extremely halotolerant and alkaliphilic *Oceanobacillus iheyensis*. This chapter will provide the comparative analysis of the alkaliphilic bacillar genomes with those of three other phylogenetically related mesophilic and neutrophilic bacilli in order to highlight the commonality between two alkaliphiles and diversity of the bacillar genome.

Phylogenetic Placement of Alkaliphilic Bacilli Among *Bacillus*-Related Species

Since genus *Bacillus* defined as Gram-positive, aerobic, spore-forming, and motile rod-shaped bacteria is a very broad genus (Sneath et al. 1986), some of *Bacillus* species have been designated in new genera such as *Alicyclobacillus* (Wisotzkey et al. 1992), *Amphibacillus* (Niimura et al. 1990), *Brevibacillus* (Shida et al. 1996), *Geobacillus* (Nazina et al. 2001), *Halobacillus* (Spring et al. 1996), *Oceanobacillus* (Lu et al. 2001), and *Salibacillus* (Wainø et al. 1999). Phylogenetic analysis of 16S rRNA for species in genus *Bacillus* and species in the new genera reclassified from *Bacillus* (*Bacillus*-related species) shows clustering of species possessing similar phenotypic properties such as alkaliphily, halophily, and thermophily (*Fig. 2.9.1*). However, each of the clusters is phylogenetically close to each other, even for those exhibiting different extremophilic phenotypes. Thus, we are very intrigued by questions of how the adaptive capabilities, which are reflected in their genomes, to extreme environments especially, alkaline environments were acquired and what intrinsic genomic structure of *Bacillus*-related species allows them to adapt to such a wide range of environments.

Determination of Genome Sequences of Alkaliphilic Bacilli

Whole-Genome Shotgun Sequencing of Alkaliphilic Bacilli

The genomes of *B. halodurans* C-125 and *O. iheyensis* HTE831 were basically sequenced by the whole-genome sequencing method (Fleischmann et al. 1995). Briefly, aliquots (10–20 μg) of chromosomal DNA were fragmented into 1–2 kb pieces by a Bioruptor UCD-200TM (Tosho Denki, Tokyo, Japan) or a HydroShear (GeneMachines, CA, USA) and then prepared and sequenced to produce a whole-genome shotgun library as previously described (Takami et al. 2000; Takami et al. 2002). DNA sequences determined by a MegaBace1000 (GE Healthcare, NJ, USA) and ABI PRISM377 DNA sequencer (Applied Biosystems, CT, USA) were assembled into contigs using Phrap (http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) with

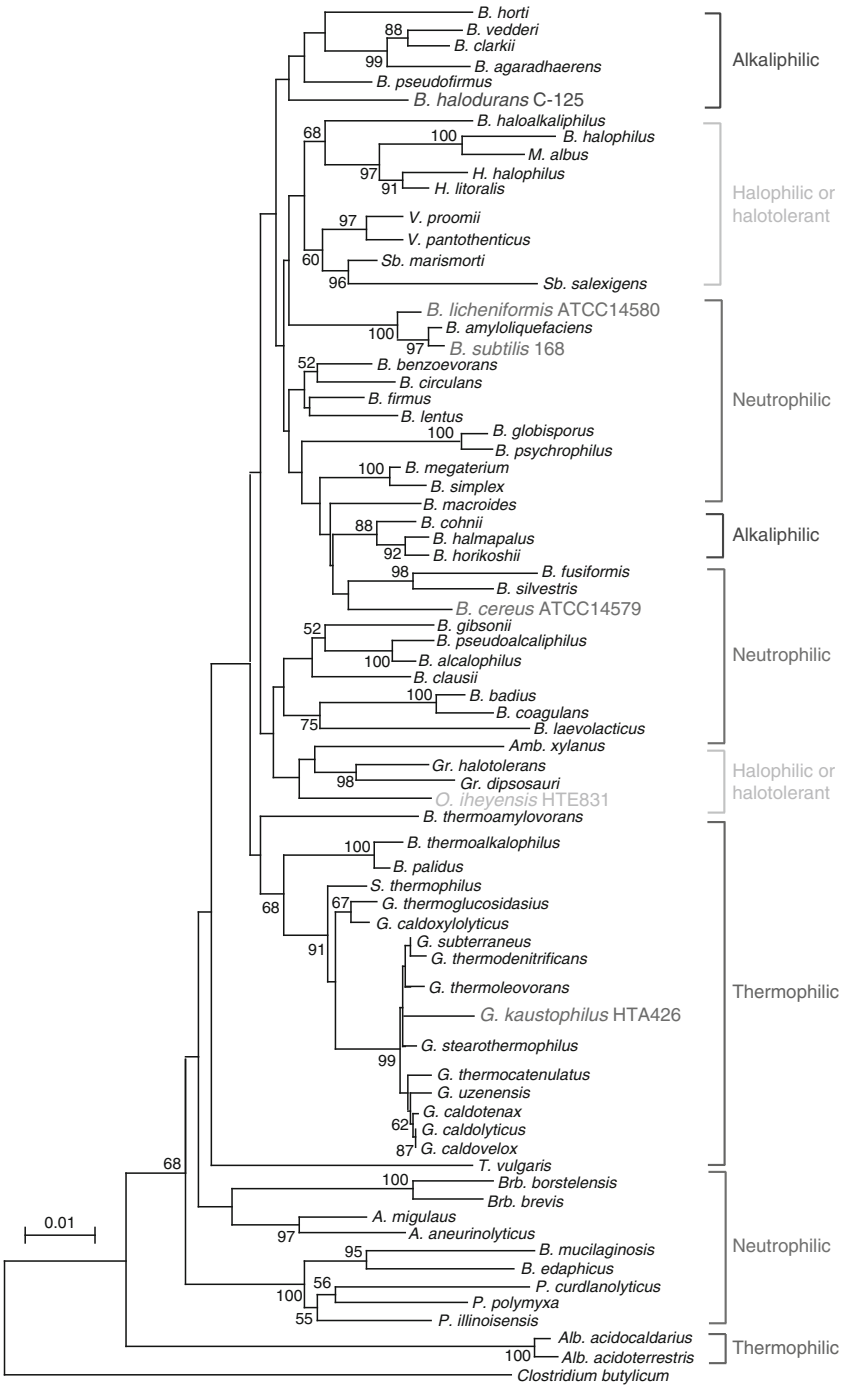


Fig. 2.9.1 (Continued)

default parameters. The assembly using Phrap yielded 330 contigs at a statistical coverage of 5.9-fold. The 2,000 sequences obtained from reverse-end shotgun cloning and from both ends of genomic libraries ranging from 4 to 6 kb. In addition, 1,000–2,000 sequences from both ends of 20-kb insert in λ-phage clones were also assembled to bridge the remaining contigs. These sequences were assembled with consensus sequences derived from the contigs of random-phase sequences using Phrap and then the contigs were reduced to 105 by this step. Gaps between contigs were closed by shotgun sequencing of large fragments, which bridged the contigs of random-phase sequences. The final gaps were closed by direct sequencing of the products amplified by long accurate polymerase chain reaction (PCR).

Gene Finding and Annotation

The predicted protein-coding regions were initially determined by searching for open reading frames (ORFs) longer than 100 codons using the GenomeGambler program, a semiautomated genome analysis system developed to support this whole-genome sequencing project (Sakiyama et al. 2000). GenomeGambler reduces the time and effort required to annotate thousands of protein coding sequences (CDSs) identified in the microbial genome by automating three major routines: analyzing assembly results provided by genome assembler software, annotating CDSs, and searching for homology. All processes and options are manipulated through a WWW browser that enables scientists to share their genome analysis results without choosing a computer operating system. A new GenomeGambler system called MetaGenomeGambler ^{LITE}, which was developed in a collaborative project with In silico Biology Inc. (http://www.insilicobiology.co.jp; Yokohama, Japan) and has already been released through this company, is a stand-alone system that makes it unnecessary to communicate with a server in the annotation process.

Coding potential analysis of the entire genome was performed with the GeneHackerPlus program using hidden Markov models (Yada et al. 2001) trained with a set of ORFs longer than 300 nucleotides from either *B. halodurans* or *O. iheyensis* before analyzing sequences from the respective species. Searches of protein databases for amino acid similarities were performed using BLAST2 sequence analysis tools (Altschul et al. 1997) with subsequent comparison of CDSs showing significant homology ($>10^{-5}$ significance) using Lipman–Pearson algorithm (Pearson and Lipman 1988). Significant similarity was defined as at least 30% identity observed over 60% of the CDS, although those CDSs showing <30% identity over >60% of the protein were also included in analysis.

Fig. 2.9.1

Phylogenetetic tree of Bacillaceae based on 16S rDNA sequences. Phylogenetic positions were generated using the neighbor-joining method with available sequences of Bacillaceae. The numbers indicate bootstrap probability values supporting the internal branches after 1,000 replications. Bootstrap probability values less than 50 were omitted. Bar = 0.01 Knuc unit. Abbreviations: *A., Aneurinibacillus; Alb., Alicyclobacillus; Amb., Amphibacillus; B., Bacillus; Brb., Brevibacillus; G., Geobacillus; Gb., Gracilibacillus; H., Halobacillus; M., Marinococcus; O., Oceanobacillus; P., Paenibacillus; S., Saccharococcus; Sb., Salibacillus; T., Thermoactinomyces; V., Virgibacillus*

Genomic Features of Alkaliphilic Bacilli

B. halodurans

Alkaliphilic *B. halodurans* strain C-125 (JCM9153) (Takami and Horikoshi 1999) was isolated from soil in 1970 (Ikura and Horikoshi 1978) and is characterized as a β-galactosidase (Ikura and Horikoshi 1979) and xylanase producer (Honda et al. 1985). Among our collection of alkaliphilic *Bacillus* isolates, this strain has been the most thoroughly characterized, physiologically, biochemically, and genetically (Horikoshi 1999a), and it was used as a representative strain for genetic analysis. Generally, alkaliphilic *Bacillus* strains cannot grow at pH below 6.5, but grow well at pH above 9.5. Facultative alkaliphilic *B. halodurans* can grow at pH 7–10.8 if sodium ions are supplied at a sufficiently high concentration (1–2%) in the medium. Over the past 3 decades, our studies have focused on the enzymology, physiology, and molecular genetics of alkaliphilic microorganisms. Industrial applications of these microbes have been investigated and some enzymes such as proteases, amylases, cellulases, and xylanases have been commercialized (Horikoshi 1999b; Takami and Horikoshi 2000).

General Features

The genome of *B. halodurans* is a single circular chromosome (Takami et al. 1999b) consisting of 4,202,352 bp with an average G+C content of 43.7% (coding region, 44.4%; noncoding region, 39.8%) (*Fig. 2.9.2* and *Table 2.9.1*). Based on analysis of skew in the ratio of G−C to G+C (G−C/G+C), we estimated that the site of termination of replication (*terC*) is located nearly 2.2–2.3 Mb (193°) from the replication origin, but we could not identify the gene encoding the replication termination protein (*rtp*) (Takami et al. 2000). Several A+T- and G+ C-rich islands are likely to reveal the signature of transposons or other inserted elements. We identified 4,066 CDSs (*Table 2.9.1*) with an average size of 879 bp.

Coding sequences covered 85% of the chromosome, and we found that 78% of the genes in *B. halodurans* started with ATG, 10% with TTG, and 12% with GTG, as compared with 87%, 13%, and 9%, respectively, in the case of *B. subtilis*. Proteins of *B. halodurans* predicted from 4,066 CDSs were estimated to range in size from 1,188 to 199,106 Da for a total of 32,841 Da: nonredundant proteins with assigned biological role, 2,141 (52.7%); conserved proteins of unknown function, 1,182 (29.1%); and no database match for 743 (18.3%) in comparisons with protein sequences from other organisms, including *B. subtilis*. Among the CDSs found in the *B. halodurans* genome, 2,310 (56.8%) were widely conserved in organisms including *B. subtilis* and 355 (8.7%) matched protein sequences found only in *B. subtilis*. The ratios CDSs conserved among the various organisms including *B. subtilis* were 80.5% and 49.7% for functionally assigned CDSs and CDSs matched with hypothetical proteins from other organisms, respectively. Of the nonredundant CDSs with a biological role, 23.8% matched hypothetical proteins found only in the *B. subtilis* database, showing relatively high similarity values.

Transcription and Translation

Genes encoding the three subunits (α, β, β′) of the core RNA polymerase have been identified in *B. halodurans* along with genes for 20 sigma factors (Haldenwang 1995). Sigma factors belonging to the σ^{70} family (σ^{A}, σ^{B}, σ^{D}, σ^{E}, σ^{F}, σ^{G}, σ^{H}, and σ^{K}) are required for sporulation

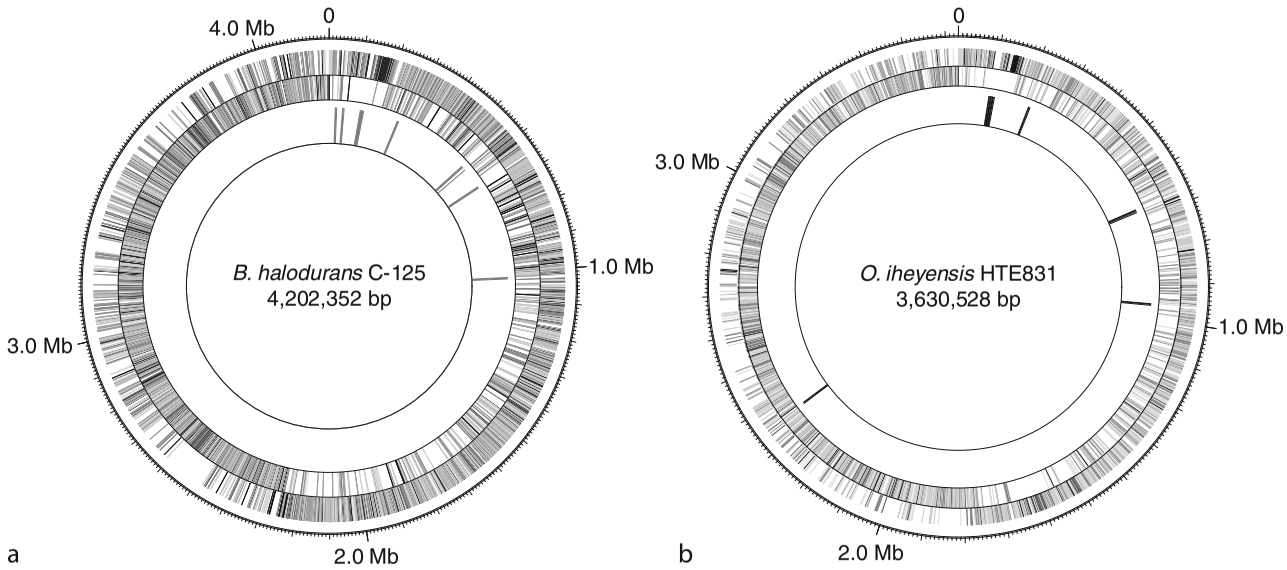


Fig. 2.9.2

Circular representation of the extremophilic bacillar genomes. (a) *Bacillus halodurans* C-125. (b) *Oceanobacillus iheyensis* HTE831. The *outer circle* is the plus strand and the *inner circle* is the minus strand. The *third* and *fourth circles* indicate the distribution of rRNA and tRNA in the genome, respectively

Table 2.9.1

General features of the genomes of alkaliphiles and their comparison with those of non-alkaliphilic bacilli

General features	*B. halodurans* C-125	*O. iheyensis* HTE831	*G. kaustophilus* HTA426[a]	*B. cereus* ATCC14579[b]	*B. anthracis* Ames[c]		*B. subtilis* 168
Chromosome							
Size (base pairs)	4,202,352	3,630,528	3,544,776	5,411,809	5,227,293		4,214,630
G+C content (mol%)							
Total genome	43.7	35.7	52.1	35.3	35.4		43.5
Coding region	44.4	36.1	52.9	35.9	36.0		43.4
Noncoding region	39.8	31.8	47.0	32.7	32.6		43.6
Predicted CDS number	4,066	3,496	3,498	5,234	5,311		4,106
Average length (bp)	879	883	862	835	794		896
Coding region (%)	85	85	86	81	81		87
Initiation codon (%)							
AUG	78	79.5	75.2	75.3	83.1		78
GUG	12	7.8	13.5	11.9	9.1		9
UUG	10	12.7	11.3	12.8	7.8		13
Stable RNA (%)	1.02	1.04	1.70	1.2	1.09		1.27
Number of *rrn* operon	8	7	9	13	11		10
Mean G+C content	54.2	52.7	58.5	52.6	52.6		54.4
Number of tRNA	78	69	87	108	86		86
Mean G+C content	59.5	58.8	59.1	59.2	59.1		58.2
Plasmid			pHTA426	pBClin15	pX01	pX02	
Size (base pairs)	–	–	47,890	15,100	181,677	94,829	–
G+C content (mol%)	–	–	44.2	38.1	32.5	33.0	–
Noncoding region	–	–	44.5	38.4	33.7	34.2	–

Table 2.9.1 (Continued)

General features	*B. halodurans* C-125	*O. iheyensis* HTE831	*G. kaustophilus* HTA426[a]	*B. cereus* ATCC14579[b]	*B. anthracis* Ames[c]		*B. subtilis* 168
Coding region	–	–	43.8	35.4	29.7	30.6	–
Predicted CDS number	–	–	42	21	217	113	–
Average length (bp)	–	–	906	645	645	639	–
Coding region (%)	–	–	79.5	89.8	77.1	76.2	–
Initiation codon (%)							
AUG	–	–	64.3	75.3	75.3	75.3	–
GUG	–	–	21.4	11.9	11.90	11.9	–
UUG	–	–	14.3	12.8	12.8	12.8	–

[a]Takami et al. 2004b, [b]Ivanova et al. 2003, [c]Read et al. 2003

and σ^L is well-conserved between *B. halodurans* and *B. subtilis*. Of the 11 sigma factors of the extracytoplasmic function (ECF) family identified in *B. halodurans*, only σ^W is also found in *B. subtilis* while the other 10 (BH640, BH672, BH1615, BH2026, BH3117, BH3216, BH3223, BH3380, BH3632, and BH3882) are unique to *B. halodurans* (Takami et al. 2000). These unique sigma factors may play a role in the special physiological mechanisms by which *B. halodurans* is able to adapt to alkaline environments, as it is well known that ECF sigma factors are present in a wide variety of bacteria and that they serve to control the uptake or secretion of specific molecules or ions and to control responses to a variety of extracellular stress signals (Lonetto et al. 1994).

The 79 tRNA species were organized into 11 clusters involving 71 tRNA genes plus 8 single genes without composing the cluster (*Table 2.9.1*), of which 6 clusters were associated with ribosomal RNA (rRNA) operons. Eight rRNA operons are present in the *B. halodurans* C-125 genome and they are organized identically to those of *B. subtilis* (tRNA-16S-23S-5S, 16S-tRNA-23S-5S, and 16S-23S-5S-tRNA). With respect to tRNA synthetases, the *B. halodurans* C-125 genome lacks the glutaminyl-tRNA synthetase gene (*glnS*), one of two threonyl-tRNA synthetase gene (*thrZ*), and one of two tyrosyl-tRNA synthetase genes (*tyrS*). The *B. subtilis* genome only lacks the glutaminyl-tRNA gene. It is likely that glutaminyl-tRNA synthetase aminoacylates $tRNA^{Gln}$ with glutamate followed by transamidation by Glu-tRNA amidotransferase in both of these *Bacillus* species.

Cell Walls

The peptidoglycan of alkaliphilic *B. halodurans* C-125 appears to be similar to that of neutrophilic *B. subtilis*. However, the cell wall components in C-125 are characterized by an excess of hexosamines and amino acids, compared to those of *B. subtilis*. Glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, meso-diaminopimelic acid, and acetic acid were found in cell wall hydrolysates (Horikoshi 1999a). Although some variation was found in the amide content of peptidoglycan isolated from alkaliphilic *B. halodurans* C-125,

the pattern of variation was similar to that known to occur in *B. subtilis*. All genes related to peptidoglycan biosynthesis such as *mraY, murC-G, cwlA, ddlA*, and *glnA* and which confirmed to be present in the *B. subtilis* genome were also conserved in the C-125 genome (Horikoshi 1999a). A bacitracin-resistance gene found in the *B. subtilis* genome is duplicated in the C-125 genome (BH474 and BH1538). On the other hand, although *tagH* and *tagG* genes were identified in *B. halodurans* C-125, 13 other genes for teichoic acid biosynthesis found in *B. subtilis* (*dltA-E, ggaA, ggaB, tagA-C, tagE, tagF*, and *tagO*) are missing in the *B. halodurans* genome. *B. halodurans* also lacks six genes (*tuaB-tuaF* and *tuaH*) for teichuronic acid biosynthesis, but retains *tuaA* and *tuaG* that are absent in *B. subtilis* (Takami et al. 2000). In addition to peptidoglycan, the cell wall of alkaliphilic *B. halodurans* is known to contain certain acidic polymers, such as galacturonic acid, glutamic acid, aspartic acid, and phosphoric acid. A teichuronopeptide (TUP) is present as a major structural component of the cell wall of *B. halodurans* C-125 and is a copolymer of polyglutamic acid and polyglucuronic acid.

Thus, the negative charges on acidic non-peptidoglycan components may permit the cell surface to absorb sodium and hydronium ions and to repel hydroxide ions, and, as a consequence, may contribute to permitting the cells to grow in alkaline environments. A mutant defective in TUP synthesis grows slowly at alkaline pH. The upper limit of pH for growth of the mutant is 10.4, whereas that for the parental strain C-125 is 10.8. The *tupA* gene encoding TUP has been cloned from *B. halodurans* C-125 chromosomal DNA (Aono et al. 1999), and in this study, it has been clarified that *B. halodurans* C-125 has no paralog of *tupA* in its genome and that the ortholog of *tupA* cannot be found in the *B. subtilis* genome.

Membrane Transport and Energy Generation

For growth under alkaline conditions, *B. halodurans* C-125 requires Na^+ in the surrounding environment for effective solute transport across the cytoplasmic membrane. According to the chemiosmotic theory, a proton-motive force is generated across the cytoplasmic membrane by electron transport chain or by extrusion of H^+ derived from ATP metabolism through the action of ATPase. We identified four types of ATPases (preprotein translocase subunit, class III heat-shock ATP-dependent protease, heavy metal-transporting ATPase, and cation-transporting ATPase). These ATPases are well-conserved between *B. halodurans* and *B. subtilis*.

Through a series of analyses such as a BLAST2 search, clustering analysis by the single linkage method examining all 8,166 CDSs identified in the *B. halodurans* C-125 and *B. subtilis* genomes, and multiple alignments, 18 CDSs were grouped into the category of antiporter- and transporter-related protein genes in the *B. halodurans* C-125 genome. In this analysis, five CDSs were identified as candidate Na^+/H^+ antiporter genes: BH1316, BH1319, BH2844, BH2964, and BH3946 (Takami et al. 2000). However, we could not identify any gene encoding antibiotic-resistance proteins in the *B. halodurans* C-125 genome, whereas the *B. subtilis* genome has nine such different genes. Eleven genes encoding multidrug resistance proteins were identified in the *B. halodurans* C-125 genome, six fewer than in the *B. subtilis* genome. A non-alkaliphilic mutant strain (mutant 38154) derived from *B. halodurans* C-125, which is useful as a host for cloning genes related to alkaliphily, has been isolated and characterized (Kudo et al. 1990). A 3.7-kb DNA fragment (pALK fragment) from the parent strain restored the growth of mutant 38154 under alkaline pH conditions. This fragment was found to contain CDS BH1319, which is one of the Na^+/H^+ antiporter genes in *B. halodurans*. The transformant was able to maintain an intracellular pH lower than the external pH and the cells expressed an

electrogenic Na^+/H^+ antiporter driven only by Dy (membrane potential, interior negative) (Horikoshi 1999a; Horikoshi 1999b). *B. subtilis* has an ortholog (*mprA*) of BH1319 and a *mprA*-deficient mutant of *B. subtilis* has been shown to be a sodium-sensitive phenotype (Kosono et al. 1999). On the other hand, a mutant of strain C-125 with a mutation in BH1317 adjacent to BH1319 has been isolated and shown to have an alkali-sensitive phenotype, although whether the Na^+/H^+ antiporter encoded by BH1317 is active in this mutant has not yet been confirmed experimentally. In addition, it has been reported that BH2819, the function of which is unknown and which is unique to the *B. halodurans* C-125 genome, is also related to the alkaliphilic phenotype (Aono et al. 1992).

B. halodurans C-125 has a respiratory electron transport chain and its basic gene set is conserved compared with that of *B. subtilis*, but the gene for cytochrome *bd* oxidase (BH3974 and BH3975) is duplicated in the *B. halodurans* C-125 genome. It is also clear that the two genes for *bo3*-type cytochrome *c* oxidase (BH739 and BH740) absent from *B. subtilis* are present in the *B. halodurans* C-125 genome. The *B. halodurans* C-125 genome has an F_1F_0-ATP synthase operon with an identical gene order (ε subunit-β subunit-γ subunit-α subunit-δ subunit-subunit β-subunit χ-subunit α) to that seen in *B. subtilis*. In addition to the F_1F_0-ATP synthase operon, the operon for a Na^+-transporting ATP synthase and the operon for flagellar-specific ATP synthase are also conserved between *B. halodurans* and *B. subtilis* (Takami et al. 2000).

ABC Transporters

Members of the superfamily of adenosine triphosphate (ATP)-binding-cassette (ABC) transport systems couple the hydrolysis of ATP to the translocation of solutes across biological membranes (Schneuder and Hunke 1998). ABC transporter genes are the most frequent class of protein-coding genes found in the *B. halodurans* genome, as in the case of *B. subtilis*. They must be extremely important in Gram-positive bacteria such as *Bacillus*, as these bacteria have an envelope consisting of a single membrane. ABC transporters protect such bacteria from the toxic action of many compounds. Through a series of analyses described above, 75 genes coding for ABC transporter/ATP-binding proteins were identified in the *B. halodurans* genome. In this analysis of the *B. halodurans* genome, 67 CDSs were categorized as ATP-binding protein genes, although 71 ATP-binding protein genes have been identified in the *B. subtilis* genome (Kunst et al. 1997). We found that *B. halodurans* has eight more oligopeptide ATP-binding proteins, but four fewer amino acid ATP-binding proteins, than *B. subtilis*. We could not find any other substantial difference between *B. halodurans* and *B. subtilis* in terms of the other ATP-binding proteins although it should be noted that the specificity of some of these proteins is not known. The genes for oligopeptide ATP-binding proteins (BH27, BH28, BH570, BH571, BH1799, BH1800, BH2077, BH2078, BH3639, BH3640, BH3645, BH3646, AppD, and AppF) are distributed throughout the *B. halodurans* C-125 genome (Takami et al. 2000).

IS Elements

Sixteen types of new insertion sequence (IS) elements with or without terminal inverted repeats (IRs) and with or without target site duplication (TSD), which is a direct repeat (DR) were identified and characterized in the *B. halodurans* genome. Ten kinds of IS elements were found to have IRs and flanked by TSD. The IS element designated as IS*641* was flanked

by a 9-bp TSD. IS*641*, which is 1,405 bp in length, shows 68% identity with the nucleotide sequence of IS*4Bsu*1 (Nagai et al. 2000) belonging to the IS*4* family (Klaer et al. 1981) and the Tpase of IS*641* shows 70.3% similarity to that of IS*4Bsu*1. The DDE (asparatic acid-asparatic acid-glutamic acid) motif, which is conserved in most Tpases and other enzymes capable of catalyzing cleavage of DNA strands (Mahillon and Chandler 1998; Plasterk 1993), was found in the Tpase of IS*641*: D (amino acid (a.a.) 124) D (a.a. 193), E (a.a. 293), and K (a.a. 300). These findings support the view that IS*641* should be categorized as a new member of the IS*4* family (Table 2.9.2). The *B. halodurans* genome of strain C-125 has two other copies of IS*641* (IS*641*-01 and IS*641*-03) with truncation and deletion, respectively, in an IS*641* segment.

The IS element (1,142 bp), designated as IS*642*, was flanked by DRs with a TA sequence. IS*642* showed 43.5% identity with the nucleotide sequence of IS*630*, which duplicates the TA sequence at the target site (Takami et al. 2001). There are two ORFs overlapping at 545 ~ 597 bp in IS*642*. It is evident that this occurred due to a frameshift mutation because the first and second ORFs are both similar to the Tpase of IS*630*, showing 23.5% and 27.2% similarity, respectively. The DDE motif was found in the Tpase segment encoded by the region straddling the frameshift mutation, as in the case of IS*630*. These results support the view that IS*642* is a new member of the IS*630* family (Table 2.9.2).

In addition, eight other new IS elements with terminal IRs that generate a TSD were identified: IS*643* (2,485 bp, IS*21* family) (Reimmann et al. 1989); IS*651* (1,384 bp, IS*L3* family); IS*652* (1,461 bp, 43.3% identity to IS*651*); IS*654* (1,384 bp, IS*256* family); IS*655* (1,221 bp,

Table 2.9.2

New IS elements and group II intron identified in the *B. halodurans* genome

IS	Size (bp)	TSD[a] (bp)	IR (bp)	No. of IS elements			Family[c]
				Total IS	IS with ends[b]	Truncated IS	
IS*641*	1405	9	18	3	2 (0)	1	IS*4*
IS*642*	1142	2 [TA]	26	1	1 (0)	0	IS*630*
IS*643*	2485	5	64	2	1 (0)	1	IS*21*
IS*650*	1929	0	–	2	1	1	IS*650*/IS*653**
IS*651*	1384	8	23	28	22 (5)	6	IS*L3*
IS*652*	1461	8	27	19	19 (6)	0	IS*L3*
IS*653*	1805	0	–	7	7	0	IS*650*/IS*653**
IS*654*	1384	8	31	9	9 (1)	0	IS*256*
IS*655*	1221	3	38	5	5 (0)	0	IS*3*
IS*656*	1558	4	15	4	4 (1)	0	IS*656*/IS*662**
IS*657*	734	2 [TT]	–	9	8 (0)	1	IS*200*/IS*605*
IS*658*	1058	4	27	4	3 (0)	1	IS*30*
IS*660*	1963	0	16	23	6	17	IS*1272**
IS*662*	1566	4	15	2	2 (0)	0	IS*656*/IS*662**
IS*663*	1980	0	14	2	1	1	IS*1272**
Bh.Int	1883	0		7	5	2	Group II intron

[a]The target site sequence is shown in [ ].
[b]IS elements with two intact ends. Numbers in parentheses show the IS elements without a target site duplication.
[c]New IS families proposed are shown by asterisks.

IS*3* family); IS*658* (1,058 bp, IS*30* family) (Dalrymple et al. 1984); IS*656* (1,558 bp, 67.7% identity to IS*662*); and IS*662* (1,566 bp). Two ISs (IS*656* and IS*662*) did not show significant similarity to any other IS elements reported to date (Takami et al. 2001), and were consequently categorized as members of a new IS family designated IS*656*/IS*662* family (Table 2.9.2). Note that some intact members of the IS elements described above were found to lack the flanking DRs of target site sequence (Table 2.9.2). This indicates that genome rearrangements have occurred through transpositional recombination mediated by IS elements. The existence of truncated members of each of the IS elements described above and below indicates the occurrence of internal rearrangements of the genome, probably through illegitimate recombination.

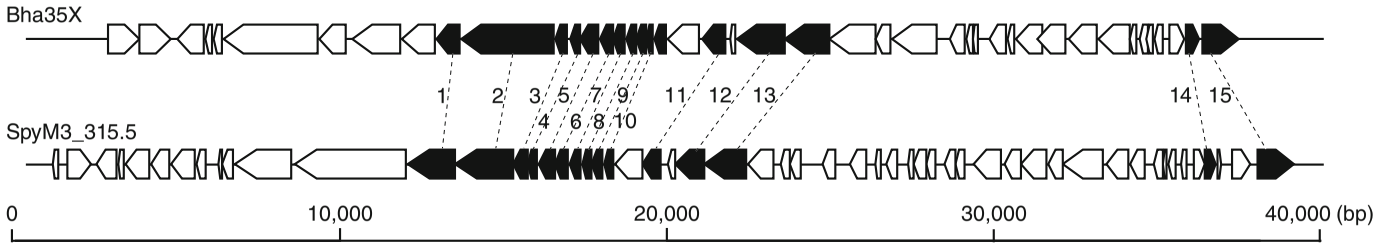
The IS element, designated as IS*663*, with IRs of 14 bp in length is present in the *B. halodurans* C-125 genome. An intact element (1,980 bp) shows 42.2% identity to the nucleotide sequence of IS*660* (1,963 bp) with IRs of 16 bp in length. The putative Tpase of IS*663* shows 45.5% similarity to that of IS*660*, suggesting that these two IS elements are related to each other. IS*660* and IS*663* show 52.7% and 59.5% identity, respectively, with the nucleotide sequence of an unclassified IS element, IS*1272*, from *Staphylococcus haemolyticus* (Archer et al. 1996). In addition, these two ISs (IS*660* and IS*663*) show 42% and 49% identity, respectively, with an unclassified IS element, IS*1182* from *Staphylococcus aureus*. These results suggest that IS*660* and IS*663*, as well as IS*1272* and IS*1182*, can be grouped into a new IS family (designated IS*1272* family; Table 2.9.2). It is notable that many copies of IS*660*, including various truncated forms, are widely distributed throughout the *B. halodurans* genome, suggesting that IS*660* may be the oldest IS element present in the genome and that its wide distribution may have occurred through complicated internal rearrangements of the genome.

Group II Introns

Group II introns are catalytic RNAs that function as mobile genetic elements by inserting themselves directly into target sites in double-stranded DNA (Abarca and Toro 2000; Bao et al. 2002; Michel and Ferat 1995). The IS element designated Bh.Int lacks both IRs and TSD. This element (1,883 bp) shows 47.6% identity to the nucleotide sequence of the group II intron of *Clostridium difficile* (Mullant et al. 1996). The CDS of Bh.Int is similar to the putative reverse transcriptase-maturase-transposase of group II introns of *C. difficile*, showing 47.5% similarity. The CDS of Bh.Int also showed significant similarity to group II introns from *Sphingomonas aromaticivorans* (38.4%) (Romine et al. 1999) and *Pseudomonas putida* (25.7%). Among these putative reverse transcriptase-maturase-transposases (RT), the amino acid sequence GTPQGG is well-conserved as a consensus sequence. Thus, IS*653* should be categorized as a new member of the group II introns. The *B. halodurans* C-125 genome contains four other copies of the element (Bh.Int-02~05) and two truncated copies of Bh.Int (Bh.Int-06~07) (Table 2.9.2).

Bacteriophage

Sequence analysis of the *B. halodurans* genome revealed a complete prophage, showing the typical genome organization of the Sfi11-like siphovirus with sequence matches over the head and tail genes to *Streptococcus pyogenes* M3 prophage 315.5 (Fig. 2.9.3) (Canchaya et al.



Bha35X DR

3617756 -> 3617736 GTTCGACTCCCACCGTCTCCA

3652280 -> 3652260 GTTCGATTCCCACCGTCTCCA

SpyM3_315.5 (NC_004588)

Bha35X	Product	CDS No.	SpyM3_315.5	Product
BH3517	Hypothetical protein	1	SpyM3_1312	Putative minor structural protein
BH3518	Phage-related protein	2	SpyM3_1313	Putative platlet-binding protein, minor tail fiber protein
BH3519	Hypothetical protein	3	SpyM3_1314	Hypothetical protein
BH3520	Conserved hypothetical protein	4	SpyM3_1315	Hypothetical protein
BH3521	Conserved hypothetical protein	5	SpyM3_1316	Hypothetical protein
BH3522	Hypothetical protein	6	SpyM3_1317	Hypothetical protein
BH3523	Hypothetical protein	7	SpyM3_1318	Hypothetical protein
BH3524	Hypothetical protein	8	SpyM3_1319	Hypothetical protein
BH3525	Hypothetical protein	9	SpyM3_1320	Hypothetical protein
BH3526	Hypothetical protein	10	SpyM3_1321	Conserved hypothetical protein
BH3529	Hypothetical protein	11	SpyM3_1323	Hypothetical protein
BH3531	Conserved hypothetical protein	12	SpyM3_1325	Hypothetical protein
BH3532	Conserved hypothetical protein	13	SpyM3_1326	Conserved hypothetical protein
BH3550	Conserved hypothetical protein	14	SpyM3_1351	Putative cI-like repressor, metallo-prtoeinase motif
BH3551	Phage integrase	15	SpyM3_1354	Putative phage integrase

Fig. 2.9.3

Comparison of bacteriophage region identified in the alkaliphilic *B. halodurans* genomes with that of *Streptococcus pyogenes* M3

2003a, b). As in a number of other prophages, an isolated adenine methyltransferase gene was detected between the DNA replication module and the DNA-packaging module. More interestingly, however, the presence of a type II restriction endonuclease and associated cytosine-specific methyltransferase located between the phage lysinand the *attR* site was confirmed. Possession of the prophage thus confers a potentially new restriction modification system on the lysogenic cell.

On the other hand, the genomes of *B. halodurnas* do not contain intact prophages, such as SPβ, PBSX, and skin, as have been found in the *B. subtilis* genome (Kunst et al. 1997). It was confirmed that *B. halodurans* contain the gene for sigma K in a complete form, which in *B. subtilis* is divided into two parts, *spoIVCB* (N-terminal) and *SpoIIIC* (C-terminal) by the prophage (skin element).

O. iheyensis

Extremely halotolerant and alkaliphilic *O. iheyensis* strain HTE831 (JCM 11309, DSM 14371) was isolated from deep-sea sediment collected at a depth of 1,050 m on the Iheya Ridge (Takami et al. 1999a; Takami 1999) and characterized as Gram positive, strictly aerobic, rod shaped, motile by peritrichous flagella, and spore forming. Strain HTE831 grows at salinities of 0–21% (3.6 M) NaCl at pH 7.5 and 0–18% (3.1 M) at pH 9.5 (Lu et al. 2001). The optimum concentration of NaCl for growth is 3% at both pH 7.5 and 9.5. Based on phylogenetic analysis using 16S rDNA sequencing, chemotaxonomy, and physiological characters of strain HTE831, this organism was validated in 2002 as being a member of a new species in a new genus, for which the name *O. iheyensis* was proposed (Lu et al. 2001; Validation List 2002).

General Features

The genome of *O. iheyensis* is single circular chromosome consisting of 3,630,528 bp with an average G+C content of 35.7% (coding region, 36.1%; noncoding region, 31.8%) (*Fig. 2.9.2* and *Table 2.9.1*). Based on skew analysis in the ratio of G−C to G+C (G−C/G+C), we estimated that the site of termination of replication (*terC*) is located nearly 1.77–1.78 Mb (176°) from the replication origin. We identified 3,496 CDSs, with an average size of 883 bp (Takami et al. 2002). Coding sequences covered 85% of the chromosome, and we found that 78% of the genes in *O. iheyensis* started with ATG, 8% with GTG, and 12% with TTG, which are quite similar to values for *B. subtilis* and *B. halodurans*, whose whole genomic sequences have been completely elucidated (*Table 2.9.1*). Proteins of *O. iheyensis* predicted from coding sequences were estimated to range in size from 2,714–268,876 Da for a total of 32,804 Da. Following comparison with sequences in a nonredundant protein database, 1,972 (56.4%) had an assigned biological role, 1,069 (30.6%) were identified as conserved proteins of unknown function, and 456 (13%) had no database match. The 69 tRNA species organized into 10 clusters involving 63 genes plus 6 single genes (*Table 2.9.1*), of which 5 clusters were associated with rRNA operons. Seven rRNA operons are present in the *O. iheyensis* HTE831 genome and they are organized identically to those of *B. subtilis* and *B. halodurans* (16S-23S-5S, 16S-23S-5S-tRAN, and tRNA-16S-23S-5S). On the other hand, no active prophages identified

in the *B. subtilis* genome were found in the *O. iheyensis* genome, although the genome also contains at least 27 putative phage-associated genes.

IS Elements and Transposon

Six kinds of new ISs, IS*667* to IS*672*, a group II intron (Oi.Int), and an incomplete transposon (Tn*8521oi*) were identified in the 3,630,528-bp genome of the extremely halotolerant and alkaliphilic *O. iheyensis* HTE831 (Takaki et al. 2004). Of 19 ISs identified in the *O. iheyensis* HTE831 genome, seven were truncated, indicating the internal rearrangement of the genome. All ISs, except IS*669*, generated a 4- to 8-bp duplication of the target site sequence, and these ISs carried 23- to 28-bp IRs. Sequence analysis newly placed four ISs (IS*669*, IS*670*, IS*671*, and

Table 2.9.3

IS family identified in the genome of *Bacillus*-related species

IS family	No. of members identified in genomes of:		
	O.iheyensis	Other *Bacillus*-related strains	
IS*3*	1(IS*672*)	*B. thuringiensis* sub. *aizawai*	4
		B. thuringiensis ,*B. halodurans*	
IS*4*	0	*B. thuringiensis* sub. *thuringiensis*,	15
		B. halodurans, *B. subtilis natto*,	
		G. stearothermophilus[a]	
IS*5*	1(IS*671*)		0
IS*6*	0	*B. thuringiensis* sub. *israelensis*	4
		B. thuringiensis sub. *fukuokaensis*	
		B. cereus	
IS*21*	0	*B. thuringiensis* sub. *thuringiensis*	5
		B. halodurans, *G. stearothermophilus*[a]	
IS*30*	1(IS*670*)	*B. halodurans*	1
IS*110*	0	*B. halodurans*	2
IS*200*/IS*605*	1(IS*669*)	*B. halodurans*	1
IS*256*	0	*B. halodurans*	1
IS*481*	0	*G. stearothermophilus*[a]	1
IS*630*	0	*B. halodurans*, *G. stearothermophilus*[a]	2
IS*650*/IS*653*	0	*B. halodurans*	1
IS*656*/IS*662*	0	*B. halodurans*	1
IS*660*/IS*1272*	0	*B. halodurans*	1
IS*982*	0	*B. thuringiensis*, *B. stearothermophilus*	2
ISL*3*	2 (IS*667*) (IS*668*)	*B. halodurans*	2

[a]*Geobacillus stearothermophilus*

IS*672*) in separate IS families (IS*200*/IS*605*, IS*30*, IS*5*, andIS*3*, respectively). IS*667* and IS*668* were also characterized as new members of the IS*L3* family. Most ISs found in the *O. iheyensis* HTE831 genome belonged to known IS families reported in other *Bacillus* strains, but aside from the IS5 family, IS*671* was the first to be identified in a bacillus genome (Table 2.9.3). On the other hand, IS belonging to the IS*4* family, the most common type of ISs found in bacilli, were not found in the *O. iheyensis* HTE831 genome. IS elements belonging to the IS*L3* family have been reported only in *B. halodurans* C-125 to date, but two other new members (IS*667* and IS*668*) showing significant similarities to those identified in strain *B. halodurans* C-125 were found in the *O. iheyensis* HTE831 genome in this study.

A trace of the 8,521-bp transposon unit, designated Tn*8521oi*, is present in the *O. iheyensis* HTE831 genome. A *ger* gene cluster comprising three CDSs (OB481, OB482, and OB483) was found in the Tn*8521oi* element (Fig. 2.9.4a). The *O. iheyensis* HTE831 genome has two other paralogous gene clusters located at 73° and 114° (*oriC* region at 12 o'clock assigned to 0°), respectively, on the circular chromosome. The genome of *B. subtilis* 168 has five gene clusters (*gerA*, *gerB*, *gerK*, *yndDEF*, and *yfkQRT*) orthologous to the *ger* gene cluster introduced into the *O. iheyensis* HTE831 genome by Tn*8521oi*, although the *gerBC* gene product did not show significant homology to the amino acid sequence of the putative corresponding protein (OB481) in the fifth gene cluster located at 315° (Fig. 2.9.4b). The spores are thought to recognize germinants such as L-alanine, L-valine, L-asparagine, glucose, fructose, and KCl through receptor proteins encoded by the *gerA* family of operons, which includes *gerA*, *gerB*, and *gerK* (Moir and Smith 1990). The *O. iheyensis* HTE831 genome has only three paralogous *ger* gene clusters, even when one acquired by the insertion of Tn*8521oi* is included, in contrast to five in *B. subtilis* and four in *B. halodurans* (Fig. 2.9.4b). Although it is unclear what role these three *gerA*-like operons identified in the *O. iheyensis* HTE831 genome play in spore germination, and no experimental data for the frequency of germination from *O. iheyensis* spores has been obtained, these genomic characteristics of the *ger* gene cluster presumably imply a low frequency of spore germination in *O. iheyensis*. On the other hand, a low frequency of spore formation in generally rich media has been observed in strain HTE831 (Lu et al. 2001). Thus, *O. iheyensis*, a low-frequency spore producer, may have no serious problems in spore germination under enriched conditions, even if the number of paralogous *gerA*-like operons is smaller than that of high-frequency spore formers.

Group II Introns

Group II introns are catalytic RNAs that function as mobile genetic elements by inserting themselves directly into target sites in double-strand DNA. The element, designated Oi.Int, has no IRs or TSDs. This element (1,889 bp in length) is 66% identical to the nucleotide sequence of the group II intron of *B. halodurans* designated Bh.Int (Takami et al. 2001). The amino acid sequence deduced from the CDS of Oi.Int was 62% identical to the putative RT of the Bh.Int. It is known that all group II introns are classified into two major subgroups: IIA and IIB (Kuroda et al. 2001), and Oi.Int is placed in subgroup IIB. Domain I is the largest among all intron domains, if the optional ORF looping out in domain IV is excluded from consideration. Domain I is involved in the alignment of the 5′ splice site (GUGUG) and the recognition of intron-specific target DNA, EBS1 and EBS3. Domain V is the most highly conserved primary sequence within the RNA core of the group II intron and plays a key role in splicing. Domain V binds domain I extensively to form a catalytic core with long-range tertiary interaction. In domain V of Oi.Int, potential tertiary parings ($\zeta-\zeta'$, $\lambda-\lambda'$, and $\kappa-\kappa'$) were identified in a consensus model (Lehman and Schmidt 2003).

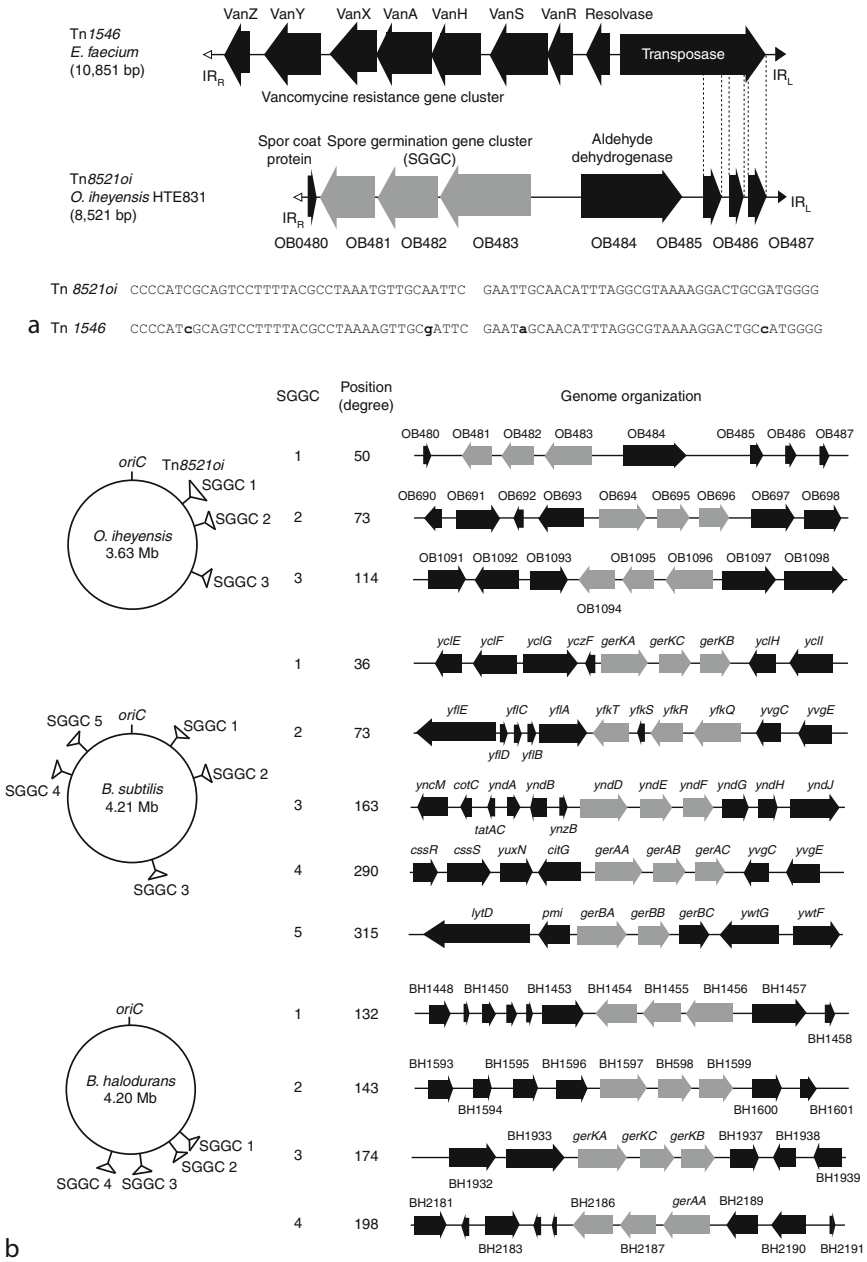


Fig. 2.9.4

Structure of the transposon-like element containing the spore germination gene cluster (SGGC), and the distribution of SGGCs in bacilli genomes. (a) Structure of the transposon-like element identified in the HTE831 genome and comparison with that of Tn*1546*. *Gray arrows* show spore germination (*ger*)-related genes. (b) Distribution of SGGCs in the HTE831 genome and comparison with those of *B. subtilis* 168 and *B. halodurans* C-125. The 12 o'clock position of the *oriC* region is assigned 0°. *Gray arrows* indicate the *ger*-related gene

Bacilli-Wide Distribution of IS Elements and Group II Introns Derived from *B. halodurans* C-125

Thirteen kinds of IS elements and Bh.Int were amplified by PCR from the genome of *B. alkalophilus* with the same primer set as for *B. halodurans* C-125, although two other ISs, IS*643* and IS*658*, were not detected, even by Southern blot analysis. IS*651*, which had the greatest number of copies (Germond et al. 1995) distributed throughout the *B. halodurans* C-125 genome, was only detected in the *B. alkalophilus* genome. In addition, two ISs (IS*656* and *IS662*) belonging to the IS*656*/IS*662* family, which was proposed as a unique new IS family in the previous study, were detected from *B. halodurans* C-125 (Takami et al. 2001) by PCR only in *B. alcalophilus*, whereas IS*650* and IS*653* belonging to another new IS family (IS*650*/IS*653*) were also detected by Southern blot analysis in other bacilli, such as *B. clausii*, *B. vedderi*, *B. firmus*, and *B. thermoalkalophilus* (Takami et al. 2004a). Thus, these findings suggest that *B. alkalophilus* is very closely related to *B. halodurans*, based on the distribution of ISs and group II introns, although the phylogenetic relationship between the two species based on 16S rDNA sequence analysis is not very close (*Fig. 2.9.5*). A total of 19 *Bacillus*-related species, excluding *B. alkalophilus*, were found to lack the five IS elements IS*642*, IS*651*, IS*654*, IS*656*, and IS*662*. No ISs and Bh.Int identified in the *B. halodurans* genome were not detected in five other *Bacillus*-related species: alkaliphilic *Bacillus gibsonii*, thermophilic *G. stearothermophilus*, neutrophilic *Paeniobacillus illinoisensis*, *Paeniobacillus polymyxa*, and *Paeniobacillus lautus* (*Fig. 2.9.5*).

On the other hand, three ISs (IS*641*, IS*643*, and IS*653*) and Bh.Int were amplified by PCR with the same size as those from *B. halodurans* C-125 from the genome of neutrophilic *B. firmus*, which is phylogenetically distant from *B. halodurans* (*Fig. 2.9.5*). Although IS*652* was not amplified from the genomes of 19 *Bacillus*-related species, excluding *B. alkalophilus*, by PCR it was detected by Southern blot analysis in eight species: *B. clausii*, *B. clarkii*, *B. halmapalus*, *B. horikoshii*, *Bacillus chonii*, *B. thermoalkalophilus*, *Bacillus horti*, and *O. iheyensis* (Takami et al. 2004a). Bh.Int with the exactly same size as that from *B. halodurans* C-125 was amplified by PCR in 11 out of 20 *Bacillus*-related species, in contrast to the IS elements, of which most were not detected by PCR (*Fig. 2.9.5*). From these findings, it is clear that five kinds of IS elements, IS*650* (IS*650*/IS*653* family, Takami et al. 2001), IS*652* (IS*L3* family, Germond et al. 1995), IS*653* (IS*650*/IS*653* family), IS*657* (IS*200*/IS*605* family, Beuzon and Casadesus 1997; Censini et al. 1996), and IS*660* (IS*1272*/IS*660* family, Archer et al. 1996; Takami et al. 2001), and Bh.Int are widely disseminated among at least four *Bacillus*-related species, without correlation to the phylogenetic relationships based on 16S rDNA sequences or to their growth environments.

Orthologous Analysis of the Genome *Bacillus*-Related Species

The genomes of extremophilic *Bacillus*-related species represent a unique opportunity to investigate the genes that underlie the capabilities to adapt to extreme environments. The first issue is addressed by comparing the orthologous relationships among the proteins deduced from all CDSs identified in the genomes of six bacilli and two other Gram-positive bacteria, *S. aureus* (Kuroda et al. 2001) and *Clostridium acetobutylicum* (Nölling et al. 2001). Orthologous groups in the Gram-positive bacterial species were established based on the all-against-all similarity results of applying the clustering program on the

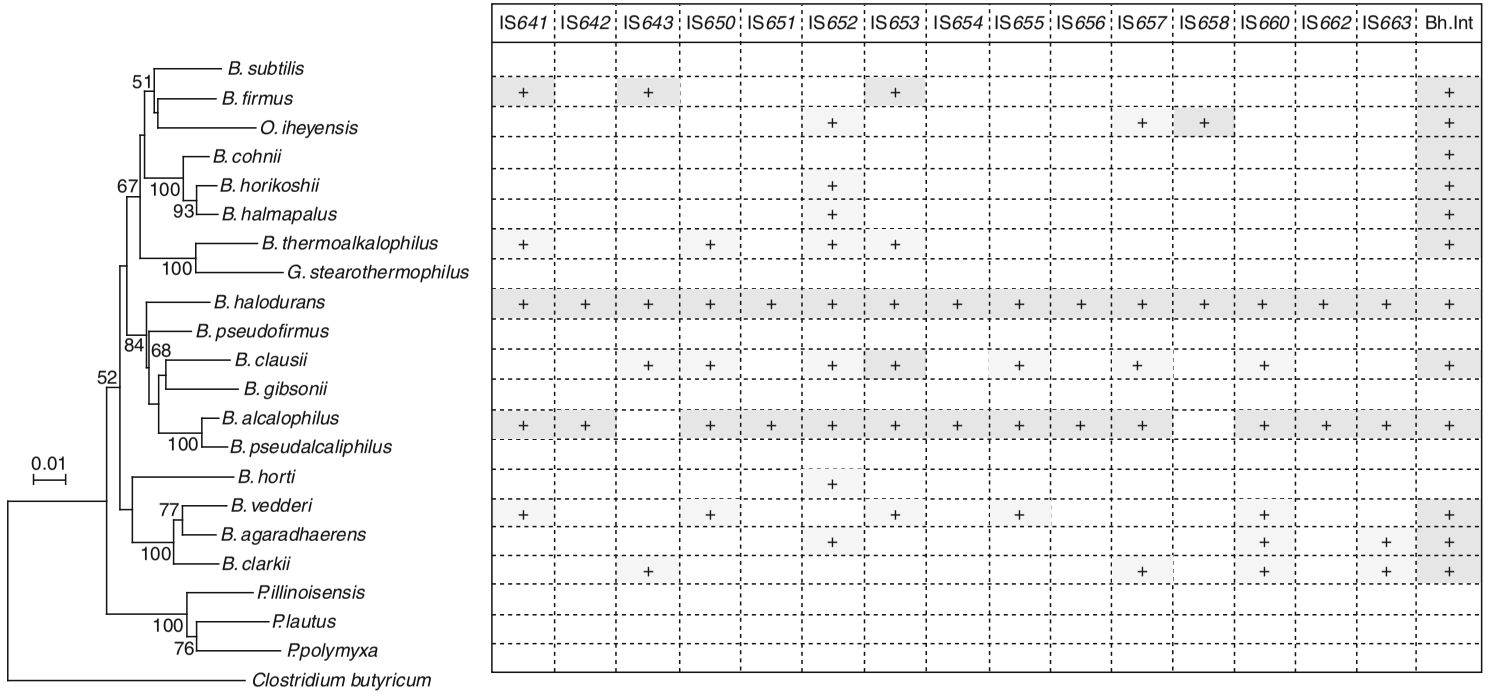


Fig. 2.9.5

Phylogenetic relationship based on 16S rDNA sequences of *Bacillus*-related species possessing IS elements and a group II intron identified in *B. halodurans* C-125. The detected ISs and a group II intron are indicated by plus. Each element confirmed by PCR and Southern blot analysis is marked with light and dark gray, respectively. Bootstrap probability values of less than 50% were omitted. *Clostridium butyricum* MW8 served as an outgroup. Bar = 0.02 Knuc unit

microbial genome database (MBGD) (Uchiyama 2003). The common orthologs conserved in all *Bacillus*-related genomes were then identified for use in the construction of multiple alignments. Since these orthologs generally display complicated mutual relationships, such as many-to-many correspondences and fusion or fission of domains, we selected only those orthologs with one-to-one correspondences without domain splitting, in order to simplify the analysis.

Out of 3,496 proteins identified in the *O. iheyensis* genome, 838 putative proteins (24.0%) had no orthologous relationship to proteins encoded in the four other genomes, 793 proteins (22.7%) were orthologous to five Gram-positive bacterial species, 354 (10.1%) were identified as common proteins only among *Bacillus*-related species, and 243 putative proteins (7.0%) were common only to the two alkaliphiles, *O. iheyensis* and *B. halodurans*. As shown in *Fig. 2.9.6*, the trend of orthologous relationships was almost the same in the case of analyses based on each *Bacillus*-related species, although the genome size of *O. iheyensis* is 600 kb smaller than that of the other two *Bacillus* genomes. In addition, orthologous relationships emerged in comparisons with all combinations among the five genomes used in this study. The putative proteins characterized on the basis of orthologous relationships were assigned to the functional categories used for *B. subtilis*.

Out of 838 putative proteins lacking orthologous relationship to the four other Gram-positive species, 390 were orphans showing no significant similarity of amino acid sequence to any other protein and 448 were conserved in other organisms, of which 174 had no known function. A total of 60 proteins were classified as transport/binding proteins and lipoproteins (category 1.2), the most abundant category. Nearly half of these proteins are ABC transporter-related proteins (*Fig. 2.9.6*). Many of the orthologs, which were shared between two to four species in the comparison were also grouped into this category, indicating that some of these transport-related proteins contribute to the distinguishing characteristics of subsets of Gram-positive bacteria. Further, 1,803 putative proteins identified as orthologs by comparative analyses with the three genomes of *Bacillus*-related species represent about 44.0–51.2% of each genome, and of these, about 980 orthologs are located at similar positions in each genome. Recently, it was shown that 271 genes are indispensable for the growth of *B. subtilis* in nutritious conditions (Kobayashi et al. 2003). Out of the 1,308 common genes, 233 show correspondences to the *B. subtilis* essential genes. Through a series of orthologous analyses of the six bacilli used in this study, four essential genes, *ymaA*, *ydiO*, *tagB*, and *tagF*, associated with purine/pyrimidine biosynthesis, DNA methylation, and teichoic acid biosynthesis, were found to be unique to the *B. subtilis* genome. Teichoic acids are composed of cell-wall teichoic acid and lipoteichoic acid (Neuhaus and Baddiley 2003). Six genes (*tagA*, tag*B*, *tagD, tagE*, *tagF*, and *tagO*) are known to be associated with teichoic acid biosynthesis in *B. subtilis,* with all genes except t*agE* being essential for growth.

Candidate Genes Involved in Alkaliphily

Generally, alkaliphilic *Bacillus* strains cannot grow or grow poorly under conditions of neutral pH, but grow well at pH above 9.5 (Horikoshi 1999a). *O. iheyensis* grows in pH up to 10 with a broad optimum pH range for growth from 7.0 to 9.5 (Lu et al. 2001). Over the past 3 decades, alkaliphilic microorganisms have been studied to elucidate their adaptation mechanisms to alkaline environments. As shown in *Fig. 2.9.6c*, 243 orthologs were identified between the only two alkaliphiles (*O. iheyensis* and *B. halodurans*) and of these, 76 genes were grouped into

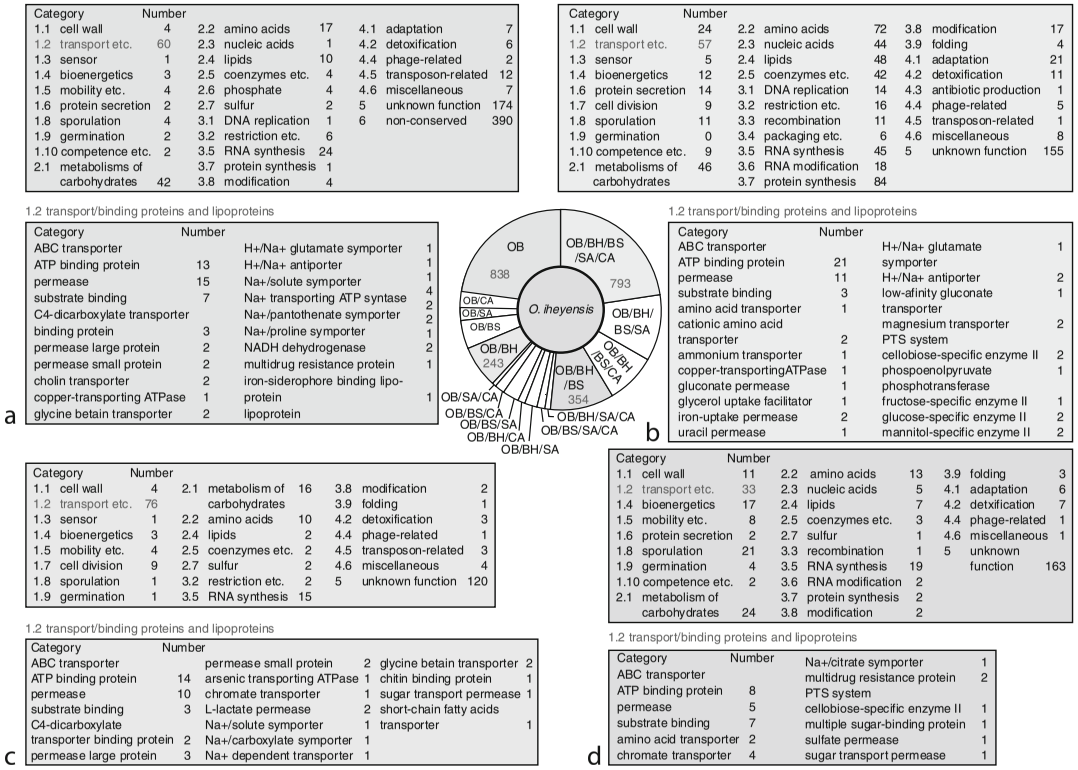


Fig. 2.9.6

Functional assignment of protein coding sequences (CDSs) based on the orthologous relationships. (a) Orphans, which have no orthologous relationship to four other Gram-positive species. (b) Orthologs identified among the genomes of five Gram-positive bacterial species. (c) Orthologs identified between *O. iheyensis* and *B. halodurans*, both alkaliphiles. (d) Orthologs identified among the genomes of three *Bacillus*-related species. The functional categories used at the *B. subtilis* genome database SubtiList: http://www.pasteur.fr/Bio/SubtiList.html) were used in this study

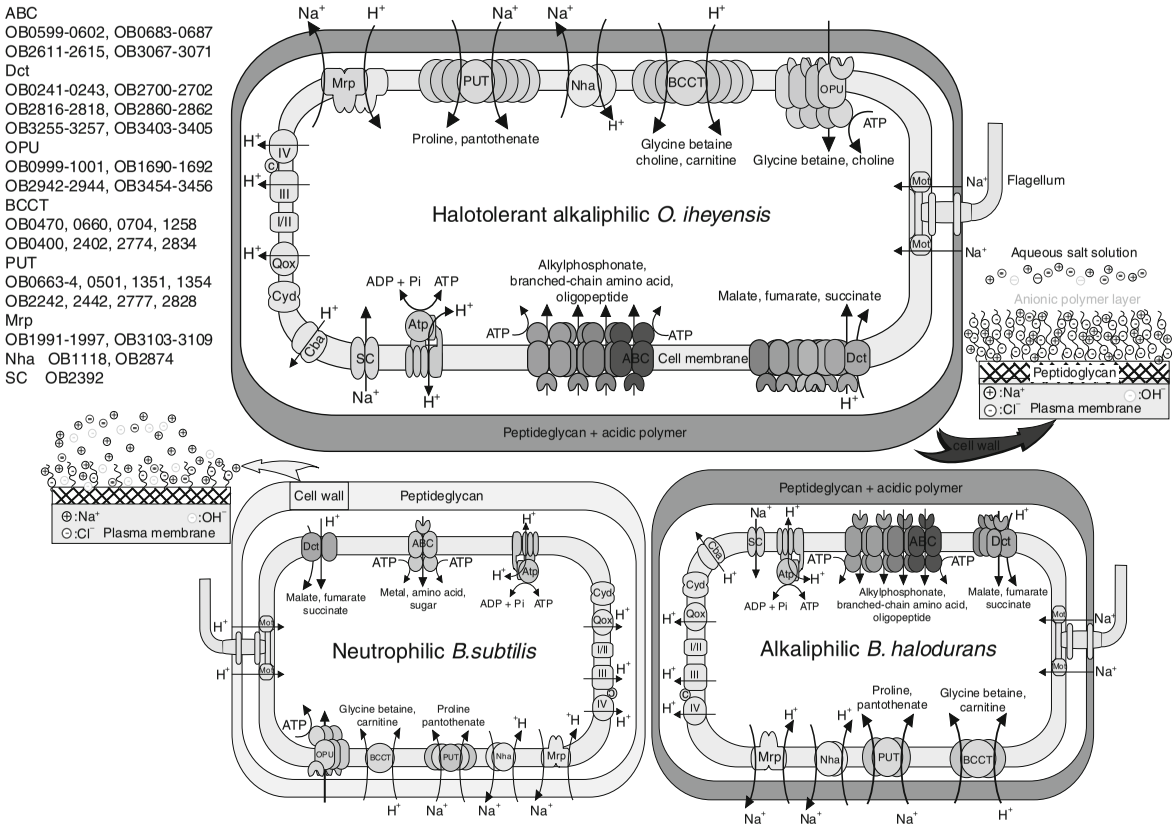


Fig. 2.9.7 (Continued)

category 1.2 (Takami et al. 2002). These included various ABC transporters, and transporters associated with C_4-dicarboxylate, organic osmotic solute transport, and Na^+ uptake (Fig. 2.9.7). The ABC transporters could be categorized into three groups based on substrate specificity. All five genes encoding ABC transporters for branched-chain amino acids identified in the *O. iheyensis* genome showed orthologuous relationships to only *B. halodurans* among the five Gram-positive species compared. These particular transporters could be important for alkaliphily. Branched-chain amino acids such as leucine, isoleucine, and valine are believed to be converted to L-glutamate in the presence of 2-oxoglutarate and pyridoxal 5-phosphate by a branched-chain amino acid aminotransferase (Kagamiyama and Hayashi 2000; Schadewaldt et al. 1995). This protein is widely distributed in various organisms including *O. iheyensis*. Since L-glutamic acid should be negatively charged at pH higher than its pKa (3.9 or 4.3), the converted L-glutamic acid and its accompanying proton could contribute to the acidification of the alkaliphilic *Bacillus* cytoplasm, the pH of which is maintained at around 8–8.5, even though pH outside of the cell is around 10.5 (Horikoshi 199a; Krulwich et al. 2001). Twenty-one genes encoding oligopeptide ABC transporters were identified in the *O. iheyensis* genome compared to 28 for *B. halodurans*, 29 for *C. acetobutylicum*, and in contrast to only 11 genes in *B. subtilis* (Nölling et al. 2001). However, only six of the genes identified in *O. iheyensis* showed orthologous relationships between only the two alkaliphiles. Similar to the case of ABC transporters for branched-chain amino acids, these transport systems are presumably active in the uptake of oligopeptides during growth under alkaline pH conditions. Thus, they can contribute to the acidification of the cytoplasm if acidic amino acids, such as glutamic acid and aspartic acid, are released by digestion of the incorporated oligopeptide by a peptidase, or if they are converted to free amino acids by digestion by aminotransferase. These acidic amino acids are an important resource of protons and for acidic polymers in the cell wall.

The cell wall is crucial in alkaliphilic *Bacillus* species because their protoplasts lose stability in alkaline environments (Horikoshi 1999a). Alkaliphilic *Bacillus* species contain certain acidic polymers containing galacturonic acid, gluconic acid, glutamic acid, asparatic acid, and phosphoric acid. The amount of acidic polymers is known to increase during growth under alkaline pH conditions. The Donnan equilibrium theory of electrolytes between an anionic polymer layer and the bulk aqueous phase was applied to cell wall systems, with a reduction in pH at the cell wall-membrane boundary (Tsujii 2002). According to this calculation, the pH values estimated inside the polymer layer (cell wall) are more acidic than those of the surrounding environment by 1–1.5 units. Teichuronopeptide (TUP) is a copolymer of polyglutamic acid, which is one of important components in the cell wall of the alkaliphilic

Fig. 2.9.7

Overview of putative major transport systems that govern alkaliphily and extreme halotolerance in *O. iheyensis*. ABC transport systems are shown as composite figures of *oval*, *circular*, and *sickle* shape. The gene number of *O. iheyensis* corresponding to each component of the transporter is described below or above each figure. Abbreviations: Atp, F_1F_0 ATP synthase; Abc, ABC transporter; Bcct, glycine betaine, carnitine/H^+ symporter; Cba, cytochrome *c* oxidase *bo3*-type; Cyd, cytochrome *bd* oxidase; I, NADH dehydrogenase, II, succinate dehydrogenase; III, menaquinol cytochrome *c* oxidoreductase; IV, cytochrome *caa3* oxidase; Dct, C_4-dicarboxylate transport system; Mrp, Na^+/H^+ antiporter; Mot, channel for energization of motility; Nha, Na^+/H^+ antiporter; Opu, glycine betaine ABC transporter; Pan, Pantothenate/Na^+ symporter; Put, proline/Na^+ symporter; Qox, cytochrome *aa3* quinol oxidase; Sc, voltage-gated sodium channel

B. halodurans and actually contributes to the regulation of pH homeostasis in the cytoplasm. The putative protein (OB2920) showing significant similarity to *tupA* gene product involved in TUP biosynthesis of *B. halodurans* (Takami et al. 2000) is shared only between the two alkaliphiles (Fig. 2.9.7). To date, no homolog of *tupA* has been identified in any other organism, except for two alkaliphiles.

Likewise, alkylphosphonate ABC transporters common to only the two alkaliphiles are not found in the three other Gram-positive species. In all, four genes encode alkylphosphonate ABC transporters (two permeases and one ATP-binding and one phosphonate-binding protein), and these are shared between *O. iheyensis* and *B. halodurans*. *E. coli* has been known for some time to cleave carbon-phosphorus (C-P) bonds in unactivated alkylphosphonates (Chen et al. 1990). When an alkylphosphonate, such as methylphosphonate, is degraded by bacterial C-P lyase ($CH_3PO_3^{2-} \rightarrow CH_4 + HPO_4^{2-}$), the resultant monohydrogen phosphate ion is utilized as a source of phosphorus for growth (Murata et al. 1988). On the other hand, in the case of mitochondria, monohydrogen phosphate ion is an important substrate and exchanges itself for dicarboxylates, such as malate and succinate, by means of the dicarboxylate carrier (Krämer and Palmieri 1992). It is unclear whether these alkylphosphonate ABC transporters contribute to the regulation of pH homeostasis of the bacterial cytoplasm because the C-P lyase gene has not been identified in the genomes of either alkaliphile.

O. iheyensis possesses a total of 18 genes encoding six sets of C_4-dicarboxylate carriers, which are all grouped into the DctA family (Janausch et al. 2002). Of these, seven genes encoding two sets of C_4-dicarboxylate carriers and one permease large protein are shared only between *O. iheyensis* and *B. halodurans* (Fig. 2.9.7). The other 11 genes encoding three C_4-dicarboxylate binding proteins, two permease large proteins and two permease small proteins are unique among the five Gram-positive species. Although the seven shared proteins showed highest similarity among the alkaliphiles, the other 11 unique proteins showed significant similarity to those of Gram-negative strains such as *Sinorizobium meiloti*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. In aerobic bacteria, dicarboxylate transport carriers catalyze uptake of C_4-dicarboxylates in a H^+- or Na^+ symport (Janausch et al. 2002). This transport system may play a role in the regulation of pH homeostasis and the sodium cycle of the alkaliphilic bacterial cytoplasm.

The Na^+ cycle plays an important role in the remarkable capacity of aerobic alkaliphilic *Bacillus* species for pH homeostasis. The capacity for pH homeostasis directly reflects the upper pH limit of growth. The first major player in the alkaliphile Na^+ cycle is the Na^+/H^+ antiporter, which achieves net H^+ accumulation coupled to Na^+ efflux. Na^+/H^+ antiport activity also represents an important mechanism for maintaining low intracellular sodium concentrations in halophilic or halotolerant aerobic bacteria (Ventosa et al. 1998). The major antiporter for pH homeostasis is thought to be the Mrp-encoded antiporter, first identified in *B. halodurans*. Mrp could function as part of a complex with six other gene products. The *O. iheyensis* genome possesses 14 genes encoding two sets of Mrp-related proteins and all 14 genes showed various orthologous relationships among 4/5 Gram-positive species but not *C. acetobutylicum*. The genome contains two genes for additional antiporters including NhaC, a property, which is shared among all five Gram-positive species. These antiporters presumably play fundamental roles in the pH homeostasis of the bacterial cell.

The second major player of the Na^+ cycle is Na^+ reentry via the Na^+/solute symporter and presumably the ion channel associated with the Na^+-dependent flagella motor (Krulwich et al. 2001). Two genes encoding a Na^+/solute symporter and a Na^+-dependent transporter are shared only between *O. iheyensis* and *B. halodurans*. These alkaliphile-specific transporters

could also be important for Na^+ reentry, although there is no experimental evidence for such a function under alkaline pH conditions. One of the new findings of the genome sequencing project of *B. halodurans* was the discovery of the first prokaryotic voltage-gated sodium channel in the alkaliphilic *B. halodurans* initially based on the sequences of transmembrane domains (Ren et al. 2001) and later confirmed experimentally to be activated by voltage and selective for sodium, although it is inhibited by calcium channel blockers. A putative protein (OB2392) identified in *O. iheyensis* shows significant similarity to this sodium channel. Because this sequence is hardly detected in other prokaryotic genomes except for the two alkaliphiles, voltage-gated channels could be a common characteristic of alkaliphilic *Bacillus*-related species. This channel likely provides a transient supply of Na^+ for the sodium cycle under both alkaline and neutral pH conditions (Chahine et al. 2004). Recently, it has been confirmed that the voltage-gated sodium channel deficient mutant lost alkaliphily in another alkaliphilic *Bacillus* species, *B. pseudofirmus* (Ito et al. 2004).

Totally, 32 putative proteins associated with flagella were identified in the *O. iheyensis* genome and all of these proteins, except for one, were well-conserved among the three *Bacillus*-related species. Alkaliphile motility is Na^+-coupled and is observed only in cells growing at highly alkaline pH ranges. In fact, *O. iheyensis* is extremely motile under alkaline pH conditions. Therefore, these flagella-associated proteins identified in the *O. iheyensis* genome are likely to work as Na^+-channels for Na^+ entry at high pH to ensure an adequate supply of Na^+ for the increased antiport coupled to H^+ accumulation (*Fig. 2.9.7*). This is in contrast to H^+-driven flagella motors present in neutrophilic *B. subtilis* (Shioi et al. 1980). On the other hand, there are no major differences in the proteins associated with the terminal oxidase of respiratory chain between neutrophilic *B. subtilis* and the two other alkaliphilic bacilli, although *B. subtilis* is missing cytochrome *bd* oxidase while this enzyme is conserved in both alkaliphiles as well as the thermophile *G. stearothermophilus* (http://www.genome.ou.edu/qbstearo.html).

We highlighted the genes involved in alkaliphilic phenotype based on comparative analysis with three *Bacillus* species and two other Gram-positive species through this chapter. However, out of 243 genes shared only between the two alkaliphiles (OB/BH), the functions of 120 genes still remain unknown, as shown in *Fig. 2.9.6c*. Therefore, it will be necessary to analyze gene expression patterns under alkaline and neutral pH conditions to clarify the genes responsible for alkaliphilic phenotype.

Conclusion

Since phylogenetically related *Bacillus*-related species share many genes despite each species having a different capability to adapt to not only alkaline but also other extreme environments, it is expected that comparative analysis with *Bacillus*-related species is useful for identifying candidate genes associated with the mechanisms of adaptation to these environments. Thus, we attempted to identify the genes involved in alkaliphily based on comparative analysis with the genomes of six closely related *Bacillus*-related species and two other Gram-positive bacteria. Consequently, we were able to identify strong candidate genes responsible for alkaliphily that were unique or common genes among the *Bacillus*-related species. However, nearly half of the genes in each of the sets are of unknown function, and other candidate genes for alkaliphily may yet be discovered among these genes. Further comparative analyses using other *Bacillus*-related species with various phenotypic properties are required as a second step in uncovering hidden capacity for alkaliphily.

Through a series of bacillar genome analyses, the presence of transposable elements such as ISs, group II introns, and transposons in the genomes of extremophilic *Bacillus*-related species contrasted to their absence in the *B. subtilis* 168 genome. Some of those elements were common to all bacilli, without correlation to phenotypic properties or phylogenetic placement using 16S rDNA sequences. Thus, it is thought that the transposable elements are certainly one of the important factors responsible for genomic diversity in *Bacillus*, as well as in other bacterial genera.

Cross-References

- 2.1 Introduction and History of Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 2.7 Bioenergetics: Cell Motility and Chemotaxis of Extreme Alkaliphiles

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2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic *Bacillus* for the Production of Beta-Cyclodextrin

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Isolation of Alkaliphilic Bacteria Producing β-CGTases

Cyclomaltodextrin glucanotransferase (CGTase) [systematic name:1,4-α- D- glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing)], which is also called as cyclodextrin glycosyltransferase or cyclomaltodextrin glycosyltransferase, is a member of the α-amylase superfamily and belongs to the transferase group of enzymes (EC.2.4.1.19). This enzyme catalyzes the intramolecular transglycosylation to form cyclodextrins (CDs), which are nonreducing and cyclic maltooligosaccharides composed of 6–12 glucose molecules linked by the α-1,4-glucopyranosidic linkage, from starch and related carbohydrates (French 1957, 1962; Thoma and Stewwart 1965; Pulley and French 1961). Major products obtained from starch by the enzyme action are cyclomalto-hexaose (α-CD), -heptaose (β-CD), and -octaose (γ-CD), and larger CDs (δ-, ε-, ζ-, η-CD, and so on) are also formed during the reaction as minor products (French et al. 1965). Small amounts of maltooligosyl-CDs branched by the α-1,6-glucopyranosidic linkage were also found in the reaction product from starch (French et al. 1965; Kobayashi et al. 1977, 1984). Recently, cyclic α-1,4-glucans with sizes less than 60 glucose units were found in the product from amylose in the early stage of reaction by this enzyme (Terada et al. 1997, 2001). These larger cyclic α-glucans will be subsequently converted to smaller CDs due to their susceptibilities to the transferring action of CGTase during longer reaction times. The enzyme also catalyzes the disproportionating reaction to form a series of linear maltooligosaccharides with a variety of carbohydrates as substrates (French 1957). The minimum size of substrate for this reaction, which is maltotriose or maltotetraose probably, is ambiguous because of the difficulty to obtain each of them as an ultimate pure form. Many saccharides, for example, D-xylose, D-glucose, 6-deoxy-D-glucose, L-sorbose, maltose, a series of maltooligosaccharides, trehalose, and sucrose, and a number of glycosides bearing the same configuration structure of OH groups at C-2, C-3, and C-4 in the pyranose ring of their molecules as that of glucose are known as good acceptors for the coupling reaction (French et al. 1954; Kitahata et al. 1992; Kitahata 1995; Kobayashi 1996). The enzyme can also use smaller branched maltooligosaccharides as good acceptors and does not remain as the limit dextrin after the prolonged reaction on starch. These observations show that branched structures in starch and maltooligosaccharides do not disturb the above mentioned three reactions.

In the late nineteenth century, Villiers (1891) found small amounts of two nonreducing and acid-stable crystalline saccharides, which he named as cellulosin(s), in the culture broth of *B. amylobacter* containing probably heat-resistant spores of *B. macerans*. This was the first discovery of CDs. Twelve years later, Schardinger (1903) also found two cyclic maltooligosaccharides showing almost same properties as those of cellulosin(s), which were α- and β-CD, in the fermented product from starch with *B. macerans*. Since then, these saccharides were called as Schardinger dextrins. On the basis of many works reported previously, Tilden and Hudson (1939, 1942) investigated on the enzymatic conversion of starch to CDs with CGTase from the culture broth of *B. macerans* obtained by an anaerobical cultivation at 37–40°C with a liquid medium containing sliced potato and oatmeal. These were the pioneering reports probably describing about the enzyme productivity of this bacterium and the enzyme dosage to produce CDs from starch. Although many works relating to methods for the production, procedures for the purification, and enzymatic characteristics of CGTase in the culture broth of *B. macerans* were done in detail (Schwimmer and Garibaldi 1952; Depinto and Campbell 1968; Lane and Pirt 1971), no one tried to find other CGTases until some Japanese scientists discovered novel enzymes.

In the beginning of 1970s, some strains of alkaliphilic microorganisms producing amylolytic enzymes extracellularly were isolated from soils by the method to select a bacterium with a clear halo zone around the colony on Horikoshi-II medium (pH 10.3) composed of 1.0% (w/v) potato starch, 0.5% Polypepton (Nihon Seiyaku Co., Japan), 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% agar, and 1.0% Na_2CO_3. These microorganisms were aerobic, spore-forming, motile, and rod-shaped. All strains belong to the genus *Bacillus*. Further, these enzymes were classified into four types by their different pH profiles for the action on starch determined as the dextrinogenic activity (Horikoshi 1971; Yamamoto et al. 1972). This was thought as the first finding of alkali-amylases. Of them, the enzyme of type 1 from the isolated bacterium (*B. pseudofirmus* A-40-2, ATCC 21592) was a typical α-amylase with an optimum for the action at around pH 10.5, and others were identified with CGTases by the thin layer chromatograpy of their reaction products from starch. It was also confirmed by the formation of water-insoluble inclusion complexes with a few specific organic precipitants for β-CD such as toluene and trichloroethylene from the same reaction product. This was also the first discovery of novel β-CGTases capable of producing β-CD predominantly from starch. Among them, the strain No. 38-2 (alkaliphilic *Bacillus* sp., ATCC 21783) was selected as the enzyme producer to produce β-CD on an industrial scale by following useful characteristics of the crude enzyme, that is, a high ability to act in the broad pH range from acidic to alkaline sides (*Fig. 2.10.1*), a high temperature optimum for the action to prevent the microbial infection during the reaction (*Fig. 2.10.2*), a relatively low thermal stability to inactivate easily by heating as compared with those of bacterial liquefying-type α-amylases being used in industries, and a higher productivity of β-CD from starch as compared with that by CGTase of *B. macerans*, which was the only enzyme known in the literature until the discovery of β-CD forming-type CGTases of alkaliphilic microorganisms. To produce β-CGTase of the strain No. 38-2, the best medium composition was examined by Nakamura and Horikoshi (1976a). As a result, the maximum enzyme yield was achieved by the aerobical cultivation with an alkaline medium containing starch, corn steep liquor, and small amounts of salts at around 37°C.

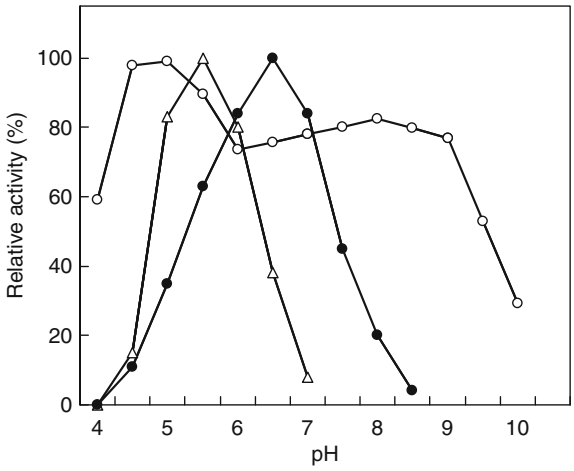

Fig. 2.10.1

Effects of pH on activities of crude CGTases from a species of alkaliphilic *Bacillus, B. macerans* and *B. coagulans*. Symbols show crude CGTases of alkaliphilic *Bacillus* sp. No. 38-2 (○), *B. macerans* (Δ) and *B. coagulans* (●)

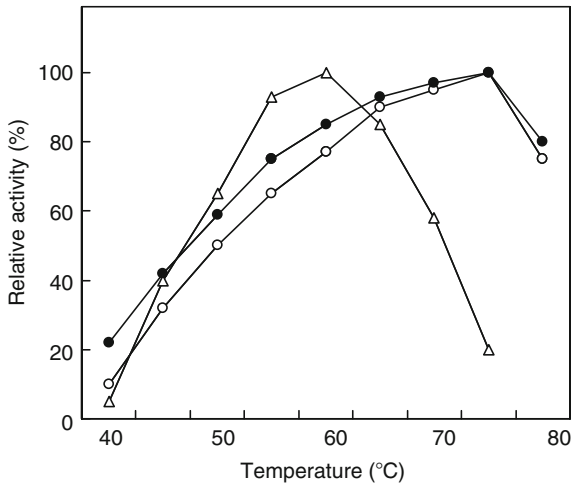

Fig. 2.10.2

Temperature optima for the action of crude CGTases from a species of alkaliphilic *Bacillus*, *B. macerans* and *B. coagulans*. Symbols show crude CGTases of alkaliphilic *Bacillus* sp. No. 38-2 (○), *B. macerans* (Δ) and *B. coagulans* (●)

Nowadays, the crude β-CGTase, which is a concentrated culture filtrate of a hyper-productive mutant from the isolated bacterium with an ultrafilter up to 1/5–1/10 of the original volume for the convenience of preservation before using, is utilized to produce β-CD industrially.

At almost the same time as the first discovery of extracellular β-CGTases of alkaliphilic bacteria, a few Japanese scientists also found novel β-CGTases in culture supernatants of *B. circulans* and *B. megaterium* and an α-/β-CGTase in that of *B. stearothermophilus* (Kitahata et al. 1974; Kitahata and Okada 1974, 1982). Of them, the latter enzyme is utilized practically for the glycosylation of some natural glycosides and others by its higher transferring ability as those shown below.

For references, pH and temperature optima for actions of crude CGTases from culture filtrates of *B. macerans* and *B. coagulans*, of which the former enzyme was classified later into one of α-CGTases producing α-CD predominantly from starch and the latter moderate thermophile was one of α-/β-CGTase producers isolated from soils in the mid-1980s by us, were also shown in *Figs. 2.10.1* and *2.10.2*. The former α-CGTase was most active at around pH 5.5 and 60°C, and the latter enzyme was at around pH 6.5 and 75°C, respectively. These enzymes were stabilized strikingly with Ca^{2+} (data not shown). On the other hand, the crude β-CGTase of the strain No. 38-2 was active in the broad pH range from acidic to alkaline sides with two apparent optima at around pH 4.8 and 8.5 when its dextrinogenic activity was determined at 40°C, and was most active at 75°C when the activity was determined at pH 7 in the presence of 5 mM Ca^{2+}. These observations suggested strongly a higher usefulness of this crude enzyme to produce CDs and others on an industrial scale. Characteristics of this crude enzyme, especially in its unique pH-activity profile causing probably by the combined action of three CGTases (acid-, neutral-, and alkali-CGTases) in the culture filtrate (Nakamura and Horikoshi 1976a, b, c, d) as shown in *Fig. 2.10.3*, were investigated further by Kaneko et al. (1988, 1989) with various chimeric β-CGTases prepared with enzyme genes from two strains (No. 38-2 and 17-1) of alkaliphilic *Bacillus* species. It was shown an obvious fact that the

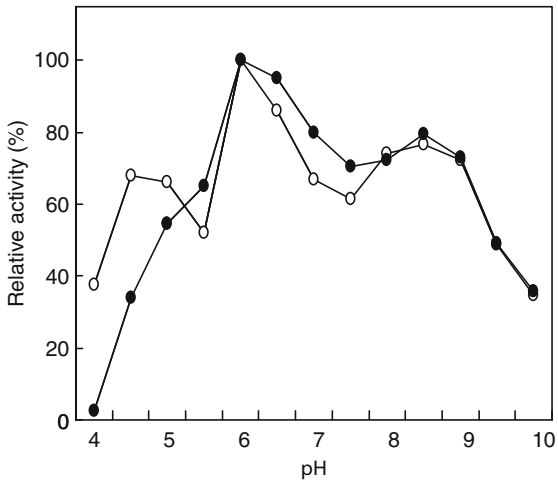

Fig. 2.10.3

Effect of pH on the CD-forming activity of the crude CGTase from a species of alkaliphilic *Bacillus*. The CD-forming activity was determined at 60 (●) and 40 (○)°C in the absence of Ca^{2+}

pH-activity profile for the action was affected by the C-terminal region of the enzyme molecule, though a question about the reason why these three enzymes show different pH optima for their actions is yet to be answered.

To confirm the practical usefulness of the crude β-CGTase, a high concentration of potato starch suspension was liquefied with the concentrated culture filtrate at a high temperature. As shown in *Fig. 2.10.4*, a remarkable reduction of the viscosity of potato starch solution as same as those observed by actions of bacterial liquefying-type α-amylases and a little decrease of the color intensity of the starch/iodine complex were observed in the final stage of reaction, though any significant amounts of reducing sugars were not formed. These inferences showed that this crude enzyme was stable enough even if the reaction was performed at relatively high temperatures to prevent the microbial infection and the presence of other amylolytic enzymes, which were unnecessary for the efficient production of CDs from starch, in the culture filtrate was negligible. To evaluate the crude enzyme further, the formation of CDs was examined under practical conditions with a high concentration of liquefied potato starch as a starting raw material. As it was of common knowledge in the industry producing sweeteners from starch that most of microbial infections during the enzymatic reaction were able to prevent by keeping at higher temperatures than 72°C, the reaction was performed at 73–74°C accordingly. As shown in *Fig. 2.10.5*, about 36% of the slightly hydrolyzed starch was converted to three CDs, of which the ratio of α:β:γ was 1:7:2 approximately, in the final stage of reaction at around pH 6.5. Almost same amounts of these CDs were also formed from the same liquefied starch by reacting at pH 5 and 8.5 under almost same conditions, respectively (data not shown). Small amounts of unknown larger saccharides were remained after the hydrolysis of the reaction product by the simultaneous action of bacterial liquefying-type α-amylase and fungus glucoamylase (data also not shown). As a result of these experiments, the crude β-CGTase of the strain No. 38-2 was thought as the best producer of β-CD from starch at that time.

For references, profiles of the formation of CDs from almost same liquefied potato starch by actions of other types of crude CGTases from *B. macerans* and *B. coagulans* were shown in

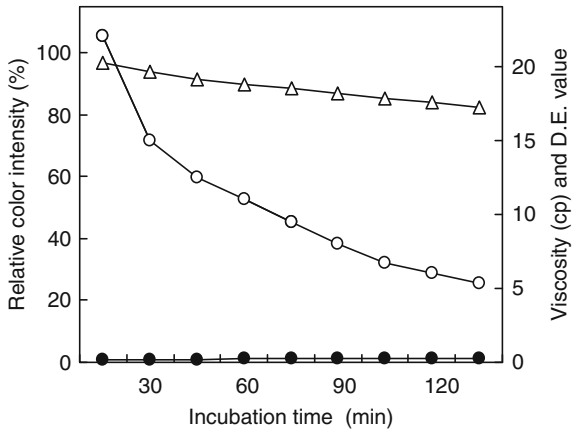

Fig. 2.10.4

Liquefaction of potato starch by the crude CGTase from alkaliphilic *Bacillus* No. 38-2

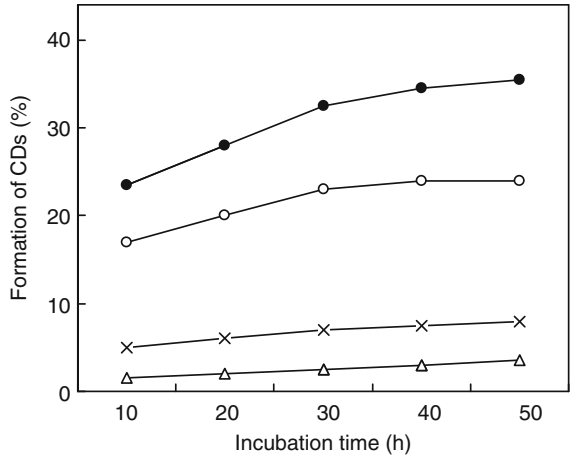

Fig. 2.10.5

Formation of CDs from starch by the crude CGTase from alkaliphilic *Bacillus* No. 38-2. Symbols show α-CD(Δ), β-CD(○), γ-CD(x) and the total of three CDs(●), respectively

Figs. 2.10.6 and *2.10.7*, respectively. The major product by the former α-CGTase from starch was α-CD, though a remarkable decrease of this CD and gradual increases of other CDs were observed by the prolonged reaction. The ratio of α:β:γ during the reaction at the maximum yield of α-CD was 65:25:10 approximately. On the other hand, that by the latter α-/β-CGTase was β-CD though the yield of this CD was relatively low as compared with that by β-CGTase of the strain No. 38-2. Maximum yields of the three CDs by actions of these three enzymes were almost same when the reaction was performed with the same liquefied starch as a starting raw material.

Since some species of alkaliphilic *Bacillus* producing extracellular β-CGTases were discovered, the isolation of various alkaliphilic bacteria producing CGTases and the characterization

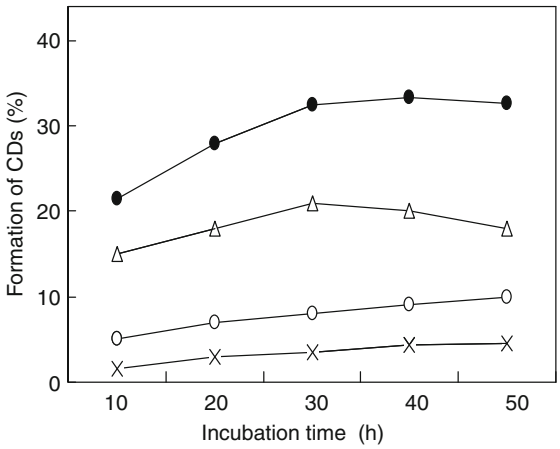

Fig. 2.10.6

Formation of CDs from starch by the crude CGTase from *B. macerans*. Symbols show α-CD(Δ), β-CD(○), γ-CD(x) and the total of three CDs(●), respectively

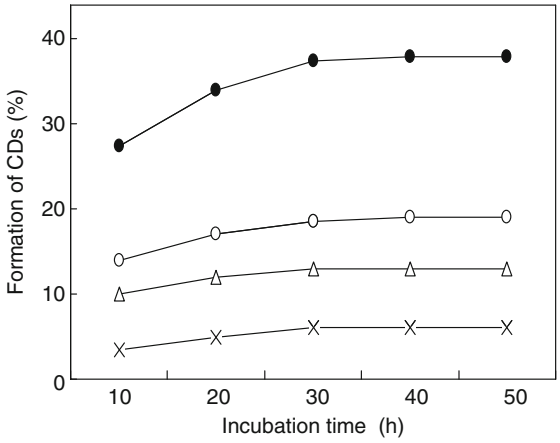

Fig. 2.10.7

Formation of CDs from starch by the crude CGTase from *B. coagulans*. Symbols show α-CD(Δ), β-CD(○), γ-CD(x) and the total of three CDs(●), respectively

of those enzymes were performed by some other scientists (Nomoto 1984, 1986; Yagi et al. 1986; Kimura et al. 1987; Georganta et al. 1993; Abelyan et al. 1994a, b; Martins et al. 2001; 2002; Mahat et al. 2004). These works were reviewed later by Horikoshi (2006). Some novel microbial γ-CGTases predominantly producing γ-CD from starch were also found earlier (Kato and Horikoshi 1986; Mori et al. 1994; Parsiegla et al. 1998; Yang et al. 2001; Takada et al. 2003; Wang et al. 2004, 2006; Nakagawa et al. 2006). Some of them are useful for the production of γ-CD though maximum yields of the total of CDs from starch by the reaction in the absence of precipitating agent(s) are relatively low as compared with those by other types of CGTases.

Industrial Production of β-CD with the Crude β-CGTase of a Species of Alkaliphilic *Bacillus*

In the beginning of 1950s, Cramer (1951) reported on the stabilization of some dyes by the complexation with CDs in aqueous solutions. Then, he demonstrated the protection of easily oxidizable substances against atmospheric oxidation, the enhancement of solubility in water of poorly soluble drugs, the reduction of the loss of highly volatile substances, etc., by the complexation with CDs. These works were reviewed later by Cramer and Hettler (1967). Since these advanced works have been done, chemical and pharmacological properties of many inclusion complexes of CDs with various organic compounds and the enzyme-like action of CDs and their derivatives during the complexation were investigated in detail by many scientists. These works were reviewed later by Bender and Komiyama (1978), Saenger (1980) and Szejtli (1988), respectively. Although some of those complexes and reactions were thought as useful materials and tools, they were not utilized actually in industries because of the difficulty to obtain sufficient amounts of CDs at reasonable prices for practical applications at that time. To produce large amounts of CDs at a cheaper rate, French (1957) investigated on the preparation of these saccharides from starches with the enzyme of *B. macerans* by the method using various specific organic precipitants such as toluene, trichloroethylene, tetrachloroethane, bromobenzene, and cyclohexane to separate CDs as water-insoluble inclusion complexes from reaction products (the organic solvent method). It was thought as a better method to produce each CD at a relatively high yield from starch by the following mechanism that the enzyme reaction was directed to the synthetic way by excluding CD(s) immediately out of the reaction system as a resistant form against the further degradation to other noncyclic maltooligosaccharides. French et al. (1963) also made a trial to produce CDs from starches without using any harmful complexing agents during the reaction on a larger-than-laboratory scale with this enzyme. However, the yield of each CD was too low to produce on an industrial scale. Their works including others performed with the enzyme of *B. macerans* were reviewed later by Rendleman (1999). Bender (1977, 1986) also reported on the method for producing α-CD with the enzyme of *K. pneumoniae* and n-decanol as a specific precipitant. On the basis of these works, a few companies in the USA and Japan produced independently small amounts of crystalline β-CD as samples for comprehensive surveys of the marketability of their products with the enzyme of *B. macerans* on a pilot plant scale by the organic solvent method using toluene or trichloroethylene in the first half years of 1970s. But any of them could not get a favorable result, because their products were too expensive for practical utilization in industries at that time. Many researchers in industries producing foods, beverages, feeds, and even those in the pharmaceutical field were very anxious about organic solvents remained probably in their products as harmful impurities, because serious social problems were caused in the past by the pollution of many fresh and processed foods such as fishes, shells, vegetables, rice, milk for babies, rice oil, and others with trace amounts of toxic chemicals in Japan.

In 1976, Nihon Shokuhin Kako Co. Ltd., one of Japanese manufacturers of corn starch and sweeteners, started the industrial production of crystalline β-CD and two by-products containing CDs with the crude β-CGTase of a species of alkaliphilic *Bacillus* (strain No. 38-2) without using any harmful organic complexing agents (the nonorganic solvent method) with the permission of utilizing these saccharides in food and beverage industries as food additives by Japanese government (Matsuzawa et al. 1975; Nakamura and Horikoshi 1977; Horikoshi 1979). Before anything, the best starting raw material was selected from some liquefied starches by several trials of the production of pure β-CD on a pilot plant scale, because the yield of CDs from

starch by the action of CGTase in the absence of complexing agent was higher when the reaction was performed with the lower concentration and the larger size of substrates in general (data not shown). As a result of these trials, about 10–15% (w/w) of starch from potato, tapioca, or sweet potato, which was hydrolyzed slightly up to 1–2 of the value of dextrose equivalent (D.E.) with a thermostable bacterial liquefying-type α-amylase before using, was thought as the best substrate to produce this saccharide without causing any technological troubles. In addition to these, starches from waxy corn, sago, wheat, rice, and glutinous rice were also better for the production. *Figure 2.10.8* shows the outline of production procedures developed at that time. Starch suspension, of which the pH value was adjusted to about 6.5 with $Ca(OH)_2$ before using, was liquefied slightly by mixing vigorously at about 105°C with an α-amylase of *B. licheniformis*, and the reaction was terminated by heating at 125–130°C for 15–30 min. After cooling immediately to 73–74°C, the liquefied starch solution was mixed with the crude β-CGTase of the strain No. 38-2, and the reaction was maintained for a certain period until the yield of β-CD was reached to the maximum. Normally, about 35–40% of starch (dry weight) was converted to CDs, of which about 70% was β-CD, in the final stage of reaction without causing the marked lowering of pH value during the reaction by the microbial infection. Depending on the size of reaction vessel and capacities of apparatuses, the concentration of starch, the reaction time and the enzyme dosage were altered flexibly. After the inactivation of CGTase by heating at 90–95°C for several hours, other saccharides without α-, β-, and γ-CD in the reaction product were hydrolyzed to smaller noncyclic ones at the same temperature with α-amylase of *B. amyloliquefaciens* or that of *B. licheniformis*. Any significant decrease of the yield of each CD was not observed during the reaction by these α-amylases. The reaction was terminated immediately by adjusting pH to about 4 with HCl solution when certain amounts of reducing sugars were formed. Water-insoluble, colored, smelled, and ionic materials including salts in the product were removed by passing successively through layers of diatomaceous

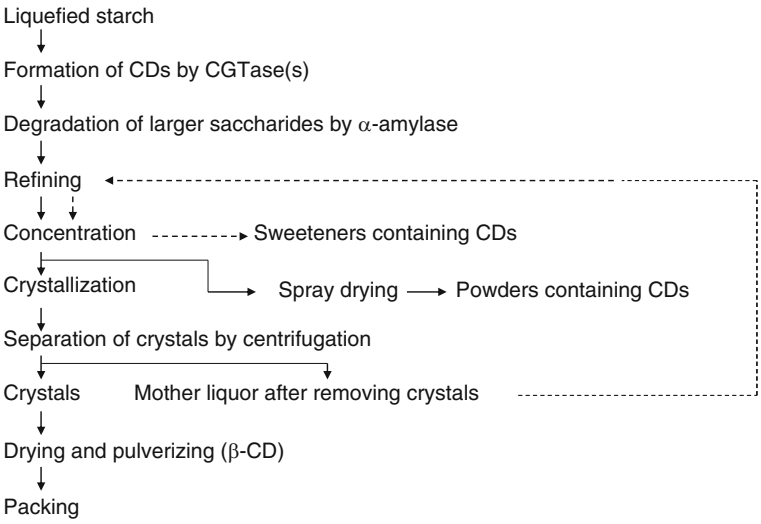

Fig. 2.10.8

Outline of the production procedure for crystalline β-CD and sweeteners containing CDs by the non-organic solvent method

earth clay and active charcoal and columns packed with ion exchange resins. The refined product thus obtained was passed again through the layer of active charcoal to refine up further. The refined product was concentrated up to 45–50% (w/w) under reduced pressure and then cooled gradually from 75–80°C to 20–25°C with stirring gently for 1–2 days to crystallize β-CD. About 80–90% of β-CD present in the mother liquor was crystallized by this procedure. After collecting crystals of β-CD by centrifugation with a basket type centrifuge, crystals were purified further up to 98.5% of the purity by washing with small amounts of pure water and followed by drying with hot air. On the other hand, the mother liquor after removing crystals of β-CD containing about 15–20% as dry basis of CDs, of which the D.E. value was around 20 or 30, was refined again and concentrated up to 70% or 75% (w/w) under the reduced pressure.

The process for producing crystalline β-CD and by-products described above was relatively simple as compared with those reported previously with α-CGTase from *B. macerans*. Operations and maintenances of facilities and apparatuses for this process were performed without causing any serious troubles, because those were almost same as those to produce familiar and conventional sweeteners such as crystalline glucose and corn syrups from starch by the enzymatic reaction. About 10 years later from the starting of an industrial production of crystalline β-CD by the method described above, a trial to produce this saccharide with the crude α-/β-CGTase of *B. coagulans* was made under almost same conditions. But, it was very hard to obtain pure crystals because of the technical difficulty to purify crude crystals, of which the purity after washing under the standard condition was about 93% by weight, up to 98.5% even if those were washed with the excess volume of additional water to remove other saccharides including α-CD as a major impurity.

Nowadays, α-CD, β-CD, and some syrups and powders containing CDs are produced regularly with three crude CGTases shown above by the modified nonorganic solvent method using the RO membrane in Japan. In addition to these products, branched CDs and a few starch hydrolysates containing them are produced with a concentrated mixture of CDs and maltose by the condensing action of bacterial pullulanase (Abudullah and French 1966; Abdullah and French 1970; Shiraishi et al. 1989) to utilize in various industrial fields at present. In other countries, especially in the USA and China, crystalline CDs are produced regularly at relatively high yields (40–60%) from starches by the organic solvent method with 1-decanol for α-CD, toluene (USA) or cyclohexane (China) for β-CD, and cyclohexadec-8-en-1-one for γ-CD. Mother liquors after removing each CD are utilized probably as raw materials to produce ethanol (USA) and a kind of fertilizer (China), respectively.

Applications of CDs and CGTases in Industries

Unmodified CDs including branched CDs are designated as food additives in many countries now. Recently, the application of them in foods was reviewed by Astray et al. (2009). Many unmodified and chemically modified CDs are also utilized in other fields now (Duchene 1987, 1991; Szejtli 1998, 2004; Qingsheng and Zimmerman 2005; Li et al. 2007; Loftsson and Duchen 2007; Brewster and Loftson 2007). Of them, the utilization of hydroxypropyl β-CD as a deodorant for clothes, furniture, window curtains, floor mats, and others is thought as one of best applications of chemically modified CDs. This material is also utilized to elongate the sweet-smelling time of fragrances on human skins in the cosmetic industry and as a stabilizer against the atmospheric oxidation and the degradation by UV-lights of the sun and illuminators or to increase the bioavailability of active components of drugs and cosmetics.

For references, some actual applications of unmodified CDs including branched CDs in Japanese food and beverage industries are described below. These CDs are utilized to stabilize volatile and labile substances such as docosahexanoic acid, eicosapentaenoic acid, loyal jelly, ethanol to preserve foods and flavors of confectioneries, black tea, juices (fruits and vegetables), dried tea extracts (green, black, and oolong) by spraying and dried cut vegetables by freezing, and to keep tastes and flavors of condiment pastes and their powders ("wasabi," garlic, mustard, and ginger) freshly, and to protect natural colors in colored foods and beverages against the atmospheric oxidation and the degradation by UV-lights of the sun and illuminators, and to keep original fresh colors of pickles (eggplant and radish), and to improve tastes and odors of soy milk, boiled rice and extracts (meats, vegetables, yeasts, ginseng, catechins, curcumin, Gymnema sylvestre, and Ganodema licidum), and to improve the solubility in water of poorly soluble materials such as naringin and hesperidins in canned citrus fruits, rutin, α-lipoic acid, soybean isoflavones, Co-Q10, boiled bamboo shoots, and tannins in bottled black tea, and to reduce the hygroscopicity of dried laver, hard candies, grated cheeses, and powders (granules) of nuts, soups, and seasonings, and as an emulsifier for oily seasonings. Cyclodextrins are also utilized to reduce a foul breath as deodorants in gargles, chewing gums, and edible tablets. The volume of egg white after the frothing increases markedly with small amounts of β-CD, though the mechanism is not made clear yet. The inclusion complex of CD (s) with allylisothiocyanate is utilized as an antiseptic agent to prevent microbial infection during preservation by mixing with foods and confectioneries or by coating onto wrapping films for them.

Coupling and disproportionating reactions of CGTases are utilized to produce various glycosylated compounds (Maitani et al. 2001). Actual applications of both reactions are described below. Since the industrial production of maltooligosyl-sucrose (Coupling Sugar), which was one of tooth-friendly sweeteners, using the mixture of liquefied starch and sucrose as a substrate was done with α-/β-CGTase of *B. stearothermophilus* by one of Japanese manufacturers of sweeteners (Kitahata 2000), some natural glycosides, for example, steviosides (Fukunaga et al. 1989; Jung et al. 2007), rutin (Suzuki and Suzuki 1991), glycyrrhizin (Maitani et al. 2001) and isoflavones (Kim et al. 2001) were glycosylated with CGTases to improve their tastes, solubilities in water, and physiological properties. Hesperidin, neohesperidin, and naringin were also modified efficiently with starch or β-CD as a donor by the action of CGTase from an alkaliphilic *Bacillus* species under alkaline conditions (Kometani et al. 1994, 1996). All these glycosylated compounds are utilized in food and beverage industries as food additives in Japan. p-Nitrophenyl-α-D-maltoheptaoside, which was one of useful substrates to determine the activity of human α-amylases for the diagnosis, was prepared by the coupling reaction of α-CGTase from *B. macerans* with α-CD as a donor and p-nitrophenyl-α-D-glucoside as an acceptor (Wallenfels et al. 1978; Pereira et al. 1985). Recently, a remarkable increase of the yield of trehalose from starch by the dual enzyme system with maltooligosyltrehalose trehalohydrolase and maltooligosyltrehalose synthase was observed when the reaction was performed in the simultaneous presence of isoamylase of *Pseu. amyloderamosa* and α-/β-CGTase of *B. stearothermophilus* (Kubota et al. 2004). This was also one of excellent industrial applications of CGTase.

In 1970s, the first discovery of β-CGTases in culture supernatants of some species of alkaliphilic *Bacillus* and an industrial production of β-CD from starch by the nonorganic solvent method using the crude enzyme of an isolated bacterium (strain No. 38-2) were done in Japan. Nowadays, the large amount of CDs is produced regularly with various CGTases including genetically modified enzymes by nonorganic and organic solvent methods in

several countries to utilize actually in various industries in the world. Furthermore, the industrial production of other useful substances with these enzymes is increasing gradually at present.

Cross-References

- 2.1 Introduction and History of Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.8 Enzymes Isolated from Alkaliphiles
- 2.9 Genomics and Evolution of Alkaliphilic *Bacillus* Species

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2.11 Alkaline Enzymes in Current Detergency

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_2.11,

Various enzymes are widely used in industrial fields such as detergent, food, and feed production; leather and textile processing; pharmaceutical production; diagnostics; and waste management. The largest world market for industrial enzymes is the detergent industry. Detergent enzymes account for approximately 30–40% of the total worldwide enzyme production except for diagnostic and therapeutic enzymes. Alkaline enzymes, such as protease, α-amylase, cellulase (endo-1,4-β-glucanase), mannanase and lipase, are incorporated into heavy-duty laundry and dishwashing detergents (Ito et al. 1998; Horikoshi 1999). Most of the alkaline enzymes for detergents were first found by Horikoshi between the 1960s and 1980s. Owing to his discovery of the world of alkaliphiles, detergents containing such alkaline enzymes have been expanded worldwide and established their importance and necessity in the detergent industry.

In 1959, the first detergent that contained a bacterial protease appeared on the market. The enzyme used was subtilisin Carlsberg from a neutrophilic strain of *Bacillus licheniformis* (Guntelberg and Ottesen 1954). After the switch from tripolyphosphate to zeolites in detergents in the early 1980s, manufacturers and consumers were not satisfied with the cleaning performance of detergents. Further, especially in Europe and the USA, consumers were using low washing temperatures to save energy, and highly alkaline detergent enzymes were extensively incorporated into detergents to compensate for low energy. In Asian countries, including Japan, the wash temperature has long been low at lower than 30°C. At present, some alkaline enzymes are recognized as the main biobuilders in detergents.

Alkaline Serine Proteases (Subtilisins)

Alkaline Protease Producers and Types of Their Enzymes

Alkaline proteases can facilitate the release of proteinaceous stains such as those due to grime, blood, and milk. To withstand the alkaline conditions in detergents, many alkaline proteases have been isolated from alkaliphilic *Bacillus* spp. Nielsen et al. (1995) proposed nine new alkaliphilic *Bacillus* spp.: *B. agaradhaerens, B. clausii, B. clarkii, B. gibsonii, B. halmapalus, B. halodurans, B. horikoshii, B. pseudoalkaliphilus*, and *B. pseudofirmus*. These bacilli exoproduce subtilisins. Proteases are classified into serine, aspartate, cysteine, and metal proteases, based on their catalytic residues and cofactors. Serine proteases are usually named subtilisins and characterized by three conserved catalytic residues Ser, Asp, and His (Markland and Smith 1971). Suntilisin family A is one of the six families (A–F) of subtilisin-like serine proteases, the subtilases (Siezen and Leunissen 1997). Subtilisin in the family A is further classified into three subfamilies: true subtilisin, high-alkaline protease, and intracellular protease. Classical subtilisins BPN' from *B. amyloliquefaciens* and Carlsberg from *B. licheniformis* are included in the member of true subtilisins. Generally, powder detergents are different from liquid detergents in washing pH; they are usually formulated to adjust pH to 9–11 in wash water. Under the extreme conditions, high-alkaline proteases offer superior wash performance to true subtilisins.

High-Alkaline Protease Producers

A high-alkaline protease, H-221, from *Bacillus* sp. no. 221 (*B. clausii* ATCC 21522) was the first enzyme form alkaliphilic *Bacillus* to be identified (Horikoshi 1971b). The optimal pH of H-221

was around 12.3, and 75% of the activity was maintained at pH 13 or more, with casein as a natural substrate. Since then, variants of high-alkaline protease, such as Savinase™ (Betzel et al. 1992), Maxacal™ (PB92; van der Laan et al. 1992) and M-protease (KAP™; Hakamada et al. 1994), were screened for use in detergents from alkaliphilic *Bacillus* spp., all of which now belong to *B. clausii* (Nielsen et al. 1995; Kageyama et al. 2007). Recently, we have completed the whole genome sequence of *B. clausii* KSM-K16 (M-protease producer; AP006627).

Nowadays, Protein-engineered variants of the family A subtilisins, especially high-alkaline proteases, from alkaliphilic *Bacillus* spp. have been hyperproduced on an industrial scale and incorporated into current detergents as the major biobuilder (Estell et al. 1985; Wells et al. 1987; van Ee 1991; Egmond 1997; Saeki et al. 2002).

Oxidatively Stable Alkaline Protease Producers

To survive the extremes of high alkalinity and high chelator concentration in detergents, subtilisins have been improved with respect to thermal stability, resistance to chelating reagents (chelators), and alteration of pH- and temperature-activity profiles of the enzymes (Bryan 2000). Subtilisins, including high-alkaline proteases, are inactivated seriously by chemical oxidants (Stauffer and Etson 1969, Bott et al. 1988) because all the enzymes contain a conserved Met residue in the vicinity of their catalytic Ser (Siezen and Leunissen 1997). In some cases, granulation devices can partly protect from the oxidative inactivation of enzyme. To improve the Achilles' heel of the enzymes, the Met residue was replaced with nonoxidizable amino acids such as Thr and Leu by site-directed mutagenesis to acquire resistance to oxidants and atmospheric air. However, the mutation at this position reduces catalytic efficiency significantly (Estell et al. 1985; Bott et al. 1988).

To meet with bleach-based detergent formulations, highly alkaline, oxidatively stable serine protease (OSP) has long been the target for enzymologists of the detergent industry. Fortunately, Horikoshi and Yoshida reported in a Japanese patent (JP 740710 from 1974) that a subtilisin-like serine protease E-1 from alkaliphilic *Bacillus* sp. D-6 was resistant to sodium perborate. Encouraged by this patent, we screened for OSPs very extensively; consequently several OSPs could be isolated from strains of alkaliphilic *Bacillus* isolated from soil samples (Saeki et al. 2000, 2002). Among them, KP-43 (FERM BP-6532) was selected for use as a detergent additive, and presently it is incorporated into compact powder and liquid detergents.

Since the patent of Horikoshi and Yoshida, a number of OSP-like enzyme-producing alkaliphilic *Bacillus* spp. were reported in patents, which include FERM P1592 (strain D-6), FERM BP-6534 (strain KSM-KP9860; PCT JP9804528), FERM BP-1029 (strain Y; EP 204342 A2), NCIB 12289 (WO 8801293 A1), and FERM P-11162 (strain SD521; JP 91191781 A2). Saeki et al. (2000) purified the five *Bacillus* proteases, designated E-1 (from strain D-6), KP-9860 (from strain KSM-KP9860), NP-1 (from strain NCBI 12289), LP-Ya (from strain Y), and SD-521 (from strain SD521) to homogeneity on SDS-PAGE, together with KP-43 (Saeki et al. 2002). All of the purified enzymes were found to resist oxidative inactivation by H_2O_2. These results were consistent with the oxidation stable E-1 from *Bacillus* sp. D-6. The comparison of the 16S rRNA sequences placed strains D-6, Y, and SD521 in *B. cohnii* and NCIB 12289 in *B. halmapalus* (Saeki et al. 2000). Strain KSM-KP43 was closely related to *B. halmapalus* (98.8% identity). However, the G + C content of the *B. halmapalus*

genomic DNA was 36.8 mol%, and that of KSM-KP43 was 41.6%. It was found that the DNA-DNA hybridization of KSM-KP43 with *B. halmapalus* revealed less than 20% association, indicating that KSM-KP43 was a novel species of the genus *Bacillus* (Saeki et al. 2002).

Purification and Enzymatic Properties of High-Alkaline Proteases and OSPs

Purification Procedures

When usual purification procedures were used, three bands of protease in a culture of *B. clausii* KSM-K16 frequently moved on SDS-PAGE (Kobayashi et al. 1996). Such multiple electrophoretic forms of alkaline proteases were also observed for other alkaline proteases such as H-221 and commercially available Savinase, Esperase, and Maxatase. Then, the major component, M-protease, was purified to homogeneity as follows (Kobayashi et al. 1995). A 2-day-old culture was centrifuged, and the supernatant obtained was dialyzed against a large volume of 5 mM Tris/HCl buffer (pH 8) plus 2 mM $CaCl_2$ at 4°C overnight. After the retentate was concentrated by ultrafiltration on a membrane, the concentrate was applied to a column of DEAE-Bio-Gel A equilibrated with 10 mM Tris/HCl buffer (pH 8) plus 5 mM $CaCl_2$. The column was washed with the equilibration buffer, and proteins were eluted with a linear gradient of KCl up to 0.1 M in the buffer. The active fractions eluted were combined and concentrated by ultrafiltration, and the concentrate was loaded on a CM-Bio-Gel A column equilibrated with 10 mM borate/NaOH buffer (pH 9.5) plus 2 mM $CaCl_2$. Proteins were eluted with a linear gradient of 0–0.1 M triethanolamine HCl in the same buffer, and the active fractions were concentrated by ultrafiltration. After the concentrate was dialyzed against 10 mM Tris/HCl buffer (pH 8) plus 2 mM $CaCl_2$, the retentate was used as the final preparation of purified enzyme.

The phylogenetic analysis showed that KP-43 and other OSPs were evolutionary close with each other. OSPs, including E-1 and KP-43, were purified individually to homogeneity by the same purification procedure as follows (Saeki et al. 2000, 2002). Each enzyme in a 2-day-old culture was precipitated by adding fine powders of ammonium sulfate (90% saturation), and the precipitates formed were dissolved in a small volume of 10 mM Tris/HCl buffer (pH 7.5) plus 2 mM $CaCl_2$ (buffer A). The retentate was loaded on a column of DEAE-Bio-Gel A equilibrated with buffer A, and the column was washed with buffer A. Non-adsorbed active fractions were pooled and concentrated by ultrafiltration. The concentrate was applied to a column of SP-Toyopearl 550W equilibrated with buffer A, and proteins were then eluted using a gradient of 0–50 mM NaCl in the buffer. The active fractions eluted were combined and concentrated by ultrafiltration, and the concentrate was used as the final preparation of purified enzyme.

Enzymatic Properties

The enzymatic properties of purified M-protease were characterized by Kobayashi et al. (1995). The molecular mass and p*I* of the enzyme was approximately 28 kDa by SDS-PAGE and around pH 10.6 by electrofocusing PAGE, respectively. The N-terminal amino acid

sequence was Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-Leu-Thr-Gly. M-protease showed the maximal caseinolytic activity at pH 12.3 (actual pH in the reaction mixture) in 50 mM phosphate/NaOH buffer; more than 50% of the maximal activity was detectable between pH 6 and pH 12.8. It was stable over a range between pH 6 and pH 12 when preincubated at 55°C for 10 min in buffers at various pH values. The optimal temperature for the reaction was around 55°C at pH 10 in 50 mM borate/NaOH buffer. The presence of 5 mM $CaCl_2$ shifted the optimal temperature to 70°C, and the activity was 1.4-fold greater than that at 55°C. The enzyme was stable up to 50°C after heating at various temperatures for 10 min in 20 mM borate/NaOH buffer (pH 9). These properties are essentially similar to those of H-221 and other high-alkaline proteases.

Casein was the most favorite substrate for M-protease among natural proteinaceous substrates. When the activity against casein was taken 100%, the rates of hydrolysis of hemoglobin, keratin, a-keratin, elastin, and egg yolk were 34%, 52%, 79%, 12%, and 2.1%, respectively. The scleroproteins, keratin and elastin, were hydrolyzed by the enzyme much more efficiently than BPN' and Carlsberg. M-protease efficiently hydrolyzed *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (-*p*NA) and *N*-succinyl-Ala-Ala-Pro-Met-*p*NA, while *N*-Cbz-Ala-Ala-Leu-*p*NA, N-Cbz-Gly-Gly-Leu-*p*NA, and *N*-Cbz-Gly-Pro-Cit-*p*NA (cit, citruline) were poor substrates, which are good substrates for subtilisins (Lyublinskaya et al. 1974). Phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphates (DPF) (1 mM each) and chymostatin (50 ppm) strongly inhibited the activity, being consistent with the classification of M-protease as a serine protease. The number of cleavage sites of the oxidized insulin B-chain was 21 with the preferentially initial cleavage site between Leu^{15} and Tyr^{16}.

Several OSPs were purified to homogeneity by SDS-PAGE (Saeki et al. 2000, 2002). The purified KP-43 had a molecular mass and p*I* were approximately 43 kDa by SDS-PAGE and around pH 8.9–9.1 by electrofocusing PAGE, respectively. The N-terminal amino acid sequence was Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala-Gln-Ser-Ser-Tyr-Gly-Leu-Tyr-Gly. In the absence of calcium ions, the caseinolytic activity at 35°C was observed at wide pH range from 4–13, with an optimum at around pH 11–12 in 20 mM glycine-NaOH buffer (actual pH in the reaction mixture). The enzyme was stable between pH 6–12 but unstable below pH 5 and above pH 13 after a 24-h incubation at 25°C. The optimal temperature for activity at pH 10 was observed at 60°C in the absence of and 70°C in the presence of 5 mM $CaCl_2$ and the activity was 1.3-fold greater than that at 60°C. The enzyme was stable up to 55°C in the absence and to 65°C in the presence of 5 mM $CaCl_2$ after a 30-min incubation at pH 10. PMSF and DPF (1 mM each) abolished the activity completely, indicating that KP-43 belongs to subtilisin (serine protease). The striking feature of KP-43 was very resistant to excess H_2O_2 (greater than 50 mM), EDTA and EGTA (5 mM each), and to various surfactants. Similar enzymatic properties were observed for other OSPs, E-1, KP-9860, LP-Ya, SD-521, and NP-1, which efficiently hydrolyzed *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and *N*-succinyl-Ala-Ala-Pro-Met-*p*NA (Saeki et al. 2000).

Amino Acid Sequences and Structures

Cloning and Sequencing

The gene for M-protease was cloned by the shotgun method (Hakamada et al. 1994). Genomic DNA of *B. clausii* KSM-K16 and plasmid pHY300PLK were both digested with *Hin*dIII.

After agarose gel electrophoresis, the digested DNAs (3.5–4.5 kb) were electroeluted from the gel, and the eluate was ligated with T4 ligase into the plasmid digested with *Hin*dIII and alkaline phosphatase. *B. subtilis* ISW 1214 cells were transformed with the ligation mixture and grown on DM3 agar plus 5 μg/ml tetracycline at 37°C for 2 days. The colonies formed were spread on nutrient agar containing 1% skim milk. After incubation at 37°C for 16 h, transformants that had formed clear zone around their margins were picked up from the agar. Plasmid DNA was isolated by the alkaline extraction procedure and sequenced directly by the dideoxy chain-termination method. The nucleotide sequence of the M-protease gene and its flanking regions (1,441 bp) was determined (Q99405). An internal amino acid sequence (Ala1 to Gly20) deduced from the open reading frame (ORF) was identical to the N-terminal amino acid sequence of the native M-protease. Thus, the proprotease consisted of 380 amino acid residues, while mature M-protease was 111 amino acids shorter, with a molecular mass of 26,723 Da, a value close to the native M-protease (28 kDa). The sequence of mature enzyme was preceded by a signal sequence of 27 amino acids adjacent to its N-terminus and a prosequence of 84 amino acids (pre-pro-sequence). Comparison of the amino acid sequence, active site, and molecular mass of M-protease with those of high-alkaline proteases, such as H-221 (P41362), Savinase (P29660) and PB92 (P27693), suggest the primary structures of these high-alkaline proteases are quite similar, but their amino acid sequences showed low similarity to Carlsberg (P00780) (∼60% identity) and BPN' (Q44684) (∼59% identity). Nevertheless, the catalytic triad Asp^{32}, His^{64}, and Ser^{221} (in BPN' numbering) is conserved as Asp^{32}, His^{62}, and Ser^{215} in M-protease.

The genes for KP-43 (AB051423), E-1 (AB046402), KP-9860 (AB046403), LP-Ya (AB046404), SD-521 (AB046405), and NP-1 (AB040406) were cloned and sequenced using appropriate primers and the respective genomes as templates (Saeki et al. 2000, 2002). The deduced amino acid sequences of these enzyme exhibited very high similarities to that of KP-43 with more than 97% identity. The catalytic triads of the OSPs are integrally conserved as Asp^{30}, His^{68}, and Ser^{255} (in KP-43 numbering). In contrast, they exhibited very limited amino acid similarities to true subtilisins BPN' and Carlsberg with 25%, high-alkaline proteases H-221 and M-protease with 25%, and intracellular proteases with less than 23% identity. The low homology is due to many peptide fragment insertions between α-helices and β-sheets in the OSPs compared with true subtilisins and high-alkaline proteases (Saeki et al. 2000). The nucleotide sequence of the KP-43 gene and its flanking regions (1,441 bp) contained the ORF encoded 640 amino acids, and the calculated molecular mass of the mature enzyme (434 amino acids) was 45,301 Da, a value close to the native KP-43. The KP-precursor had a prepropeptides with 206 amino acids, and the mature enzyme contained the very unique C-terminal extension of ∼160-amino-acid residue (the possible role of this extension is described below). Unexpectedly, oxidant-sensitive Met residues, as in the cases of true subtilisins and high-alkaline proteases, occur at positions next to the catalytic Ser residues in all the OSPs identified.

Three-Dimensional Structures

Many crystal structures of subtilisins, including true subtilisins Carlsberg (PDB code 2SEC) and BPN' (PDB code 2ST1), have been reported to date. High-alkaline proteases have a molecular mass of ∼30 kDa and fold into a conserved structure, named the subtilisin fold

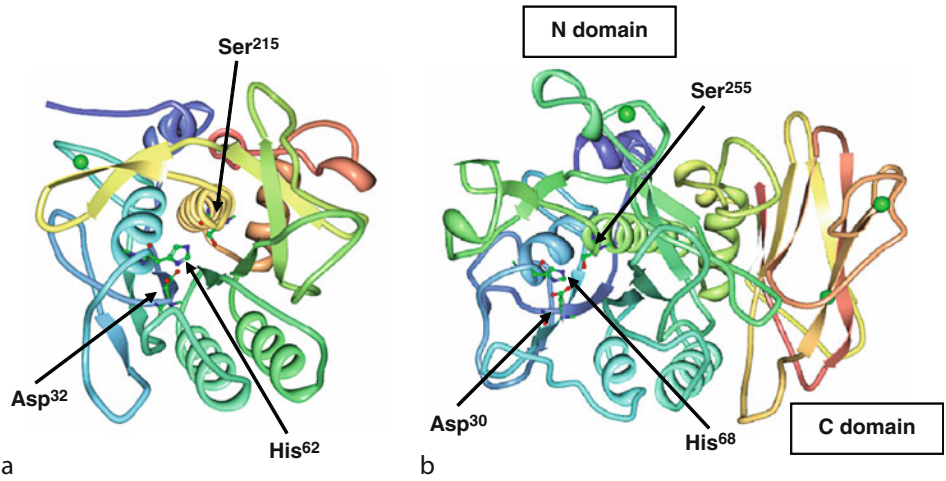

Fig. 2.11.1

Subtilin-fold structures of alkaline proteases. (a) M-protease (1WSD); (b) KP-43 (1WMD). The catalytic triads are shown in ball-stick model, and the Ca^{2+} ions in spheres. The structure of KP-43 is composed of N and C domains. Protein structures in this chapter were downloaded from the Worldwide Protein Bank (http://www.wwpdb.org/) and drawn by Protein Workshop

(Murzin et al. 1995), characterized by layers of the central α-helix, parallel β-sheet and outer helices (Betzel et al. 1992; van der Laan et al. 1992).

To analyze the stability against alkaline pH of high-alkaline proteases in detergent solution, we refined the crystal structure of M-protease (PDB code 1WSD; Yamane et al. 1995, Shirai et al. 1997), as shown in *Fig. 2.11.1a*. The catalytic triad residues were well conserved as Asp^{32}, His^{62}, and Ser^{215}, located close to the N terminus of the central α-helix. The pairs of neighboring oppositely charged amino acid residues in folded proteins are known to interact to generate hydrogen bonds or salt bridges and thus acquire thermal stability and/or structural rigidity (Vogt et al. 1997; Kumar et al. 2000; Bryan 2000; Ozawa et al. 2001). The analysis of the crystal structure of M-protease revealed that the enzyme contains the unique salt bridge triad Arg^{19}-Glu^{265}-Arg^{269}, where such triad is not found in BPN' and the corresponding residues are Gln^{19}, Gln^{271}, and Gln^{275}, respectively, as shown in *Fig. 2.11.2*. We proved by site-directed mutagenesis that the salt bridges between the N and C termini on the surface of M-protease is very important to retain its higher thermal stability especially in alkaline pH than true subtilisins (Kobayashi et al. 2005).

As shown in *Fig. 2.11.1b*, the crystal structure of KP-43 is composed of two domains, a subtilisin-like α/β domain (N domain) and a C-terminal jelly roll β-barrel domain (C domain) (PDB code 1WMD; Nonaka et al. 2004). When the C domain (from Pro^{424} to His^{433} is truncated by site-directed mutagenesis, the mutant recombinant enzyme was not expressed as an active form in *Escherichia coli* cells (unpublished result). The N domain consists of 11 α-helices and 10 β-sheets and includes the catalytic triad Asp^{30}, His^{68}, and Ser^{255}. The C domain showed limited amino acid sequence homology to the C-terminal prodomain of aqualysin precursor (Terada et al. 1990), which functions as an intramolecular chaperone to stabilize the unfolded precursor during translocation through the cytoplasmic membrane (Kim et al. 1997; Kim and Matsuzawa 2000). The C-terminal prodomain of aqualysin I is

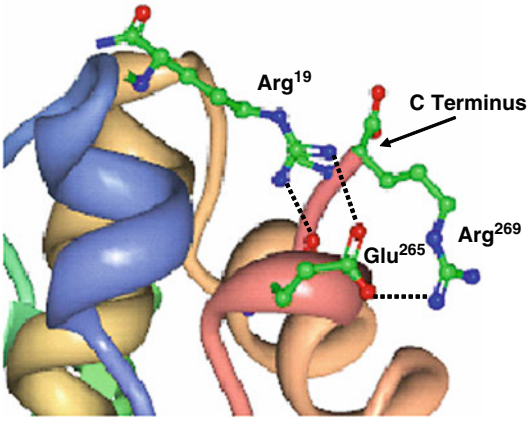

Fig. 2.11.2

Contribution of salt bridges to the thermal stability of M-protease. This figure is a close-up view around the salt bridge triad of M-protease (1WSD). The salt bridge is composed of Arg^{19}, Glu^{265}, and Arg^{269}

also assumed to consist of β-sheets (MacGuffin et al. 2000). Therefore, it is possible that the C domain of KP-43 contributes to protein folding and maintenance of the overall structural integrity of the enzyme. KP-43 contains Met^{256} adjacent to catalytic Ser^{255} residues similarly with other subtilisins, including all the OSPs reported so far. When fully oxidized by H_2O_2, sulfoxides of Met^{251} and Met^{256} were formed as detected in the electron density map (Nonaka et al. 2004). A possible mechanism for the oxidative stability of KP-43 would be the slow oxidation of Met^{256} in the vicinity of the catalytic Ser. It might be due to the longer distance between Met^{251} and the oxianion hole and/or to the existence of Met^{251}. However, further study is required to understand the detailed mechanism underlying the oxidative stability of OSPs.

Alkaline Cellulases

Alkaline Cellulase Producers

Horikoshi and coworkers found that alkaliphilic *Bacillus* strains N-4 (Horikoshi et al. 1984) and no. 1139 (N-1; *B. wakoensis*) (Fukumori et al. 1985; Nogi et al. 2005), both of which had been described in a patent (US patent 3844890 from 1974), produced alkaline cellulases (or endo-1,4-β-glucanases, CMCases). His discovery of the alkaline endo-1,4-β-glucanases (Egls) led us to the eventual incorporation of a Egl in a new compact-type detergent (Attack^{TM}) in 1987 (Ito et al. 1989). According to the method of Horikoshi, a number of alkaline Egl-producing bacilli have been isolated from soil samples (Fukumori et al. 1985; Fukumori et al. 1989; Ito et al. 1989; Okoshi et al. 1990; Shikata et al. 1990; Sumitomo et al. 1992; Hakamada et al. 1997). A thermophilic fungus, *Humicola insolens* DSM 1880, also produces cellulase (Celluzyme^{TM}), which is active under a pH rage of 7–9 (WO 91/17243). Among them, the detergent enzymes industrially produced are Egl-K (KAC^{TM}) from alkaliphilic *Bacillus* sp. KSM-635 (a relative of *B. subtilis*) (Ito et al. 1989) and Egl-237 (or S-237) from

alkaliphilic *Bacillus* sp. KSM-S237 (AB035091, *B. pseudofirmus*) (Hakamada et al. 1997), which were later reclassified as a novel *Bacillus* species (not deposited) and found to be a relative of *B. akibai* (AB043858, Nogi et al. 2005) alignment, respectively. The *H. insolens* cellulase (rich in Egl activity; Carezyme^{TM}) is used for color revival and softening of cotton fabrics in detergents (Kottwitz and Upadek 1997).

The Egl (CMCase)-producing strains of alkaliphiles can easily be picked up as colonies around which shallow craters or clear zones had been formed on alkaline CMC-agar plates or on alkaline plates that contain CMC and Trypan blue (or Congo red) dye, respectively.

Purification and Enzymatic Properties

Purification Procedures

Egl-K is industrially hyperproduced by a mutant form of *Bacillus* sp. KSM-635 and is currently incorporated into compact-type detergents as an effective additive showing good wash performance. It can be purified to homogeneity by a simple procedure as follows (Yoshimatsu et al. 1990). A small volume of culture supernatant was concentrated by ultrafiltration on a membrane and dialyzed against a large volume of 10 mM Tris/HCl (pH 7.5) plus 5 mM $CaCl_2$ (buffer B). The retentate was applied to a column of DEAE-Toyopearl 650S equilibrated with buffer B plus 0.2 M NaCl, and proteins were eluted with a linear gradient of 0.2–0.4 M NaCl in the buffer. The active fractions were pooled and concentrated by ultrafiltration, and the concentrate was loaded on a Bio-Gel A0.5m column equilibrated with buffer B plus 0.1 M NaCl. The activity was separated into two peaks, and the first peak was designated E-H and the next E-L. Each active fraction was combined, diluted with buffer B, and then concentrated to a small volume by ultrafiltration. The concentrates thus obtained were used as the final preparations of purified enzyme. The degrees and purification and total recovery were 2.1-fold and 56% for E-L and 3.7-fold and 12% for E-L.

We purified Egl-237 to homogeneity from the spent medium of *Bacillus* sp. KSM-S237 (Hakamada et al. 1997). The culture supernatant was treated with ammonium sulfate, and the fraction precipitated between 60% and 90% saturation were collected by centrifugation. A small volume of 10 mM Tris/HCl buffer (pH 7.5) (buffer C) was added to the precipitate, and the solution was then dialyzed against the same buffer. Firstly, the retentate was applied to a column of DEAE-Toyopearl 650M equilibrated with buffer C, and proteins were eluted with a linear gradient of 0–1 M NaCl in the equilibration buffer. Secondly, the active fractions were pooled and diluted with buffer C, and the dilute was directly loaded on a column of DEAE-Toyopearl 650S equilibrated with the same buffer, and proteins were eluted with a linear gradient of 0–0.6 M NaCl in the equilibration buffer. The active fractions were combined, diluted with buffer C, and then concentrated to a small volume by ultrafiltration. The concentrate thus obtained was used as the final preparation of purified enzyme.

Protein bands associated Egl activity can be visualized very sensitively by staining with Congo red after non-denaturing and SDS-PAGE, by laying the slab gels at 30 for 30 min on top of a CMC-agar sheet. The CMC-agar sheet with 5-mm thickness is composed of 2% (w/v) CMC, 3% (w/v) NaCl, 0.1 M glycine/NaOH buffer (pH 9), and 0.8% (w/v) agar. The sheet is stained with 0.1% (w/v) Congo red solution for 15 min and destained for 10 min in 0.1–1 M NaCl solution at room temperature. The protein bands with CMCase activity are located as the clear zones on the sheets of red background. In the case of the protein bands

after SDS-PAGE, the polyacrylamide gels must be rinsed with 0.1 M NaCl solution adequately to remove SDS from the gels before activity staining.

Enzymatic Properties

The enzymatic properties of purified E-H and E-L were characterized by Yoshimatsu et al. (1990). The presence of calcium ions was essential for the separation of the two enzymes with a high overall yield (~70%). In addition to a simple protein peak at 280, both enzymes had had a shoulder at around 290 nm presumably due to absorption by Trp residues in the molecules. The molecular masses of E-H and E-L were estimated to be 500 kDa and 100 kDa, respectively, by gel filtration chromatography. SDS-PAGE gave molecular masses for E-H and E-L of 130 kDa and 103 kDa, respectively, indicating that E-H exists as a homotetramer and E-L has a monomeric form. The p*I* values of both enzymes were less than pH 4 by electrofocusing PAGE. The molecular mass and p*I* of alkaline Egl from *Bacillus* sp. no. 1139 were about 92 kDa and pH 3.1, respectively (Fukumori et al. 1985). Neither enzyme was inhibited by sulfhydryl inhibitors (1 mM each), EDTA and EGTA (0.5 mM each), zeolite (0.5%, w/v) or various surfactants used for detergents (0.05% each, w/v) at all, and they were abolished completely by *N*-bromosuccinimide (50 μM), suggesting that Trp residues involves as subsites in the catalytic pocket of Egls (Kawaminami et al. 1994, 1999).

Maximal pH and temperature toward CMC of E-H and E-L were observed at around pH 9.5 (actual pH in the reaction mixture) and at 40°C for both, respectively, in 100 mM glycine/NaOH buffer. Both enzymes were stable over a range between pH 6 and pH 11 when preincubated at 5°C for 3 h in buffers at various pH values. The two enzymes were stable up to 35°C after heating at various temperatures for 10 min in glycine/NaOH buffer (pH 9.5). The presence of Ca^{2+}, Mn^{2+}, and Ca^{2+} ions (at more than 0.1 mM) markedly protected the enzymes from the complete inactivation at 45°C. Interestingly, Na^{+} and K^{+} ions (at more than 0.2 M) protected the enzyme from thermal inactivation at 40°C almost completely. E-H and E-L efficiently hydrolyzed CMC (β-1,4-linkage) and lichenan (β-1.3; β-1,4- linkage), but crystalline celluloses, curdlan (β-13-linkage), laminarin (β-13; β-1,6-linkage), and 4-nitrophenyl-β-D-glucopyranose were practically inert as substrates. By adding the enzymes, viscosity of buffered CMC solution was rapidly lowered and the substrate was randomly hydrolyzed to form cellooligosaccharides, indicative of the enzymes being an endo-1,4-β-glucanase. His residues appear to contribute in some way to catalysis in Egl-K because the enzyme has no activity around at pH 6 (Yoshimatsu et al. 1990; Ozaki et al. 1995). This hypothesis may be supported by the pK_a value of His (around 6.3) compared with those of Glu and Asp (around 4.1).

The purified Egl-237 had molecular mass and p*I* of 86 kDa by SDS-PAGE and pH 3.8 by electrofocusing PAGE (Hakamada et al. 1997). The N-terminal amino acid sequence of the enzyme was Glu-Gly-Asn-Thr-Arg-Glu-Asp-Asn-Phe-Lys-His-Leu-Leu-Gly-Asn-Asp-Asn-Val-Lys-Arg, which is identical to those of Egls from *Bacillus* sp. strains no. 1139 (Fukumori et al. 1985) and KSM-64 (Sumitomo et al. 1992). However, the sequence was completely different from that of CelK (Ozaki et al. 1990). The optimal pH and temperature of the enzyme were around 9 in Britton-Robinson buffer (actual pH in the reaction mixture) and 45°C at pH 9 in 0.1 M glycine/NaOH buffer. In the presence of calcium ions at 5 mM, the optimal temperature was shifted to 60°C and the activity was 1.1-fold greater than that at 40°C. The activity was inhibited completely by Fe^{2+} ions but not by Hg^{2+} ions, and

stimulated by Co^{2+} ions (each at 1 mM). The stimulation by Co^{2+} ions is a common characteristic of some *Bacillus* Egls (Yoshimatsu et al. 1990; Okoshi et al. 1990). The substrate specificity of Egl-237 was similar to those of alkaline Egls so far reported. Notably, Egl-237 is more thermostable than Egls from mesophilic, alkalphilic *Bacillus* spp. In the absence of 5 mM Ca^{2+} ions, the enzyme was stable up to 45°C, and above this temperature the residual activity decreased gradually. The temperature response was unusual in that there was a shoulder around 65°C and more than 30% of the original activity remained even after heating at 100°C and at pH 9 for 10 min. Calcium ions protected the enzyme from thermal inactivation between 45°C and 55°C, and consequently the enzymes was stable up to 55°C. When recombinant enzymes were used, halves of the activity remained after heating were 70°C for Egl-237, 55°C for Egl-64, and the truncated CelK for 35°C (Hakamada et al. 2001). The detailed mechanism of thermal stability of Egl-237 has been reported in the literature (Ozawa et al. 2001).

Amino Acid Sequences and Structures of Egl-K and Egl-237

Cloning and Sequencing

The gene for CelK (E-L) in Egl-K was cloned by the shotgun method using the host-vector system. *E. coli* strains HB101 and JM 109 were used as the hosts, and plasmid pBR322 and bacteriophages M13mp18 and M13mp19 were used for cloning and sequencing. In the sequence determined (3,498 bp), an ORF of 2,823 bp that corresponded to the proenzyme of CelK with a calculated molecular mass of 104,628 (941 amino acids) was found (AAA22304) (Ozaki et al. 1990). The amino acid residues from 1 to 29 were possible signal peptide. The mature CelK exhibited amino acid homology to Egls from *Bacillus* spp. strains of KSM-64 (Sumitomo et al. 1995; AAA73189), KSM-S237 (Hakamada et al. 2000; BAB19360) and no. 1139 (Fukumori et al. 1985; AAA22305) with ~70% identity and to that from N-4 (Fukumori et al. 1989; AAA22306) with 66% identity, all of which are the member of glycoside hydrolase family (GH) 5 (http://www.cazy.org/). The carbohydrate-binding modules 17–18 were located in the C-terminal halves of the respective enzymes.

The gene for thermostable Egl-237 (BAB19360) was cloned and sequenced as follows. The *Eco*RI digest of the genomic DNA (1 μg, 2–9 kb) was ligated with T4 ligase into pUC18 treated with *Eco*RI and alkaline phosphatase. *E. coli* HB101 was then transformed with the constructed plasmid and grown on Luria-Bertini agar plus 50 μg/ml ampicillin at 37°C for 24 h. CMCase-positive colonies were selected by the soft-agar-overlay method. After the soft agar (0.8%, w/v) was incubated at 37°C for 3 h, a 1% (w/v) solution of Congo red was poured onto the soft agar. The positive clones formed a clear zone around the colonies, and were washed with 1 M NaCl solution so that the zones became clearer. After the plasmid in a positive clone was extracted and purified, the nucleotide sequence of the insert was determined. The nucleotide sequence coded Met^{1}-Ser^{120}. To sequence the entire gene, primers 5′-GATGCAACAGGCTTATATTTAGAG-3′ designed from a sequence upstream of the possible promoter region and 5′-AAATTACTTCATCATTCTATCAC-3′ designed from a sequence downstream of the Egl-1139 gene were synthesized. After PCR under a defined condition using *Pwo* DNA polymerase, the amplified fragment (3.1 kb) was purified and sequenced. The entire gene for Egl-237 harbored a 2,472-bp ORF encoding 824 amino acids, including a 30-amino-acid signal peptide. The deduced amino acid sequence of the mature enzyme

(794 amino acids, 88,284 Da) showed similarity to those of GH 5 mesophilic, alkaline Egls from *Bacillus* sp. KSM-64 with 91%, *Bacillus* sp. no. 1139 with 91% and CelK with 70% identity. Expression of the Egl-237 gene in *B. subtilis* resulted in high CMCase activity (2 g/l) under a defined culture condition, concomitant with the appearance of a protein band on an SDS gel at 86 kDa. Site-directed mutagenesis delineated the importance of Arg^{111}, His^{151}, Glu^{190}, His^{262}, Tyr^{264}, and Glu^{305} residues in catalysis and/or substrate binding of Egl-237. Among them, Glu^{190} and Glu^{305} appear to be the acid/base catalyst and nucleophile, respectively.

CMCase-positive transformants can be detected as follows. Transformed *E coli* cells are selected by growing on Luria-Bertani agar plates that contains 50 μg ampicillin/ml and CMC (2%, w/v) at 37°C. Production of Egls by the drug-resistant colonies can be visualized by staining the agar plates with Congo red (Teather and Wood 1982).

Three-Dimensional Structures

To crystallize and analyze the three-dimensional structure of CelK, we truncated the genes encoding the N- and C-terminal regions of CelK by site-directed mutagenesis (Ozaki et al. 1995), and the engineered gene was expressed by hyper-expression system using the host *B. subtilis* and vector pHSP64 (Sumitomo et al. 1995). The truncated recombinant CelK (Ala228-Thr584, 357 amino acids; 40,204 Da) was purified and then crystallized in the presence of Cd^{2+} ions (PDB code 1G01; Shirai et al. 2001). The crystal structure of CelK at 1.9 Å resolution indicated that the enzyme conserves a $(\beta/\alpha)_8$ core barrel structure with Glu^{373} as the acid/base catalyst and Glu^{485} as the nucleophile, as shown in *Fig. 2.11.3a*. The core structure is common among GH5 cellulases. Showing 47% amino acid identity of the catalytic domains, CelK is the closest match with Cel5A from *B. agaradhaerens* (AAC19169) (Davies et al. 1998; Varrot et al. 2000). By homology modeling method with the structure of CelK, the modeled Egl-237 was also shown to have a $(\beta/\alpha)_8$ core barrel structure containing the

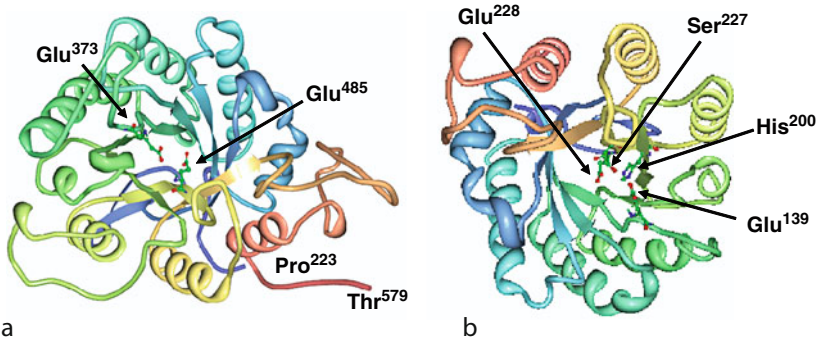

Fig. 2.11.3

$(\beta/\alpha)_8$-Barrel structures of alkaline endoglucanases. (a) CelK (1G01); (b) Cel5 (1H5V). The catalytic residues (acid/base and nucleophile) and/or possible catalytic triad are shown in *ball-stick model*. The N- and C-terminal residues of the truncated CelK are included in the figure

conserved Glu^{190} and Glu^{305} residues essential for activity (Hakamada et al. 2000; Ozawa et al. 2001).

Bacillus sp. CBS 670.93 produce a GH5 Egl, Cel5, whose pH-activity curve is broad with an optimum of around 8 (US patent 6767879-A). The amino acid sequence (CAB59165) exhibited high homology to those of Cel5A from *B. agaradhaerens* DSM 8721 (AAC19169) with 95% identity and celB from *Bacillus* sp. N-4 (ATCC 21833) (AAA22299) (Fukumori et al. 1986) with 92% identity, both of which produce a CMCase with very broad pH-activity curve profile (Fukumori et al. 1986; Hirasawa et al. 2006). The crystal structure of Cel5 (PDB codes 1H5V and 1LF1), revealed that the enzyme had Glu^{139} and Glu^{228} in close proximity to one another in the active site that are typical of retaining Egls (Varrot et al. 2001). Shaw et al. (2002) proposed that Glu^{139} would be a member of a Ser^{227}-His^{200}-Glu^{139} catalytic triad, forming a combination of catalytic machineries, as shown in *Fig. 2.11.3b*. They also suggested that this catalytic triad would control the protonation of the nucleophile Glu^{228}, facilitating the first step of the catalytic reaction, protonation of the substrate, by the proton donor Glu^{139} and that such proton relay reflect the broad pH-activity curves of the enzymes. However, their proposal has not yet been confirmed because several GH5 Egls have variations at the corresponding Ser position.

Cellulase Effects in Detergents

At present, protein-engineered variants of Egl-K, Egl-237, and *H. insolens* Egl are incorporated in laundry detergents. According to Maurer (1997), the cellulase effects in the detergents are fabric softening, antipilling, color revival, detergency, and anti-redeposition of cotton fabrics. The *Bacillus* Egls (KACs) show excellent detergency/cleaning anti-redeposition, while the fungal Egl (Carezyme; 43-kDa component of Celluzyme) is effective for antipilling, fabric softening, and color revival.

Alkaline α-Amylases

Alkaline Endo-Type α-Amylase Producers

Numerous amylolytic enzymes have been isolated from various origins and reported in the literature. The most well-known industrial α-amylases are liquefying (endo-type) ones from several neutrophilic *Bacillus* spp. such as *B. stearothermophililus* (BSA), *B. amyloliquefaciens* (BAA), and *B. licheniformis* (BLA). Among them, highly themostable BLA (Saito 1973) is used widely in industrial application fields such as in bread making, production of glucose and fructose syrup and bioethanol from starch materials and textile treatment. However, most of the *Bacillus* liquefying amylases have pH optima of between 5 and 7.5 (Saito 1973; Manning and Campbell 1961), and, therefore, they are not practically suitable for use in laundry and automatic dishwashing detergents with high alkalinity (pH 8–12). In addition, high durability under alkaline conditions containing ingredients such as surfactants, chelators, and bleaches is required for detergent enzymes.

Since Horikoshi (1971a) first found an alkaline amylase from alkaliphilic *Bacillus* sp. A-40-2, many alkaline enzymes have been reported to be produced extracellulary by, for example, *Bacillus* sp. NRRL B-3881 (Japanese patent 9,049,584), *Bacillus* sp. H-167

(Hayashi et al. 1988), *B. alcanothermophilus* A3-8 (Boyer and Ingle 1972) and *Bacillus* sp. GM8901 (Kim et al. 1995). These alkaline enzymes reported so far were of the saccharifying type (exo-type), including exo-1,4-α-D-glucosidase, β-amylase, exo-isomaltotriose hydrolase, exo-maltotetraohydrolase and exo-maltohexaohydrolase.

We found an alkaline liquefying α-amylase (AmyK, formerly designated LAMY^{TM}) for the first time from alkaliphilic *Bacillus* sp. KSM-1378 (FERM BP-3048; a relative of *B. firmus*) (Igarashi et al. 1998a). Subsequently, we also found a novel α-amylase (AmyK38) from alkaliphilic *Bacillus* sp. KSM-K38 (FERM BP6936) that is highly resistant to chelators and oxidants (Hagihara et al. 2001b). Strain KSM-K38 (AB044748) was closely related to *B. agaradhaeren* DSM 8721^{T} (X76455; 95.5% identity) but the DNA-DNA hybridization between both strains was less than 23% association, indicating that KSM-K38 was a novel species of the genus *Bacillus*. Their enzymatic properties are suitable for use in detergent biobuilders. To aid the wash performance of the liquefying alkaline α-amylases, alkaline debranching enzymes, such a pullulanase (Ara et al. 1992; Hatada et al. 2001), a neopullulanase (Igarashi et al. 1992), an isoamylase (Ara et al. 1993) and an amylopullulanase (Ara et al. 1995; Hatada et al. 1996), were found by us from alkaliphilic *Bacillus* spp.

Purification and Enzymatic Properties

Purification Procedures

AmyK was purified to homogeneity by a simple procedure as follows (Igarashi et al. 1998a). The centrifugal supernatant of culture broth was treated with ammonium sulfate, and the fraction precipitated at 60% saturation was dissolved in a small volume of 10 mM Tris/HCl plus 2 mM $CaCl_2$ (buffer D). After dialyzing against a large volume of buffer D, the retentate was applied to a column of CM Toyopearl 650M equilibrated with buffer D, and proteins were then eluted with the same buffer. Non-adsorbed active fractions were directly put on a column of DEAE-Toyopearl 650M equilibrated with buffer D. Proteins were then eluted with a linear gradient of 0–0.5 M NaCl in the buffer. The active fractions eluted were combined and concentrated by ultrafiltration on a membrane. The concentrate was dialyzed against a large volume of buffer D, and the resulting retentate was used as the final preparation of purified enzyme.

AmyK38 was purified to homogeneity by the following procedure (Hagihara et al. 2001b). The centrifugal supernatant of culture broth was treated with ammonium sulfate, and the fraction precipitated at 80% saturation was dissolved in a small volume of 10 mM Tris/HCl buffer (pH 7). After dialyzing against a large volume of the buffer, the retentate was applied to a column of DEAE Toyopearl 650M equilibrated with 10 mM Tris/HCl buffer (pH 7), and proteins were then eluted with a linear gradient of 0.3–1 M NaCl in the same buffer. Active fractions were combined and concentrated by ultrafiltration on a membrane. The concentrate was put on a column of Toyopearl HW-55 equilibrated with 10 mM Tris/HCl buffer (pH 7) plus 0.2 M NaCl. Proteins were then eluted with the equilibration buffer. The active fractions eluted were combined and concentrated by ultrafiltration. The concentrate was dialyzed against a large volume of 10 mM glycine/NaOH buffer (pH 10), and the resulting retentate was used as the final preparation of purified enzyme.

The recombinants AmyK and AmyK38 can be purified by similar methods as reported by Ikawa et al. (1998) and Hagihara et al. (2001a), respectively.

Enzymatic Properties

The molecular mass of AmyK was estimated to be 53 kDa by SDS-PAGE, a value similar to those of BAA, BSA, and BLA. The p*I* value was around 9. The N-terminal amino acid sequence was His-His-Asn-Gly-Thr-Asn-Gly-Thr-Met-Met-Gln-Tyr-Phe-Glu-Trp. Maximal pH and temperature toward soluble starch was observed at around pH 8–8.5 and at 55°C, respectively. The activity was not detectable at pH 3 and 11. The enzyme was stable over a range between pH 6 and pH 10 when preincubated at 40°C for 30 min in 10 mM Britton-Robinson buffer of various pHs. The enzyme was stable at 50°C after heating for more than 60 min in 50 mM Tris/HCl buffer (pH 8) in the presence of 0.1 mM $CaCl_2$. In the absence of Ca^{2+} ions, the residual activity decreased gradually with the incubation time and retained less than 40% of the original activity after heating for 60 min. The activity was strongly inhibited by iodoacetate (0.5 mM), EDTA (10 mM), EGTA (10 mM), and *N*-bromosuccimide (0.1 mM). However, *p*-chloromercuribenzoate (0.5 mM), *N*-ethylmaleimide (1 mM), diethyl pyrocarbonate (2 mM), and various surfactants (each at 0.1%, w/v) gave no inhibitory effect on the activity. AmyK randomly hydrolyzed soluble starch (from potato), amylopectin (from potato), amylose (from potato; degree of polymerization 17), glycogen (from oyster), and dextrin (from corn) with a relative rate of 100:114:37:83:7. Neither dextran, α-, β- and γ-cyclodextrins, pullulan, nor maltooligosaccharides in the G2–G6 range were inert as substrate.

The molecular mass of AmyK38 was determined to be 55 kDa by SDS-PAGE. The p*I* value was around 4.2. The N-terminal amino acid sequence was Asp-Gly-Leu-Asn-Gly-Thr-Met-Gln-Tyr-Tyr-Glu-Trp. Temperature and pH optima toward soluble starch were observed at 55–60°C and around pH 9.5 in 50 mM glycine/NaOH buffer. AmyK38 randomly hydrolyzed soluble starch, amylopectin, amylose, glycogen, and dextrin with a relative rate of 100:74:65:58:27 (Hagihara et al. 2001b). The most striking feature of AmyK38 is the resistance to excess H_2O_2 (more than 1.8 M) and EDTA and EGTA (more than 0.1 M). When the oxidative stability of AmyK38 was examined after incubation with 0.6 M H_2O_2 at 30°C and at pH 10 in 50 mM glycine/NaOH buffer, the enzyme retained full activity even over the course of 1 h, but the residual activity of BLA rapidly decreased (a half-life of around 3 min) under the same conditions. AmyK38 and BLA were each incubated with EDTA and EGTA up to 0.1 M in 50 mM glycine/NaOH buffer (pH 10) at 45°C for 30 min. As a result, AmyK38 maintained full activity in the presence of both chelators at as high as 0.1 M, but the activity of BLA was reduced by adding each chelator at 1 mM to 50% of the original activity (Hagihara et al. 2001b). Analysis of atomic absorption spectra indicated that the contents of Ca^{2+} and other divalent cations in the AmyK38 molecule were almost negligible (Hagihara et al. 2001a).

Amino Acid Sequences and Structures

Cloning and Sequencing

The gene for AmyK (BAA32431) was cloned and sequenced as described by Igarashi et al. (1998a). Using two primers A and B designed from two common regions Asp-Ala-Val-Lys-His-Ile-Lys and Asp-Val-Thr-Phe-Val-Asp-Asn-His-Asp, respectively, of typical α-amylases were first used for PCR of the gene fragment (0.3 kb) of AmyK. To determine the complete sequence, inverse PCR was performed using a self-circulated *Xba*I-digested genomic DNA as template and two primers C and D designed from the gene fragment amplified. A 0.7-kb fragment

amplified encoded a deduced amino acid sequence identical to the C-terminal sequence of typical α-amylases and s stop codon TAA. Then, primer E designed from the N-terminal sequence of the purified AmyK was synthesized. PCR was done with the primers C and E and the genomic DNA as template, which generated a 0.7-kb fragment. The second inverse PCR was done using a self-circulated *Hin*dIII-digested genomic DNA as template and suitably synthesized primers F and G to generate a 0.8-kb fragment. The amplified fragment contained a putative regulatory region and a sequence containing the N-terminal sequence of purified AmyK. Finally, the AmyK gene (1.8 kb) was amplified using suitably synthesized two primers and the genomic DNA. The 1.8-kb fragment contained a single ORF (1,545 bp in the 1.787-bp sequence) that encoded 516 amino acid residues of AmyK. When suitably aligned, the deduced amino acid sequence of mature AmyK (485 amino acid, 55,391 Da) exhibited 66.7%, 68.6%, and 68.9% identity to those of BAA (P00692), BSA (P06279) and BLA (P06278). It contained the four conserved regions (I, II, III, and IV) and three conserved domains (A, B, and C) of the latter three enzymes, which was first reported for porcine α-amylase (Buisson et al. 1987), and had a $(\beta/\alpha)_8$ barrel core fold. The catalytic triad Asp^{231}-Glu^{261}-Asp^{328} in BLA was conserved as Asp^{236}-Glu^{266}-Asp^{323} in AmyK.

Declerck et al. (1995, 1997) and Joyet et al. (1992) showed that two substitutions in the amino acid sequence, His133Ile (or His133Tyr) and Ala209Val (or Ala209Ile) can together increased in the half-life of BLA at 90°C up to tenfold. Further, van der Laan (WO 95/35382) and Aehle (1997) reported that two substitutions, Val128Glu/His133Tyr or His133Tyr/Asn188Asp, improved the thermostability of BLA in the presence of 1.5 mM $CaCl_2$. Interestingly, the original amino acid sequence of AmyK conserves Tyr^{135} and Ile^{214} and Glu^{130} (although the substituted Asp^{188} corresponds to Ile), respectively. Suzuki et al. (1989) reported that the thermostability of BAA was improved by deletion of Arg^{176}-Gly^{177} and substitution of Lys^{269} for Ala and suggested that an increase in hydrophobicity by changes in charged residues enhanced the thermostability of the enzyme. However, the substituted Ala^{269} in BAA corresponds to Ala^{274} for AmyK and Ala^{269} for BLA in their original sequences. Then, we deleted the corresponding dipeptide Arg^{181}-Gly^{182} from the sequence of AmyK by site-directed mutagenesis by (Igarashi et al. 1998b; Ito et al. 2002; Ozawa et al. 2006) and proved that the dipeptide-deleted mutant was thermodynamically stabilized by enhanced calcium binding. We also improved the thermal stability of AmyK drastically by double mutation of the Arg-Gly deletion and Arg124Pro substitution (Igarashi et al. 1999). Van der Laan showed in his patent (WO 95/35382) that the substitution of both His133Tyr and Ser187Asp in BLA increased the specific activity of the enzyme threefold. The specific activity of AmyK is several times greater than that of BLA (Igarashi et al. 1998a), which is very reasonable in that the original sequence of AmyK conserves as Tyr^{135} and Asp^{192}.

The ORF and its flanking regions of AmyK38 (AB051102) were cloned and sequenced by Hagihara et al. (2001a). The gene encoding an internal sequence of AmyK38 (1 kb) was PCR-amplified with the *Bacillus* sp. KSM-K38 genomic DNA as template and appropriate mix primers, which were designed form Met-Gln-Tyr-Phe-Glu-Trp and Trp-Phe-Lys-Pro-Leu-Ala-Tyr commonly found in the N-terminal and internal regions of BLA, BSA, BAA and AmyK. To determine the remaining N- and C-terminal flanking regions of the amplified fragment, the genomic DNA was digested with *Xba*I or *Eco*RI, and each digest was treated with T4 DNA ligase under the conditions that favored the formation of monomeric form. Then, inverse PCR was performed using *Xba*I-self-circularized DNA as template and appropriate mix primers, which were designed from the terminal ends of the 1-kb amplified fragment. An amplified fragment (2 kb) contained the remaining C-terminal sequence and its flanking region (0.5 kb).

The genomic DNA digested with *Eco*RI was ligated with the oligonucleotide cassette having *Eco*RI cohesive ends. Then, the gene encoding part of AmyK38 was PCR-amplified from an *Eco*RI-cassette-ligated genomic DNA as template with appropriate primers. The PCR-amplified fragment was used as template for the second round of PCR with appropriate primers, and the resulting fragment contained the remaining N-terminal sequence and its upstream region of 0.4 kb. Finally, the complete gene for AmyK38 and its flanking regions (1.8 kb) were PCR-amplified using the genomic DNA as template and appropriate primers, which were designed from the sequences 0.2-kb upstream of possible initiation codon and 0.1-kb downstream of the possible termination codon, respectively. In the 1,715-bp nucleotide sequenced, a single ORF (1,545 bp in the 1.787-bp sequence) that encoded 501 amino acid residues of AmyK. When suitably aligned, the deduced amino acid sequence of mature AmyK (480 amino acid, 55,079 Da) exhibited 59.6%, 59.2%, 62.8%, and 66.7% identity to those of BAA, BSA, BLA, and AmyK. It contained the four conserved regions (I to IV) and three conserved domains (A, B, and C) and had a $(\beta/\alpha)_8$ barrel core structure. The conserved catalytic triad was Asp^{236}-Glu^{266}-Asp^{323}.

The high oxidative stability of AmyK38 was suggested to relate to its original amino acid sequence that conserves a nonoxidizable Leu^{197}, where Met residue locates as a subsite in other α-amylases reported to date (Hagihara et al. 2003). Because AmyK38 contains no Ca^{2+} ions (Hagihara et al. 2001a), it was also suggested that the microenvironments around the metal-binding sites of AmyK38 were different from those of BLA and other related enzymes. Compared with the crystal structure of BLA, the residues involved in the metal coordination geometry in AmyK38 were completely replaced by other ones, as will be shown bellow. In addition, the specific activity of this enzyme is much higher than that of BLA, and this may also be explained by the amino acid substitutions similar to AmyK.

Three-Dimensional Structures

Recently, we solved the three-dimensional structure of AmyK (PDB code 2DIE) with special respect to the molecular-based mechanism of alkaline adaptation of proteins (Shirai et al. 2007), as shown in *Fig. 2.11.4b*. AmyK consists of three domains A, B, and C, comprising residues 5–106/210–398, 107–209, and 399–485, respectively, in the $(\beta/\alpha)_8$ core structure. As in the case of BLA (PBD code 1BLI) (*Fig. 2.11.4a*), the metal-binding sites, CaI-Na-CaII and CaIII, and the catalytic triad Asp^{236}-Glu^{266}-Asp^{323} are seen in the AmyK molecule. More than 40 crystal structures of GH13 family amylases have been deposited in the Protein Data Bank, and those close to AmyK were BHA from alkaliphilic *B. halmapalus* (PDB code 1W9X; Davies et al. 2005) and the amylase from alkaliphilic *Bacillus* sp. 707 (PDB codes 1WPC and 1WP6; Kanai et al. 2004). Although the enzymatic properties of the latter two enzymes have not yet clarified yet, they share 86% identity with AmyK in the amino acid sequences. AmyK exhibits 67% amino acid sequence identity to AmyK38.

We have solved the three-dimensional structure of AmyK38 at 2.13 Å resolution (PDB code 1UD2; Nonaka et al. 2003). The overall structure of AmyK38 is essentially similar to that of BLA, in which the molecule consists of three domains (A, B, and C) containing a $(\beta/\alpha)_8$ barrel core structure in Domain A, as shown in *Fig. 2.11.4c*. However, neither a highly conserved Ca^{2+} ion, located at the interface between domains A and B, nor any other Ca^{2+} ions existed in the AmyK38 molecule. When compared with BLA (PBD code 1BLI; Machius et al. 1998), Asp^{161}, Ala^{181}, Asp^{183}, Asp^{200}, and Asp^{204} around the CaI-Na-CaII triad were replaced with

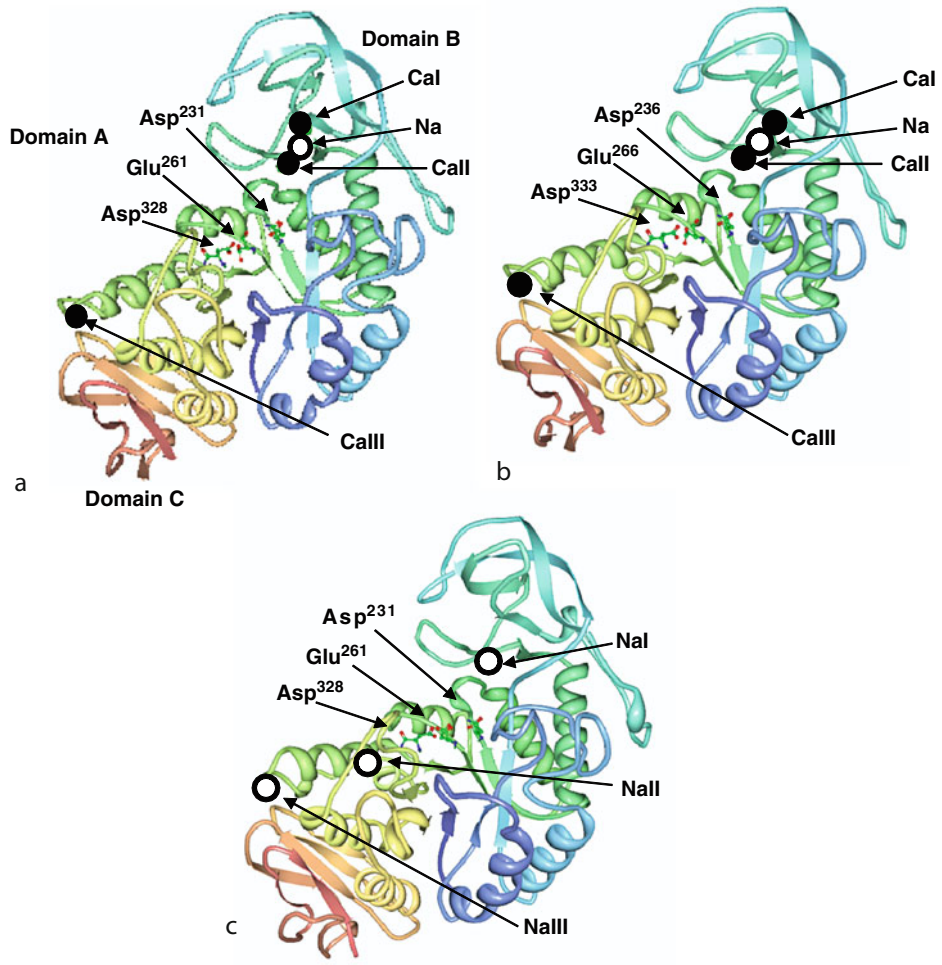

Fig. 2.11.4

Structures of α-amylases. (a) BLA (1BLI); (b) AmyK (2DIE); (c) AmyK38 (1UD2). Each structure is composed of domains A ($(\beta/\alpha)_8$-barrel core, central part), B (β-sheets, N-terminal part), and C (β-sheets, C-terminal part). The Ca^{2+} ions and Na^{+} ions in each structure are shown in *black balls* and *circled white balls*, respectively

Asn^{161}, Asn^{181}, Asn^{183}, Asn^{200}, and Ser^{204} at the corresponding site of AmyK38 (Site I), respectively. In addition, His^{406} and Asp^{430} at the CaIII of BLA were replaced with Trp^{406} and Asn^{430} at AmyK38 (Site III). Further, the residue His^{289} was replaced with Asn^{289} that is essential for newly formed Na^{+}-binding residues for AmyK38.(Site II). As a result, in the AmyK38 molecule, NaI is coordinated with Asn^{104}, Asn181, Asp^{194}, Asn^{200}, and His^{235}; NaII with Asn^{289}, Val^{324}, Asp^{325}, and Ser^{337}; and NaIII with Gly^{300}, Tyr^{302}, Trp^{403}, Asp^{404}, and Asn^{427}, as shown in *Fig. 2.11.5*. Exchange experiments using Na^{+}, Li^{+}, and Rb^{+} ions also showed that the sites I, II, and III were Na ion-binding sites (NaI, NaII, and NaIII, respectively)

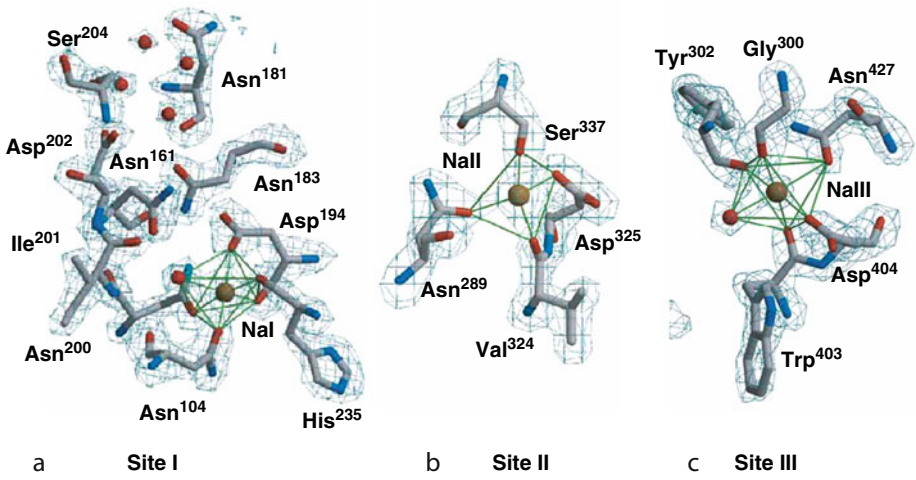

Fig. 2.11.5

Three sodium ion-binding sites of AmyK38. Residues that contribute to the hydrogen-bonding cages with Ca^{2+} and/or Na^{+} ions are shown in ball-stick model. The sodium-binding sites in AmyK38, NaI, and NaII between domains A and B and NaIII between domains A and C, are shown in *sphere*

and that NaI was bound more tightly than NaII and was the most important to the rigidity of the AmyK38 structure. AmyK38 is the first case in which the structure of the amylase has no Ca^{2+} ions and in which the Na^{+} ions instead of Ca^{2+} play an important role in maintaining the structure of the α-amylases. Improvement of the thermal stability of AmyK38 has been achieved by site-directed mutagenesis experiments (Hagihara et al. 2002; Ozawa et al. 2007).

Lipase

Several enzyme manufacturers have developed detergent lipases such as the genetically improved enzymes originally from a fungus *H. lanuginosa* (Lipolase™) and from *Pseudomonas* spp. (for instance, an alkaline lipase, Lipomax™). The fungal lipase-containing detergent was first launched in 1988 in Japan. The wash performance of such enzymes is marked by a multicycle effect; namely, four to five cycles of washing and, in between, rinsing and drying of the fabrics are required for removal of fatty stains (Misset 1997). According to a manufacturer, the lipase attaches to fatty stains after the first wash cycle, and during drying them, it is concentrated on fabrics to become more active and remove the fatty stains further (Wolff and Showell 1997).

Cross-References

9.3 Biochemistry

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Part 3

Extremophiles: Halophiles

3.1 Taxonomy of Halophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.1,

Introduction

Hypersaline environments are widely distributed on our planet and they are mainly represented by saline lakes and other water systems as well as saline soils. Microorganisms that inhabit those habitats are designated as halophiles and they are extremophilic organisms that must cope not only with the high ionic composition but also other environmental factors such as alkaline pH values, low oxygen availability, high or low temperatures, presence of heavy metals and/or other toxic compounds, etc. (Oren 2002; Ventosa 2006).

The relationships of different microorganisms with salt have been studied in detail and several classifications have been proposed. In this chapter, we will adopt the approach proposed by Kuhsner and Kamekura (1988) that is widely used by most scientists. This classification is based on the optimal growth of microorganisms with respect to the concentration of NaCl. Thus, two main physiological groups of halophiles can be found in hypersaline environments: extreme halophiles, able to grow optimally in media with 15–30% (2.5–5.2 M) NaCl, and moderate halophiles, growing optimally in media with 3–15% (0.5–2.5 M) NaCl. Besides, the slight halophiles, which are represented by most marine microorganisms, are able to grow optimally between 1% and 3% (0.2–0.5 M) NaCl. Non-halophilic microorganisms are those organisms that grow best in media with less than 1% (0.2 M) NaCl; however, some of the non-halophilic microorganisms are able to tolerate high NaCl concentrations and they are defined as halotolerant or extremely tolerant organisms (Kuhsner and Kamekura 1988).

In this chapter we will focus on the taxonomy of two main groups of microorganisms, the halophilic *Archaea* and halophilic *Bacteria* that are found in hypersaline habitats and thus, we will consider only those species that are extremely or moderately halophilic. Slight halophiles will not be considered on this review as well as other eukaryotic organisms that can be found on hypersaline habitats such as algae, *Dunaliella* and other representatives, fungi or the crustacean *Artemia salina* (Oren 2002; Ventosa 2006). Besides, we will focus only on those species names that have been validly described by publication on the International Journal of Systematic and Evolutionary Microbiology or on the Validations Lists that are published on this journal, the official organ of the International Committee on Systematics of Prakaryotes (ICSP). We should consider that besides the species included here, many other organisms, some of them not formally described as new taxa, have been published and their features described. On the other hand, some species have been described as halophilic or halotolerant but their relationship with NaCl has not been studied in detail or their NaCl optimal growth has not been reported on their taxonomic descriptions and they have not been included here.

The two classical groups of halophiles from hypersaline environments are the extremely aerobic halophilic *Archaea* (haloarchaea) and the moderately halophilic *Bacteria*, represented by a limited number of species. However, the current situation is quite different due to the recent efforts to characterize microorganisms from hypersaline environments, and in fact the number of species described during the recent years is growing exponentially. As we will point out later, the halophilic *Archaea* are represented not only by the haloarchaea and on the other hand, not all species of haloarchaea can be considered as extremely halophilic since some of them are able to grow optimally at NaCl concentrations within the range described for moderately halophilic microorganisms. In fact, their presence in environments with low salinities has been recently reported (Purdy et al. 2004). On the other hand, the *Bacteria* are currently represented by a large number of species included on different phylogenetic branches, reflecting their broad metabolic activities, and with salt requirements from moderately to

extremely halophilic that reflect their ecological adaptations to a wide range of habitats that in most cases are not stable and that have changing saline conditions.

In the next sections we will review the halophilic species that are currently included within the *Archaea* and *Bacteria* as well as their classification and some interesting characteristics. A more detailed information about their features can be found on the original descriptions as well as in the most recent editions of the *Bergey's Manual of Systematic Bacteriology* and *The Prokaryotes*.

Taxonomy of Halophilic *Archaea*

The halophilic *Archaea* are included on the phylum *Euryarchaeota*. They are represented by the extremely halophilic aerobic *Archaea*, also designated as haloarchaea, currently included within the class *Halomebacteria* (Cavalier-Smith 2002) or its later homotypic synonym *Halobacteria* (Grant et al. 2001). However, some halophilic methanogenic species have been described from hypersaline environments. The features of these two groups of *Archaea* are described below.

Class *Halomebacteria* (*Halobacteria*)

The haloarchaea (also named as "halobacteria") are currently included within a single family, *Halobacteriaceae*, that was proposed by Gibbons (1974) to accommodate the genera *Halobacterium* (represented by rod-like species) and *Halococcus* (spherical cells). The use of different culture media and growth conditions, exhaustive investigations on the biodiversity of a broad variety of hypersaline habitats, and the application of the most recent molecular and chemotaxonomic methods resulted in the description of a large number of species and genera within this archaeal group. At the time of writing (December 2009), the family includes 104 species whose names have been validly published, classified in 27 genera (*Table 3.1.1*). The complete descriptions of the features of these taxa can be found in the original articles, as well as in the second edition of Bergey's Manual of Systematic Bacteriology (Grant et al. 2001). The characterization of haloarchaeal species is currently based on a combination of phylogenetic analysis obtained by comparison of the 16S rRNA gene sequences, phenotypic features, and polar lipid analysis. The ICSP-Subcommittee on the taxonomy of the *Halobacteriaceae* published recommended minimal standards for describing new taxa within this family that can be very useful for the taxonomic characterization of new species of this archaeal group (Oren et al. 1997).

The most important feature of the haloarchaea is their absolute requirement for high concentrations of NaCl. They are considered as the prokaryotes best adapted to high salt concentrations and in fact they cannot grow in freshwater media where most of them are lysed. They can grow easily aerobically in media with 20–25% NaCl. They produce red to pink-pigmented colonies (except some species of the genus *Natrialba*) due to the presence of C_{50}-carotenoids designated bacterioruberins and thus they are partially responsible for the typical pink pigmentations of the saline lakes and most highly salt concentrated ponds of salterns ("crystallizers") where they predominate. Some species are not only halophilic, they are able to grow optimally at alkaline pH values and thus, are defined as haloalkaliphilic. Haloarchaea have typical archaeal characteristics such as the presence of ether-linked lipids that can be easily detected by

Table 3.1.1

Validly published genus and species names within the family *Halobacteriaceae* (as to 31 December 2009). The three-letter genus abbreviations recommended by the ICSP-Subcommittee on the taxonomy of the family *Halobacteriaceae* have been used. Basonyms/synonyms of micro-organisms that have been transferred to other genera are not included. For genera/species whose descriptions have been emended, only the most recent reference is included

Genus	Species
Halobacterium[a] (Oren et al. 2009)	*Hbt. salinarum*[b] (Gruber et al. 2004), *Hbt. jilantaiense* (Yang et al. 2006), *Hbt. noricense* (Gruber et al. 2004), *Hbt. piscisalsi* (Yachai et al. 2008)
Haladaptatus (Savage et al. 2007)	*Hap. paucihalophilus*[b] (Savage et al. 2007)
Halalkalicoccus (Xue et al. 2005)	*Hac. tibetensis*[b] (Xue et al. 2005), *Hac. jeotgali* (Roh et al. 2007b)
Haloarcula (Oren et al. 2009)	*Har. vallismortis*[b] (Torreblanca et al. 1986), *Har. amylolytica* (Yang et al. 2007), *Har. argentinensis* (Ihara et al. 1997), *Har. hispanica* (Juez et al. 1986), *Har. japonica* (Takashina et al. 1990), *Har. marismortui* (Oren et al. 1990), *Har. quadrata* (Oren et al. 1999)
Halobaculum (Oren et al. 1995)	*Hbl. gomorrense*[b] (Oren et al. 1995)
Halobiforma (Oren et al. 2009)	*Hbf. haloterrestris*[b] (Hezayen et al. 2002), *Hbf. lacisalsi* (Xu et al. 2005c), *Hbf. nitratireducens* (Hezayen et al. 2002)
Halococcus (Oren et al. 2009)	*Hcc. morrhuae*[b] (Kocur and Hodgkiss 1973), *Hcc. dombrowskii* (Stan-Lotter et al. 2002), *Hcc. hamelinensis* (Goh et al. 2006), *Hcc. qingdaonensis* (Wang et al. 2007b), *Hcc. saccharolyticus* (Montero et al. 1989), *Hcc. salifodinae* (Denner et al. 1994), *Hcc. thailandensis* (Namwong et al. 2007)
Haloferax (Oren et al. 2009)	*Hfx. volcanii*[b] (Torreblanca et al. 1986), *Hfx. alexandrinus* (Asker and Ohta 2002), *Hfx. denitrificans* (Tindall et al. 1989), *Hfx. elongans* (Allen et al. 2008), *Hfx. gibbonsii* (Juez et al. 1986), *Hfx. larsenii* (Xu et al. 2007a), *Hfx. lucentense* (Gutierrez et al. 2002), *Hfx. mediterranei* (Torreblanca et al. 1986), *Hfx. mucosum* (Allen et al. 2008), *Hfx. prahovense* (Enache et al. 2007), *Hfx. sulfurifontis* (Elshahed et al. 2004)
Halogeometricum (Montalvo-Rodríguez et al. 1998)	*Hgm. borinquense*[b] (Montalvo-Rodríguez et al. 1998)
Halomicrobium (Oren et al. 2002)	*Hmc. mukohataei*[b] (Oren et al. 2002), *Hmc. katesii* (Kharroub et al. 2008b)
Halopiger (Gutiérrez et al. 2007)	*Hpg. xanaduensis*[b] (Gutiérrez et al. 2007)
Haloplanus (Elevi Bardavid et al. 2007)	*Hpn. natans*[b] (Elevi Bardavid et al. 2007)
Haloquadratum (Burns et al. 2007)	*Hqr. walsbyi*[b] (Burns et al. 2007)
Halorhabdus (Antunes et al. 2008b)	*Hrd. utahensis*[b] (Wainø et al. 2000), *Hrd. tiamatea* (Antunes et al. 2008b)

Table 3.1.1 (Continued)

Genus	Species
Halorubrum (Oren et al. 2009)	*Hrr. saccharovorum*[b] (McGenity and Grant 1995), *Hrr. aidingense* (Cui et al. 2006b), *Hrr. alkaliphilum* (Feng et al. 2005), *Hrr. arcis* (Xu et al. 2007b), *Hrr. californiense* (Pesenti et al. 2008), *Hrr. chaoviator* (Mancinelli et al. 2009), *Hrr. cibi* (Roh and Bae 2009), *Hrr. coriense* (Oren and Ventosa 1996), *Hrr. distributum* (Oren and Ventosa 1996), *Hrr. ejinorense* (Castillo et al. 2007b), *Hrr. ezzemoulense* (Kharroub et al. 2006b), *Hrr. kocurii* (Gutiérrez et al. 2008b), *Hrr. lacusprofundi* (McGenity and Grant 1995), *Hrr. lipolyticum* (Cui et al. 2006b), *Hrr. litoreum* (Cui et al. 2007a), *Hrr. luteum* (Hu et al. 2008), *Hrr. orientale* (Castillo et al. 2006c), *Hrr. sodomense* (McGenity and Grant 1995), *Hrr. tebenquichense* (Lizama et al. 2002), *Hrr. terrestre* (Ventosa et al. 2004), *Hrr. tibetense* (Fan et al. 2004), *Hrr. trapanicum* (McGenity and Grant 1995), *Hrr. vacuolatum* (Kamekura et al. 1997), *Hrr. xinjiangense* (Feng et al. 2004)
Halosarcina (Savage et al. 2008)	*Hsn. pallida*[b] (Savage et al. 2008)
Halosimplex (Vreeland et al. 2002)	*Hsx. carlsbadense*[b] (Vreeland et al. 2002)
Halostagnicola (Castillo et al. 2006b)	*Hst. larsenii*[b] (Castillo et al. 2006b)
Haloterrigena (Oren et al. 2009)	*Htg. turkmenica*[b] (Ventosa et al. 1999), *Htg. hispanica* (Romano et al. 2007), *Htg. jeotgali* (Roh et al. 2009), *Htg. limicola* (Cui et al. 2006c), *Htg. longa* (Cui et al. 2006c), *Htg. saccharevitans* (Xu et al. 2005a), *Htg. salina* (Gutiérrez et al. 2008a), *Htg. thermotolerans* (Montalvo-Rodríguez et al. 2000)
Halovivax (Castillo et al. 2006a)	*Hvx. asiaticus*[b] (Castillo et al. 2006a), *Hvx. ruber* (Castillo et al. 2007a)
Natrialba (Oren et al. 2009)	*Nab. asiatica*[b] (Hezayen et al. 2001), *Nab. aegyptia* (Hezayen et al. 2001), *Nab. chahannaoensis* (Xu et al. 2001), *Nab. hulunbeirensis* (Xu et al. 2001), *Nab. magadii* (Kamekura et al. 1997), *Nab. taiwanensis* (Hezayen et al. 2001)
Natrinema (Xin et al. 2000)	*Nnm. pellirubrum*[b] (McGenity et al. 1998), *Nnm. altunense* (Xu et al. 2005b), *Nnm. ejinorense* (Castillo et al. 2006d), *Nnm. gari* (Tapingkae et al. 2008), *Nnm. pallidum* (McGenity et al. 1998), *Nnm. versiforme* (Xin et al. 2000)
Natronobacterium (Tindall et al. 1984)	*Nbt. gregoryi*[b] (Tindall et al. 1984)
Natronococcus (Tindall et al. 1984)	*Ncc. occultus*[b] (Tindall et al. 1984), *Ncc. amylolyticus* (Kanai et al. 1995), *Ncc. jeotgali* (Roh et al. 2007a)
Natronolimnobius (Itoh et al. 2005)	*Nln. baerhuensis*[b] (Itoh et al. 2005), *Nln. innermongolicus* (Itoh et al. 2005)
Natronomonas (Kamekura et al. 1997)	*Nmn. pharaonis*[b] (Kamekura et al. 1997)
Natronorubrum (Oren et al. 2009)	*Nrr. bangense*[b] (Xu et al. 1999), *Nrr. aibiense* (Cui et al. 2006a), *Nrr. sulfidifaciens* (Cui et al. 2007b), *Nrr. tibetense* (Xu et al. 1999)

[a]Type genus of the family.
[b]Type species of the genus.

thin-layer chromatography and they are used as a key feature for the differentiation of taxa, especially at the genus level. All haloarchaea contain phytanyl ether analogues of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Many species also have phosphatidylglycerol sulfate and one or more glycolipids and sulfated glycolipids. Haloarchaea have diphytanyl ($C_{20}C_{20}$) glycerol ether core lipids but some species may have additional phytanylsesterterpanyl ($C_{20}C_{25}$) glycerol core lipids as well as disesterterpanyl ($C_{25}C_{25}$) glycerol core lipids (Grant et al. 2001). Thus, certain polar lipid components are used for the chemotaxonomic characterization of most genera of *Halobacteriaceae*. However, currently some genera, such as the genus *Haloterrigena*, include species with different polar lipid composition, and their taxonomic status should be studied in more detail (Oren et al. 2009).

Halophilic Methanogenic Archaea

Methanogenic *Archaea* play an important role in hypersaline environments but few halophilic species have been characterized. Taxonomically the methanogenic *Archaea* are grouped within five orders, but only the order *Methanosarcinales* includes halophilic species. They are placed within the family *Methanosarcinaceae*. They are strictly anaerobic and obtain energy by formation of methane. They can grow by dismutating methyl compounds (methanol, methyl amines, or methyl sulfides). The following genera include extremely or moderately halophilic species: *Methanohalobium*, *Methanohalophilus*, and *Methanosalsum* (Boone et al. 2001).

Methanohalobium evestigatum is the only representative of the genus *Methanohalobium* and is a nonmotile, strictly methylotrophic, moderately thermophilic and extremely halophilic archaeon. It is able to grow on a NaCl range of 15–25%, showing optimal growth at 25% NaCl. It has been isolated from sediments of hypersaline lakes and salterns (Zhilina and Zavarzin 1987). The genus *Methanohalophilus* includes three moderately halophilic species: *Methanohalophilus mahii*, isolated from the sediments of Great Salt Lake (Utah, USA) (Paterek and Smith 1988), *Methanohalophilus halophilus*, isolated from bottom deposits of Shark Bay, Australia (Wilharm et al. 1991), and *Methanohalophilus portucalensis*, isolated from sediments of a saltern in Portugal (Boone et al. 1993). The genus *Methanosalsum* comprises the single species *Methanosalsum zhilinae*, a moderately halophilic archaeon isolated from alkaline and saline sediments from the Wadi Natrun, Egypt, and Lake Magadi (Boone and Baker 2001).

Finally, the species *Methanocalculus halotolerans* has been isolated from an oil-producing well in France (Ollivier et al. 1998). It has been described as a halotolerant methanogen. However, its optimal growth is at 5% NaCl and is able to grow up to 15.5% NaCl and thus, according to the classification that we have adopted on this chapter, it might be classified as a moderately halophilic species and not as halotolerant. Besides, phylogenetically this species belongs to the order *Methanomicrobiales* but it cannot be assigned to any family and is currently included as a genus *incertae sedis* within this order.

Taxonomy of Halophilic *Bacteria*

The domain *Bacteria* is currently subdivided into 28 phyla (http://www.bacterio.cict.fr/classifphyla.html). Moderately or extremely halophilic species with validly published names are included in the following phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Spirochaetes*, *Bacteroidetes*, *Thermotogae*, *Cyanobacteria*, and *Tenericutes*. In the following sections we will review the taxonomic status of these species.

Phylum *Proteobacteria*

The phylum *Proteobacteria* was proposed by Garrity et al. (2005a) on the basis of phylogenetic analysis of 16S rRNA sequences. This phylum contains five different classes of Gram-negative bacteria: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* (or *Deltabacteria*), and *Epsilonproteobacteria* (Brenner et al. 2005). Microorganisms belonging to the phylum *Proteobacteria* are very ubiquitous and heterogeneous, with diverse physiological properties. They can be isolated from diverse environments including marine, hypersaline, alkaline, and acidic habitats.

With the exception of the class *Betaproteobacteria*, all other classes include halophilic bacteria. In this section we will review the genera and species belonging to the *Proteobacteria*.

Class *Alphaproteobacteria*

The class *Alphaproteobacteria* comprises three orders that include moderately halophilic bacteria: *Rhodobacterales* (Garrity et al. 2005b), *Rhizobiales* (Kuykendall 2005), and *Rhodospirillales* (Pfenning and Trüper 1971).

From all of these, the order *Rhodobacterales* is the one most prominent with 27 genera including halophilic microorganisms. Described in 1994 by Hiraishi and Ueda, the genus *Rhodovulum* groups mesophilic bacteria that require sodium chloride for growth. From the 11 species forming the genus until now, only *R. adriaticum* (Hiraishi and Ueda 1994) is moderately halophilic. This strain was initially classified as *Rhodopseudomonas adriatica* (Neutzling et al. 1984) and later as *Rhodobacter adriaticus* (Imhoff et al. 1984), but in 1994 was definitively transferred to the genus *Rhodovulum*. Cells of this species are rod-shaped, contain bacteriochlorophyll *a* and carotenoids of the spheroidene series, and are able to oxidize sulfide and thiosulfate. Ubiquinone 10 is the major quinone, the major fatty acid is $C_{18:1}$, membrane lipids contain sulfolipids, and the G+C content of the genomic DNA ranges from 64.9 to 66.7 mol%.

Other genera and species of the order *Rhodobacterales* including moderately halophilic microorganisms are *Antarctobacter heliothermus* (Labrenz et al. 1998), *Citreimonas salinaria* (Choi and Cho 2006a), *Hypomonas hirschiana* and *H. jannaschiana* (Weiner et al. 1985), *Jannaschia seosinensis* (Choi et al. 2006b), *Maribaculum marinum* (Lai et al. 2009b), *Maribius pelagius* and *M. salinus* (Choi et al. 2007), *Maricaulis parjimensis* and *M. virginensis* (Abraham et al. 2002), *Marivita cryptomonadis* and *M. litorea* (Hwang et al. 2009), *Methylarcula marina* and *M. terricola* (Doronina et al. 2000), *Oceanibulbus indolifex* (Wagner-Döbler et al. 2004), *Oceanicola marinus* (Lin et al. 2007), *Palleronia marisminoris* (Martínez-Checa et al. 2005c), *Paracoccus halophilus* (Liu et al. 2008), *P. homiensis* (Kim et al. 2006a) and *P. saliphilus* (Wang et al. 2009e), *Ponticoccus litoralis* (Hwang and Cho 2008), *Rhodothalassium salexigens* (Imhoff et al. 1998a), *Roseinatronobacter monicus* (Boldareva et al. 2007), *Roseisalinus antarcticus* (Labrenz et al. 2005), *Roseovarius pacificus* (Wang et al. 2009a) and *R. tolerans* (Labrenz et al. 1999), *Salinihabitans flavidus* (Yoon et al. 2009a), *Salipiger mucosus* (Martínez-Cánovas et al. 2004d), *Sediminimonas qiaohouensis* (Wang et al. 2009g), *Shimia marina* (Choi and Cho 2006a), *Sulfitobacter brevis* (Labrenz et al. 2000) and *S. litoralis* (Park et al. 2007), *Tropicibacter naphthalenivorans* (Harwati et al. 2009), *Woodsholea maritima* (Abraham et al. 2004), and *Yangia pacifica* (Dai et al. 2006).

The order *Rhizobiales* contains only two genera with validly published moderately halophilic species names: *Dichotomicrobium* (Hirsch and Hoffman 1989) and *Rhodobium* (Urdiain

et al. 2008). The members of the genus *Rhodobium*, *R. gokarnense* and *R. orientalis* (Urdiain et al. 2008), are halophilic purple nonsulfur phototrophic bacteria that contain internal photosynthetic membranes as lamellar stacks parallel to the cytoplasmatic membrane, bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. The major respiratory quinones are ubiquinone-10 and menaquinone-10, the main fatty acids are $C_{18:1}$ and $C_{18:0}$, and the polar lipid profile consists of the major compounds phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylcholine. The G+C content of their DNA ranges from 61.5 to 65.7 mol% (Hiraishi et al. 1995; Urdiain et al. 2008). The most closely related species to the genus *Rhodobium* are members of *Rhizobium* and *Sinorhizobium*. The genus *Dichotomicrobium*, including one species, *D. thermohalophilum*, was isolated from a solar lake in Sinai (Hirsch and Hoffman 1989).

Belonging to the family *Rhodospirillaceae* (Pfenning and Trüper 1971), in the order *Rhodospirillales*, only the genera *Fodinicurvata*, with the species *F. fenggangensis* and *F. sediminis* (Wang et al. 2009f), *Marispirillum*, containing the single species *M. indicum* (Lai et al. 2009a), and *Rhodovibrio*, including the species *R. salinarum* and *R. sodomensis* (Imhoff et al. 1998a), as well as the species *Roseospira mediosalina* (Imhoff et al. 1998a), are moderate halophiles. Species of the genus *Rhodovibrio* accumulate glycine betaine and ectoine as compatible solutes in response to the external salinity (Severin et al. 1992; Imhoff 1992) and are able to grow under microoxic to oxic conditions in the dark (Imhoff et al. 1998a).

Class *Gammaproteobacteria*

The class *Gammaproteobacteria* contains the largest number of moderately halophilic genera (a total of 51) and species. The family *Halomonadaceae* (Franzmann et al. 1988) in the order *Oceanospirillales* is the most important one in number of halophilic species (*Table 3.1.2*) and some of its representatives are among the most halophilic bacteria (Ventosa et al. 1998). It was proposed according to the results obtained with the 16S rRNA cataloging technique, to accommodate the moderately halophilic and marine bacteria of the genera *Halomonas* (Vreeland et al. 1980) and *Deleya* (Baumann et al. 1983). However, in 1996, Dobson and Franzmann (Dobson and Franzmann 1996) unified both genera into a single genus, *Halomonas*, and proposed the transfer of the species *Halovibrio variabilis* (Fendrich 1988) and *Paracoccus halodenitrificans* (Davis et al. 1969) to the genus *Halomonas*. At the time of writing, this genus contains 58 validly published halophilic species names, which turns it into the genus with the largest number of halophilic bacteria within the *Proteobacteria*. Furthermore, other genera that contain moderately halophilic species have been described and included into the family *Halomonadaceae*, which are as follows: *Aidingimonas* (Wang et al. 2009d), *Carnimonas* (Garriga et al. 1998), *Chromohalobacter* (Ventosa et al. 1989), *Cobetia* (Arahal et al. 2002a), *Kushneria* (Sánchez-Porro et al. 2009b), *Modicisalibacter* (Ben Ali Gam et al. 2007), and *Salinicola* (Anan'ina et al. 2007). Members of the family *Halomonadaceae* have been isolated from very different habitats, such as marine water, hypersaline and/or alkaline lakes, saline soils, solar saltern, hydrothermal environment, sewage, oilfield, sand, food, plants and mural paintings. Recently, three novel *Halomonas* species, *H. hamiltonii*, *H. johnsoniae*, and *H. stevensii*, have been isolated from patients' blood and from dialysis machines of a renal care center (Kim et al. 2010). The genus *Halomonas* groups species with very heterogeneous features, but all members are rod-shaped (except *H. halodenitrificans*), non-endospore forming, and chemoorganotrophic with a mainly respiratory type of metabolism where

Table 3.1.2

Validly published moderately halophilic genus and species names within the family *Halomonadaceae* (as of 31 December 2009). Basonyms/synonyms of microorganisms that have been transferred to other genera are not included. For genera/species whose descriptions have been emended, only the most recent reference is included

Genus	Species
Aidingimonas (Wang et al. 2009d)	*A. halophila*[b] (Wang et al. 2009d)
Carnimonas (Garriga et al. 1998)	*C. nigrificans*[b] (Garriga et al. 1998)
Chromohalobacter (Arahal et al. 2001a)	*C. beijerinckii* (Peçonek et al. 2006), *C. canadensis* (Arahal et al. 2001a), *C. israelensis* (Arahal et al. 2001a), *C. japonicus* (Sánchez-Porro et al. 2007), *C. marismortui*[b] (Ventosa et al. 1989), *C. nigrandesensis* (Prado et al. 2006), *C. salexigens* (Arahal et al. 2001b), *C. sarecensis* (Quillaguamán et al. 2004a)
Cobetia (Arahal et al. 2002a)	*C. marina*[b] (Arahal et al. 2002a)
Halomonas[a] (Dobson and Franzmann 1996)	*H. alimentaria* (Yoon et al. 2002c), *H. alkaliphila* (Romano et al. 2006a), *H. almeriensis* (Martínez-Checa et al. 2005b), *H. andesensis* (Guzmán et al. 2010), *H. anticariensis*(Martínez-Cánovas et al. 2004a), *H. aquamarina* (Dobson and Franzmann 1996), *H. arcis* (Xu et al. 2007c), *H. axialensis* (Kaye et al. 2004), *H. boliviensis* (Quillaguamán et al. 2004b), *H. campaniensis* (Romano et al. 2005a), *H. campisalis* (Mormile et al. 1999), *H. caseinilytica* (Wu et al. 2008b), *H. cerina* (González-Domenech et al. 2008b), *H. cupida* (Dobson and Franzmann 1996), *H. daqingensis* (Wu et al. 2008a), *H. denitrificans* (Kim et al. 2007), *H. desiderata* (Berendes et al. 1996), *H. elongata*[b] (Vreeland et al. 1980), *H. eurihalina* (Mellado et al. 1995), *H. fontilapidosi* (González-Domenech et al. 2009), *H. gomseomensis* (Kim et al. 2007), *H. gudaonensis* (Wang et al. 2007d), *H. halmophila* (Dobson et al. 1990), *H. halocynthiae* (Romanenko et al. 2002), *H. halodenitrificans* (Dobson and Franzmann 1996), *H. halodurans* (Hebert and Vreeland 1987), *H. halophila* (Dobson and Franzmann 1996), *H. hamiltonii* (Kim et al. 2010), *H. hydrothermalis* (Kaye et al. 2004), *H. ilicicola* (Arenas et al. 2009), *H. janggokensis* (Kim et al. 2007), *H. johnsoniae* (Kim et al. 2010), *H. kenyensis* (Boltyanskaya et al. 2007), *H. korlensis* (Li et al. 2008a), *H. koreensis* (Lim et al. 2004), *H. kribbensis* (Jeon et al. 2007), *H. lutea* (Wang et al. 2008c), *H. magadiensis* (Duckworth et al. 2000), *H. maura* (Bouchotroch et al. 2001), *H. meridiana* (James et al. 1990), *H. mongoliensis* (Boltyanskaya et al. 2007), *H. muralis* (Heyrman et al. 2002), *H. neptunia* (Kaye et al. 2004), *H. nitroreducens* (González-Domenech et al. 2008a), *H. organivorans* (García et al. 2004), *H. pacifica* (Dobson and Franzmann 1996), *H. pantelleriensis* (Romano et al. 1996), *H. sabkhae* (Kharroub et al. 2008a), *H. saccharevitans* (Xu et al. 2007c), *H. salifodinae* (Wang et al. 2008d), *H. salina* (Dobson and Franzmann 1996), *H. shengliensis* (Wang et al. 2007c), *H. subglaciescola* (Franzmann et al. 1987), *H. stevensii* (Kim et al. 2010), *H. subterranea* (Xu et al. 2007c), *H. sulfidaeris* (Kaye et al. 2004), *H. taeanensis* (Lee et al. 2005), *H. variabilis* (Dobson and Franzmann 1996), *H. ventosae* (Martínez-Cánovas et al. 2004c), *H. venusta* (Dobson and Franzmann 1996), *H. xinjiangensis* (Guan et al. 2010), *H. zhanjiangensis* (Chen et al. 2009f)

Table 3.1.2 (Continued)

Genus	Species
Kushneria (Sánchez-Porro et al. 2009b)	*K. aurantia*[b] (Sánchez-Porro et al. 2009b), *K. avicenniae* (Sánchez-Porro et al. 2009b), *K. indalinina* (Sánchez-Porro et al. 2009b), *K. marisflavi* (Sánchez-Porro et al. 2009b)
Modicisalibacter (Ben Ali Gam et al. 2007)	*M. tunisiensis*[b] (Ben Ali Gam et al. 2007)
Salinicola (Anan'ina et al. 2007)	*S. halophilus* (de la Haba et al. 2010b), *S. salaria* (de la Haba et al. 2010b), *S. socius*[b] (Anan'ina et al. 2007)

[a]Type genus of the family.
[b]Type species of the genus.

oxygen is the terminal electron acceptor, although some species of the genus have been shown to grow anaerobically with nitrate, nitrite, or fumarate as an electron acceptor and with glucose on solid media in sealed jars (Mata et al. 2002). Their main respiratory quinone is ubiquinone-9; the major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylethanolamine; the major fatty acids are $C_{16:1}\omega7c$, $C_{17:0}$ cyclo, $C_{16:0}$, $C_{18:1}\omega7c$, and $C_{19:0}$ cyclo (Franzmann and Tindall 1990). The G+C content ranges from 52.0 to 74.3 mol% (Martínez-Cánovas et al. 2004c). *Halomonas* species possess diverse biochemical functions, such as exopolysaccharide production by *H. alkaliphila*, *H. almeriensis*, *H. anticariensis*, *H. caseinilytica*, *H. cerina*, *H. daqingensis*, *H. eurihalina*, *H. fontilapidosi*, *H. halophila*, *H. maura*, *H. nitroreducens*, *H. sabkhae*, *H. salina*, and *H. ventosae*; polyhydroxybutyrate production by *H. almeriensis*, *H. anticariensis*, *H. alkaliphila*, *H. aquamarina*, *H. boliviensis*, *H. campaniensis*, *H. campisalis*, *H. caseinilytica*, *H. cerina*, *H. cupida*, *H. daqingensis*, *H. desiderata*, *H. elongata*, *H. eurihalina*, *H. fontilapidosi*, *H. halmophila*, *H. halodenitrificans*, *H. halophila*, *H. magadiensis*, *H. maura*, *H. meridiana*, *H. nitroreducens*, *H. pacifica*, *H. pantelleriensis*, *H. salina*, *H. subglaciescola*, *H. variabilis*, *H. ventosae*, *H. venusta*, and *H. zhanjiangensis*; degradation of aromatic compounds by *H. campisalis*, *H. halodurans*, *H. organivorans*, and *H. venusta* denitrification by *H. alimentaria*, *H. axialensis*, *H. campisalis*, *H. cerina*, *H. denitrificans*, *H. desiderata*, *H. elongata*, *H. fontilapidosi*, *H. gudaonensis*, *H. halodenitrificans*, *H. hydrothermalis*, *H. korlensis*, *H. maura*, *H. neptunia*, *H. nitroreducens*, *H. salina*, *H. shengliensis*, *H. sulfidaeris*, and *H. ventosae*; and H_2S production by *H. halophila*, *H. maura*, *H. salina*, and *H. sulfidaeris*. Members of the family *Halomonadaceae* accumulate organic compatible solutes (mainly ectoine, hydroxyectoine, and betaine) to provide osmotic equilibrium of the cytoplasm with the surrounding medium. Compatible solutes of the species *Chromohalobacter salexigens* are the most deeply studied in this family because of their potential biotechnology applications (Wohlfarth et al. 1990; Canovas et al. 1996, 1998a, b, 2000; García-Estepa et al. 2006; Vargas et al. 2006, 2008). Moreover, the genome of this microorganism has been completely sequenced. The phylogeny of the family *Halomonadaceae* has been deeply studied based on 23S and 16S rRNA sequence analysis (Arahal et al. 2002b; de la Haba et al. 2010a). The ICSP-Subcommittee on the taxonomy of the *Halomonadaceae* published recommended minimal standards for describing new taxa within this family and they can be very useful for the taxonomic characterization of new species of this bacterial group (Arahal et al. 2007).

The order *Oceanospirillales* also groups other genera that include moderately halophilic species: *Alcanivorax borkumensis* (Yakimov et al. 1998), *A. dieselolei* (Liu and Shao 2005), *A. hongdengensis* (Wu et al. 2009), and *A. venustensis* (Fernández-Martínez et al. 2003); *Hahella ganghwensis* (not validly published) (Baik et al. 2005); *Nitrincola lacisaponensis* (Dimitriu et al. 2005); *Oleispira antarctica* (Yakimov et al. 2003); and *Saccharospirillum impatiens* (Labrenz et al. 2003) and *S. salsuginis* (Chen et al. 2009a). Besides, two genera of extreme halophiles, *Halospina* (including the species *H. denitrificans* [Sorokin et al. 2006a]) and *Salicola* (which contains the species *S. marasensis* [Maturrano et al. 2006] and *S. salis* [Kharroub et al. 2006a]) also cluster within this order. The moderately halophilic genus *Halovibrio*, with the only species *H. denitrificans* (Sorokin et al. 2006a), is not validly published up to now, but Sorokin and Tindall (2006) request that the Judicial Commission recognize that species name.

The family *Alteromonadaceae* (Ivanova and Mikhailov 2001) in the order *Alteromonadales* is another of the important groups that include moderately halophilic *Proteobacteria*. The genus *Marinobacter* (Gauthier et al. 1992) is the largest one within this family with 17 rod-shaped moderately halophilic, motile, aerobic bacteria that utilize a variety of hydrocarbons as the sole source of carbon and energy. In terms of metabolism, they are either facultatively anaerobic or strictly aerobic heterotrophs. Often, they are capable of dissimilatory nitrate reduction and, for some species, hydrocarbon degradation and/or fermentation have also been demonstrated (Handley et al. 2009). The isolation sources are quite diverse: *M. algicola* (Green et al. 2006) was isolated from laboratory cultures of dinoflagellates; *M. goseongensis* (Roh et al. 2008), *M. litoralis* (Yoon et al. 2003b), and *M. pelagius* (Xu et al. 2008) from seawater; *M. guineae* (Montes et al. 2008), *M. maritimus* (Shivaji et al. 2005), *M. mobilis* (Huo et al. 2008), and *M. segnicrescens* (Guo et al. 2007) from marine sediment; *M. koreensis* (Kim et al. 2006b) from sea sand; *M. lacisalsi* (Aguilera et al. 2009) from a hypersaline lake; *M. lipolyticus* (Martín et al. 2003) from saline soil; *M. lutaoensis* (Shieh et al. 2003) from a coastal hot spring; *M. salicampi* (Yoon et al. 2007c) and *M. szutsaonensis* (Wang et al. 2009b) from a solar saltern; *M. salsuginis* (Antunes et al. 2007) from a brine–seawater interface; *M. santoriniensis* (Handley et al. 2009) from hydrothermal sediment; and *M. vinifirmus* (Liebgott et al. 2006) from wastewater. All described members of this genus have been found to contain $C_{16:0}\omega 9c$, $C_{16:1}\omega 9c$, and $C_{18:1}\omega 9c$ as the predominant fatty acids and to have DNA G+C contents ranging from 53.0 to 59.6 mol% (Liebgott et al. 2006).

There are two other genera that contain moderately halophilic species within the *Alteromonadaceae*: *Idiomarina*, including the species *I. abyssalis* (Ivanova et al. 2000), *I. baltica* (Brettar et al. 2003), *I. fontislapidosi* (Martínez-Cánovas et al. 2004b), *I. loihiensis* (Donachie et al. 2003), *I. ramblicola* (Martínez-Cánovas et al. 2004b), *I. seosinensis* (Choi and Cho 2005), and *I. zobellii* (Ivanova et al. 2000); and *Melitea*, with the only species *M. salexigens* (Urios et al. 2008a). Furthermore, the species *Aestuariibacter salexigens* (Yi et al. 2004), *Alteromonas genovensis* (Vandecandelaere et al. 2008), *A. hispanica* (Martínez-Checa et al. 2005a), *A. stellipolaris* (Van Trappen et al. 2004a), *Glaciescola chathamensis* (Matsuyama et al. 2006), *G. nitratireducens* (Baik et al. 2006), *G. polaris* (Van Trappen et al. 2004b), *Haliea salexigens* (Urios et al. 2008b), *Marinobacterium halophilum* (Chang et al. 2007), *Microbulbifer halophilus* (Tang et al. 2008b), *Pseudidiomarina homiensis* (Jean et al. 2009), *P. sediminum* (Hu and Li 2007), *Pseudoalteromonas peptidolytica* (Venkateswaran and Dohmoto 2000), *Psychromonas ingrahamii* (Auman et al. 2006), and *P. marina* (Kawasaki et al. 2002) showed moderated salt requirement for optimal growth.

The order *Chromatiales* contains, basically, anoxygenic phototrophic bacteria, known as phototrophic purple sulfur bacteria, able to perform photosynthesis under anoxic conditions

without oxygen production. In addition, the order contains nonphototrophic, purely chemotrophic relatives. As a member of this order, the family *Ectothiorhodospiraceae* (Imhoff 1984) groups species that are able to oxidize sulfide to elemental sulfur, which is deposited outside the cells and may be further oxidized to sulfate. The species *Alkalilimnicola ehrlichii* (Hoeft et al. 2007), *A. halodurans* (Yakimov et al. 2001), *Alkalispirillum mobile* (not validly published) (Rijkenberg et al. 2001), *Ectothiorhodosinus mongolicus* (Gorlenko et al. 2004), *Ectothiorhodospira haloalkaliphila* (Imhoff and Süling 1996), *E. mobilis* (Ventura et al. 2000), *E. variabilis* (Gorlenko et al. 2009), *Thioalkalivibrio halophilus* (Banciu et al. 2004), *T. jannaschii* (Sorokin et al. 2002), *Thiohalospira alkaliphila*, and *T. halophila* (Sorokin et al. 2008d), in the family *Ectothiorhodospiraceae*, are unique among the phototrophic purple bacteria in their requirement for saline to extremely saline and alkaline growth conditions. This family also contains other non-alkaliphilic moderately halophilic species (*Aquisalimonas asiatica* [Márquez et al. 2007], *Arhodomonas aquaeolei* [Adkins et al. 1993]) and extremely halophilic species (*Halorhodospira abdelmalekii*, *H. halochloris*, and *H. halophila* [Imhoff and Süling 1996]).

Other moderate halophiles within the order *Chromatiales* are *Halochromatium glycolicum*, *H. salexigens* (Imhoff et al. 1998b), *Halothiobacillus halophilus* (Kelly and Wood 2000), *Marichromatium bheemlicum* (Anil Kumar et al. 2007), *M. purpuratum* (Imhoff et al. 1998b), *Thiohalocapsa halophila* (Imhoff et al. 1998b), and the haloalkaliphilic species *Thioalkalibacter halophilus* (Banciu et al. 2008).

Besides, the class *Gammaproteobacteria* contains other diverse members of halophilic bacteria, including the moderately halophilic species *Gilvimarinus chinensis* (Du et al. 2009), *Methylohalomonas lacus* (Sorokin et al. 2007c), *Salinisphaera shabanensis* (Antunes et al. 2003), *Salinivibrio costicola* (Mellado et al. 1996), *S. proteolyticus* (Amoozegar et al. 2008b), *S. siamensis* (Chamroensaksri et al. 2009), *Thiohalomonas denitrificans*, *T. nitratireducens* (Sorokin et al. 2007b), *Thiohalophilus thiocyanatoxydans* (Sorokin et al. 2007a), and *Thiomicrospira halophila* (Sorokin et al. 2006b); and the extremely halophic species *Thiohalorhabdus denitrificans* (Sorokin et al. 2008a). The genus *Pseudomonas*, within the order *Pseudomonadales*, contains only one halophilic microorganism, *P. halophila* (type strain DSM 3050^T). Nevertheless, in 2006, Sorokin and Tindall (2006) demonstrated that the characteristics of strain DSM 3050^T corresponded to the original description of *Halomonas variabilis* (type strain DSM 3051^T) and those of DSM 3051^T to *Pseudomonas halophila* DSM 3050^T. Therefore, the Judicial Commission has to decide if DSM 3051^T should be recognized as the type strain of *Pseudomonas halophila*, which means that it should be properly placed within the genus *Halomonas*.

Class *Deltaproteobacteria*

Among the *Deltaproteobacteria* (*Deltabacteria*), all the moderately halophilic relatives are strictly anaerobic chemoorganotrophic, chemolithoheterotrophic, or chemolithoautotrophic sulfate-reducing bacteria with respiratory metabolism. The common electron acceptor is sulfate, which is reduced to sulfide. Most of them may also reduce sulfite, thiosulfate, or elemental sulfur to sulfide. The species growing optimally at moderately salinities are *Desulfocella halophila* (Brandt et al. 1999), *Desulfohalobium retbaense* (Ollivier et al. 1991), *D. utahense* (Jakobsen et al. 2006), *Desulfonatronospira delicata*, *D. thiodismutans* (Sorokin et al. 2008b), *Desulfovermiculus halophilus* (Belyakova et al. 2006), *Desulfovibrio bastinii* (Magot et al. 2004), *D. gabonensis* (Tardy-Jacquenod et al. 1996), *D. gracilis* (Magot et al. 2004), *D. halophilus*

(Caumette et al. 1991), *D. marinus* (Ben Dhia Thabet et al. 2007), *D. salexigens* (Postgate and Campbell 1966), *D. vietnamensis* (Dang et al. 1996), and *Desulfurivibrio alkaliphilus* (Sorokin et al. 2008c). Apart from the 16S rRNA gene sequence, alternative molecular markers, *dsrAB* genes (encoding the major subunits of dissimilatory sulfite reductase), have been used within the *Deltaproteobacteria* to base the phylogenetic relationships of those microorganisms (Jakobsen et al. 2006; Foti et al. 2007; Sorokin et al. 2008c).

Class *Epsilonproteobacteria*

The class *Epsilonproteobacteria* represents a taxonomically diverse but phylogenetically distinct group of Gram-negative bacteria that occupy a variety of ecological niches, with some species living in association with various animals (including humans) and others that are free living and found in environments such as seawater and anaerobic sludge. There are only three species of moderately halophilic bacteria in this class: *Arcobacter halophilus* (Donachie et al. 2005), isolated from a hypersaline lagoon, *Sulfurimonas autotrophica* (Inagaki et al. 2003), and *Sulfurovum lithotrophicum* (Inagaki et al. 2004), all three isolated from hydrothermal environments.

Phylum *Firmicutes*

The bacterial phylum *Firmicutes* contains a very heterogeneous group of microorganisms traditionally included in the three classes *Bacilli*, *Clostridia*, and *Mollicutes*. However, the class *Mollicutes* has recently been excluded from this phylum and has been classified in the phylum "*Tenericutes*" (Ludwig and Schleifer 2005; Ludwig et al. 2008). On the other hand, two new classes, "*Erysipelotrichi*" and *Thermolithobacteria*, have been included within the *Firmicutes* (Ludwig et al. 2008; Sokolova et al. 2007).

Members of the phylum *Firmicutes* are highly diverse in morphology and physiology, being able to inhabit a wide variety of environments, such as hypersaline habitats. Within this phylum, the classes *Bacilli* and *Clostridia* contain three and two families, respectively, with a large number of halophilic species isolated from different saline and hypersaline environments (*Table 3.1.3*).

Class *Bacilli*

The class *Bacilli* is composed of two orders, *Bacillales* and "*Lactobacillales*"; however, a number of paralogous groups are found within the *Bacilli*, some of which have been reclassified (Ludwig et al. 2008). Very recently, a proposal of minimal standards for describing new taxa of aerobic endospore-forming bacteria and closely related non-endospore-formers has been published. The intention of this proposal, endorsed by the Subcommittee on the taxonomy of the genus *Bacillus* and related organisms of the International Committee on Systematics of Prokaryotes, was to guide the construction of species descriptions on the basis of current knowledge and to encourage some practically useful conformity, and so comparability, in the characterization of novel taxa (Logan et al. 2009).

Family *Bacillaceae*

The family *Bacillaceae*, belonging to the order *Bacillales*, is the most prominent with 21 genera that include obligately halophilic species.

Table 3.1.3

Taxonomic position of moderately and extremely halophilic species belonging to the *Firmicutes* (as to 31 December 2009)

Taxonomic position	Species
Class *Bacilli*	
Order *Bacillales*	
Family *Bacillaceae*	
Genus *Bacillus*[a] (Cohn et al. 1872)	*Bacillus aidingensis* (Xue et al. 2008), *B. auriantiacus* (Borsodi et al. 2008), *B. chagannoriensis* (Carrasco et al. 2007a), *B. isabeliae* (Albuquerque et al. 2008), *B. oshimensis* (Yumoto et al. 2005), *B. persepolensis* (Amoozegar et al. 2009), *B. polygoni* (Aino et al. 2008), *B. qingdaonensis* (Wang et al. 2007a), *B. salarius* (Lim et al. 2006), *B. saliphilus* (Romano et al. 2005b)
Genus *Alkalibacillus* (Jeon et al. 2005b)	*Alkalibacillus haloalkaliphilus*[b] (Jeon et al. 2005b), *A. halophilus* (Tian et al. 2009), *A. filiformis* (Romano et al. 2005c), *A. salilacus* (Jeon et al. 2005b), *A. silvisoli* (Usami et al. 2007)
Genus *Aquisalibacillus* (Márquez et al. 2008)	*Aquisalibacillus elongatus*[b] (Márquez et al. 2008)
Genus *Filobacillus* (Schlesner et al. 2001)	*Filobacillus milosensis*[b] (Schlesner et al. 2001)
Genus *Gracilibacillus* (Wainø et al. 1999)	*Gracilibacillus orientalis* (Carrasco et al. 2006), *G. lacisalsi* (Jeon et al. 2008), *G. halophilus* (Chen et al. 2008b), *G. saliphilus* (Tang et al. 2009d)
Genus *Halalkalibacillus* (Echigo et al. 2007)	*Halalkalibacillus halophilus*[b] (Echigo et al. 2007)
Genus *Halolactibacillus* (Cao et al. 2008)	*Halolactibacillus alkaliphilus* (Cao et al. 2008)
Genus *Halobacillus* (Spring et al. 1996)	*Halobacillus halophilus*[b] (Spring et al. 1996), *H. aidingensis* (Liu et al. 2005), *H. alkaliphilus* (Romano et al. 2008), *H. dabanensis* (Liu et al. 2005), *H. karajensis* (Amoozegar et al. 2003), *H. kuroshimensis* (Hua et al. 2008b), *H. litoralis* (Spring et al. 1996), *H. locisalsi* (Yoon et al. 2002a), *H. mangrovi* (Soto-Ramírez et al. 2008), *H. profundi* (Hua et al. 2008b), *H. salinus* (Yoon et al. 2003a), *H. salsuginis* (Chen et al. 2009g), *H. sehoaensis* (Yoon et al. 2008), *H. trueperi* (Spring et al. 1996)
Genus *Lentibacillus* (Jeon et al. 2005a)	*Lentibacillus salicampi*[b] (Yoon et al. 2002b), *L. halophilus* (Tanasupawat et al. 2007), *L. juripiscarius* (Namwong et al. 2005), *L. kapialis* (Pakdeeto et al. 2007a), *L. halodurans* (Yuan et al. 2007), *L. lacisalsi* (Lim et al. 2005b), *L. salarius* (Jeon et al. 2005a), *L. salinarum* (Lee et al. 2008b), *L. salis* (Lee et al. 2008a), *L. halodurans* (Yuan et al. 2007)
Genus *Oceanobacillus* (Lu et al. 2001)	*Oceanobacillus picturae* (Heyrman et al. 2003; Lee et al. 2006), *O. oncorhynchi* subsp. *incaldenensis* (Romano et al. 2006b), *O. kapialis* (Namwong et al. 2009)
Genus *Ornithinibacillus* (Mayr et al. 2006)	*Ornithinibacillus californiensis* (Mayr et al. 2006)

Table 3.1.3 (Continued)

Taxonomic position	Species
Genus *Paraliobacillus* (Chen et al. 2009d)	*Paraliobacillus quinghaiensis* (Chen et al. 2009d)
Genus *Pontibacillus* (Lim et al. 2005a)	*Pontibacillus halophilus* (Chen et al. 2009h)
Genus *Salimicrobium* (Yoon et al. 2007b)	*Salimicrobium luteum* (Yoon et al. 2007b), *S. album*[b] (Yoon et al. 2007b), *S. halophilum* (Yoon et al. 2007b), *S. flavidum* (Yoon et al. 2007b; Yoon et al. 2009b)
Genus *Salinibacillus* (Ren and Zhou 2005b)	*Salinibacillus aidingensis*[b] (Ren and Zhou 2005b), *S. kushnerii* (Ren and Zhou 2005b)
Genus *Salirhabdus* (Albuquerque et al. 2007)	*Salirhabdus euzebyi*[b] (Albuquerque et al. 2007)
Genus *Salsuginibacillus* (Carrasco et al. 2007b)	*Salsuginibacillus kocurii*[b] (Carrasco et al. 2007b)
Genus *Sediminibacillus* (Carrasco et al. 2008)	*Sediminibacillus halophilus*[b] (Carrasco et al. 2008), *S. albus* (Wang et al. 2009c)
Genus *Tenuibacillus* (Ren and Zhou 2005a)	*Tenuibacillus multivorans*[b] (Ren and Zhou 2005a)
Genus *Thalassobacillus* (García et al. 2005)	*Thalassobacillus devorans*[b] (García et al. 2005), *T. cyri* (Sánchez-Porro et al. 2009a)
Genus *Virgibacillus* (Heyndrickx et al. 1998)	*Virgibacillus arcticus* (Niderberger et al. 2009), *V. carmonensis* (Heyrman et al. 2003), *V. chiguensis* (Wang et al. 2008a), *V. dokdonensis* (Yoon et al. 2005b), *V. halodenitrificans* (Denariaz et al. 1989; Yoon et al. 2004), *V. koreensis* (Lee et al. 2006), *V. kekensis* (Chen et al. 2008a), *V. necropolis* (Heyrman et al. 2003), *V. marismortui* (Arahal et al. 1999; Heyrman et al. 2003), *V. salarius* (Hua et al. 2008a), *V. salexigens* (Garabito et al. 1997; Heyrman et al. 2003), *V. salinus* (Carrasco et al. 2009), *V. sediminis* (Chen et al. 2009c)
Family *Planococcaceae*	
Genus *Jeotgalibacillus* (Yoon et al. 2001)	*Jeotgalibacillus alimentarius* (Yoon et al. 2001)
Family *Staphylococcaceae*	
Genus *Salinicoccus* (Ventosa et al. 1990)	*Salinicoccus roseus*[b] (Ventosa et al. 1990), *S. albus* (Chen et al. 2009b), *S. alkaliphilus* (Zhang et al. 2002), *S. halodurans* (Wang et al. 2008b), *S. hispanicus* (Ventosa et al. 1992), *S. iranensis* (Amoozegar et al. 2008a), *S. jeotgali* (Aslam et al. 2007), *S. kunmingensis* (Chen et al. 2007), *S. luteus* (Zhang et al. 2007), *S. salsiraiae* (Franca et al. 2006), *S. siamensis* (Pakdeeto et al. 2007b)

Table 3.1.3 (Continued)

Taxonomic position	Species
Class *Clostridia*	
Order *Halanaerobiales*	
Family *Halanaerobiaceae*	
Genus *Halanaerobium*[a] (Zeikus et al. 1983)	*Halanaerobium praevalens*[b] (Zeikus et al. 1983; Oren 2000), *H. acetoethylicum* (Rainey et al. 1995), *H. alcaliphilum* (Tsai et al. 1995), *H. congolense* (Ravot et al. 1997), *H. fermentans* (Kobayashi et al. 2000), *H. kushneri* (Bhupathiraju et al. 1999), *H. lacusrosei* (Cayol et al. 1995), *H. saccharolyticum* subsp. *saccharolyticum* (Rainey et al. 1995; Zhilina et al. 1992), *H. saccharolyticum* subsp *senegalensis* (Cayol et al. 1994a; Rainey et al. 1995), *H salsuginis* (Bhupathiraju et al. 1994)
Genus *Halocella* (Simankova et al. 1994; Oren 2000)	*Halocella cellulosilytica*[b] (Simankova et al. 1994; Oren 2000)
Genus *Halothermothrix* (Cayol et al. 1994b)	*Halothermotrix orenii*[b] (Cayol et al. 1994b)
Family *Halobacteroidaceae*	
Genus *Halobacteroides*[a] (Oren et al. 1984)	*Halobacteroides halobius*[b] (Oren et al. 1984), *H. elegans* (Zhilina et al. 1997)
Genus *Acetohalobium* (Zhilina and Zavarzin 1990)	*Acetohalobium arabaticum*[b] (Zhilina and Zavarzin 1990)
Genus *Halanaerobacter* (Mouné et al. 1999)	*Halanaerobacter chitinivorans*[b] (Liaw and Mah 1996), *H. lacunarum* (Zhilina et al. 1991; Rainey et al. 1995), *H. salinarius* (Mouné et al. 1999)
Genus *Halonatronum* (Zhilina et al. 2001)	*Halonatronum saccharophilum*[b] (Zhilina et al. 2001)
Genus *Natrionella* (Zhilina et al. 1996a)	*Natroniella acetigena*[b] (Zhilina et al. 1996a)
Genus *Orenia* (Rainey et al. 1995)	*Orenia marismortui*[b] (Rainey et al. 1995), O. *salinaria* (Mouné et al. 2000), *O. sivhasensis* (Zhilina et al. 2000)
Genus *Selenihalanaerobacter* (Switzer Blum et al. 2001)	*Selenihalanaerobacter shriftii*[b] (Switzer Blum et al. 2001)
Genus *Sporohalobacter* (Oren et al. 1987)	*Sporohalobacter lortetii*[b], *Sporohalobacter marismortui* (Oren et al. 1987)

[a]Type genus of the family.
[b]Type species of the genus.

Genus *Bacillus* Established by Cohn in 1872 (Cohn 1872), the genus *Bacillus* is a large taxonomic group containing species of many different physiological types that has undergone considerable taxonomic changes since its creation. Several early studies with halophilic endospore-forming organisms were related to *Bacillus* as a broad group; however, on the basis of molecular and chemical analyses the majority of the halophilic species originally assigned to the genus *Bacillus* have been subsequently reclassified as members of novel genera or transferred to other genera (Stackebrandt and Liesack 1993; Spring et al. 1996; Heyndrickx et al. 1998; Wainø et al. 1999; Schlesner et al. 2001; Yoon et al. 2001, 2002b, 2004, 2007a).

Currently, ten moderately halophilic species have been recognized within this genus: *B. aidingensis* (Xue et al. 2008), *B. auriantiacus* (Borsodi et al. 2008), *B. chagannoriensis* (Carrasco et al. 2007a), *B. isabeliae* (Albuquerque et al. 2008), *B. oshimensis* (Yumoto et al. 2005), *B. persepolensis* (Amoozegar et al. 2009), *B. polygoni* (Aino et al. 2008), *B. qingdaonensis* (Wang et al. 2007a), *B. salarius* (Lim et al. 2006), and *B. saliphilus* (Romano et al. 2005b). These halophilic organisms have been isolated from a variety of saline and/or alkaline environments and thus, some of them show not only a halophilic but also an alkaliphilic response. For example, *Bacillus polygoni*, *B. oshimensis*, and *B. aurantiacus* have been described as obligately alkaliphilic, whereas *B. chagannorensis* and *B. saliphilus* are considered as alkalitolerant microorganisms. Besides their taxonomic description, other aspects have been studied in a few of these species. Since the original isolation of the type strain of *Bacillus polygoni* (Aino et al. 2008) from an indigo ball by Ota et al. in 1975 (Ota et al. 1975), the NADH dehydrogenase (Xu et al. 1991; Kitazume et al. 2006), cyanide-insensitive non-proteinaceous respiratory material (Higashibata et al. 1998), flagella rotatory system (Hirota and Imae 1983), and polyamines (Hamasaki et al. 1993) of this strain have been studied. On the other hand, some physiological studies carried out in *B. oshimensis* have reported that this species may require NaCl for pH homeostasis for adaptation in an alkaline environment or for energy production through the respiratory chain (Tokuda and Unemoto 1981, 1984) or ATPase (Ueno et al. 2000). Besides, it has been reported that this haloalkaliphilic bacterium has a relatively low content of respiratory cytochromes (Yumoto et al. 1997) and is able to produce several hydrolytic enzymes (Yumoto et al. 2005). *B. saliphilus*, the only haloalkaliphilic non-sporulating coccus included up to now in the genus *Bacillus*, accumulates under growth conditions glycine betaine, hydroxyectoine, ectoine, and glutamate as compatible solutes (Romano et al. 2005b).

Genus *Alkalibacillus* Another member of the family *Bacillaceae* that includes moderately halophilic species is the genus *Alkalibacillus*. This genus was established by Jeon et al. in 2005 and since then includes five species, all of them with a haloalkaliphilic response: *A. filiformis* (Romano et al. 2005c), *A. haloalkaliphilus* (Jeon et al. 2005b), *A. halophilus* (Tian et al. 2007), *A. salilacus* (Jeon et al. 2005b), and *A. silvisoli* (Usami et al. 2007). The studies carried out with *Alkalibacillus* species have focused mainly on the taxonomic characterization and descriptions of the species, and very little effort has been devoted about their physiology or the role that they may play in the habitats where they were isolated. Some studies of intracellular ion concentrations were carried out in *Alkalibacillus* [*Bacillus*] *haloalkalophilus*, in which apparent intracellular cation concentrations as high as those present in the growth medium were reported by Weisser and Trüper (1985); however, this species was later known to produce ectoine as the main osmotic solute (Oren 2002). To the best of our knowledge, no other studies have been reported in the other *Alkalibacillus* species.

Genus *Halobacillus* Initially, the genus *Halobacillus* (Spring et al. 1996) was created to accommodate Gram-positive, spore-forming, moderately halophilic rod-shaped or spherical to oval cells with a cell wall peptidoglycan based on L-Orn-D-Asp; however, in the last few years, three species, *H. campisalis*, *H. seohaensis*, and *H. salsuginis*, have been found to contain *meso*-diaminopimelic acid instead of L-ornithine as the amino acid in their cell wall peptidoglycan (Yoon et al. 2007a, 2008; Chen et al. 2009g). Currently, this genus comprises 17 species with validly published names. With the exception of *H. campisalis*, *H. faecis*, and *H. yeojeoni*, which are halotolerant or extremely halotolerant species, the rest of *Halobacillus* species have a moderately halophilic response and thus, this genus can be considered as the most prominent

Firmicutes genus including halophilic microorganisms (Table 3.1.3). Most physiological studies performed in this genus have been focused in the type species, *Halobacillus halophilus*. Several approaches have demonstrated the importance of chloride not only for growth of this bacterium but also for the motility and flagelar synthesis, germination of endospores, regulation of a variety of proteins, and glycine betaine transport (Dohrmann and Müller 1999; Roeβler et al. 2000; Roeβler and Müller 1998, 2001, 2002). The salt concentration in the external environments play an important role in the compatible solutes accumulated for *Halobacillus halophilus*; thus, whereas it is able to produce mainly ectoine at high salt concentrations, glutamate and glutamine are predominantly accumulated at moderate salinities (Saum and Müller 2007, 2008). On the other hand, Köcher et al. (2009) have found that *Halobacillus halophilus* accumulates carotenoids structurally related to staphyloxanthins. Very recently, these authors have performed a study about the structure, function, and organization of the genes involved in the biosynthesis of these carotenoids and have identified a carotenogenic gene cluster in which all genes necessary for the synthesis of staphyloxanthins were organized in two operons. These studies are very important in order to obtain a better understanding about the salt-dependent carotenoid synthesis and pathway regulation in this halophilic species (Köcher et al. 2009).

Genus *Lentibacillus* The genus *Lentibacillus* is formed by Gram-variable, endospore-forming rods that contain a cell wall peptidoglycan with *meso*-diaminopimelic acid, MK-7 as the predominant isoprenoid quinone, and values of DNA G+C content between 42 and 49 mol % (Yoon et al. 2002b; Lee et al. 2008a). Currently this genus includes nine moderately halophilic species, five isolated from soil sediment of hypersaline habitats: *L. salicampi* (Yoon et al. 2002b), *L. halodurans* (Yuan et al. 2007), *L. lacisalsi* (Lim et al. 2005b), *L. salarius* (Jeon et al. 2005a), *L. salinarum* (Lee et al. 2008a), and *L. salis* (Lee et al. 2008a), and three isolated from fermented fish sauces: *L. halophilus* (Tanasupawat et al. 2006), *L. juripiscarius* (Namwong et al. 2005), and *L. kapialis* (Pakdeeto et al. 2007a).

Genus *Virgibacillus* This genus was first proposed by Heyndrickx et al. (1998) to accommodate *Bacillus pantothenticus*. Members of the genus *Virgibacillus* are halotolerant or halophilic motile bacteria, catalase positive, with cell walls containing peptidoglycan of the *meso*-diaminopimelic acid type, anteiso-$C_{15:0}$ as the major cellular fatty acid, and DNA G+C contents ranging from 30.7 to 42.8 mol% (Carrasco et al. 2009). At present, the genus *Virgibacillus* includes 13 moderately halophilic species out of a total of 17 recognized. The species *V. kekensis* (Chen et al. 2008a), *V. marismortui* (Arahal et al. 1999; Heyrman et al. 2003), *V. salarius* (Hua et al. 2008a), *V. salexigens* (Garabito et al. 1997; Heyrman et al. 2003), *V. salinus* (Carrasco et al. 2009), and *V. sediminis* (Chen et al. 2009c) were described on the basis of strains isolated from different saline or hypersaline lakes. On the other hand, *V. chiguensis* and *V. halodenitrificans* were isolated from solar salterns (Wang et al. 2008a; Denariaz et al. 1989; Yoon et al. 2004), *V. carmonensis* and *V. necropolis*, from paintings (Heyrman et al. 2003), *V. arcticus*, from a permafrost core (Niderberger et al. 2009), *V. dokdonensis*, from seawater (Yoon et al. 2005b), and *V. koreensis*, from a salt field (Lee et al. 2006).

Other Genera of *Bacillaceae* Other *Bacillaceae* genera less prominent in halophilic species are *Gracilibacillus* (Wainø et al. 1999), with four moderately halophilic species isolated from saline samples located in China: *G. orientalis* (Carrasco et al. 2006), *G. lacisalsi* (Jeon et al. 2008), *G. halophilus* (Chen et al. 2008b), and *G. saliphilus* (Tang et al. 2009b); and *Oceanobacillus*

(Wainø et al. 1999; Lu et al. 2001) that contains three moderately halophilic species, *O. picturae* isolated from a painting (Heyrman et al. 2003; Lee et al. 2006), *O. oncorhynchi* subsp. *incaldenensis* from an algal mat (Romano et al. 2006b), and *O. kapialis* from fermented shrimp paste (Namwong et al. 2009).

Within the family *Bacillaceae*, there are also many genera that only contain one or two moderately halophilic species and the majority of them have been described on the basis of a single strain. They are the following: *Aquisalibacillus*, with the species *A. elongatus* (Márquez et al. 2008), *Filobacillus* (*F. milosensis*) (Schlesner et al. 2001), *Halalkalibacillus* (*H. halophilus*) (Echigo et al. 2007), *Halolactibacillus* (*H. alkaliphilus*) (Cao et al. 2008), *Ornithinibacillus* (*O. californiensis*) (Mayr et al. 2006), *Paraliobacillus* (*P. quinghaiensis*) (Chen et al. 2009d), *Piscibacillus* (*P. salipiscarius*) (Tanasupawat et al. 2007), *Pontibacillus* (*P. halophilus*) (Lim et al. 2005a; Chen et al. 2009h), *Salinibacillus* (*S. aidingensis* and *S. kushnerii*) (Ren and Zhou 2005b), *Salirhabdus*, (*S. euzebyi*) (Albuquerque et al. 2007), *Salsuginibacillus* (*S. kocurii*) (Carrasco et al. 2007b), *Sediminibacillus* (*S. halophilus* and *S. albus*) (Carrasco et al. 2008; Wang et al. 2009c), *Tenuibacillus* (*T. multivorans*) (Ren and Zhou 2005a), and *Thalassobacillus* (*T. devorans* and *T. cyri*) (García et al. 2005; Sánchez-Porro et al. 2009a).

On the other hand, some genera of the family *Bacillaceae* include species with heterogeneous physiological characteristics. It is the case of *Salimicrobium*, a genus that was created in 2007 by (Yoon et al. 2007b) to describe the species *Salimicrobium luteum* and reclassifying *Marinococcus albus* and *Bacillus halophilus* as *Salimicrobium album* and *Salimicrobium halophilum*, respectively. Currently, this genus includes four species, the three cited before and *Salimicrobium flavidum* (Yoon et al. 2007a, 2009b). These four species are facultatively anaerobic, which grow optimally at 10–15% NaCl and have a DNA G+C content in the range of 44.9–51.5 mol%. Whereas *S. album* is a Gram-positive, non-spore-forming coccus, and *S. halophilum* a Gram-positive spore-forming rod, *S. luteum* and *S. flavidum* have been described as Gram-variable staining, non-spore-forming species with coccoid, ovoid, or rod-shaped morphology.

Family *Planococcaceae*

This family is a monophyletic unit that contains the genera *Planococcus*, *Filibacter*, *Kurthia*, *Planomicrobium*, and *Sporosarcina* as well as three genera transfered from the *Bacillaceae* (*Jeotgalibacillus*, *Marinibacilllus*, and *Ureibacillus*) and *Caryophanon* (Ludwig et al. 2008). The genus *Jeotgalibacillus* includes up to now only a validly described species name *Jeotgalibacillus alimentarius* (Yoon et al. 2001). This species was based on a moderately halophilic, round-endospore-forming bacterium isolated from jeotgal, a traditional Korean fermented seafood. This organism has a cell wall containing a directly cross-linked peptidoglycan with L-lysine as the diagnostic diamino acid, MK-7 and MK-8 as the predominant menaquinones, iso-$C_{15:0}$ as the major fatty acid, and a DNA G+C content of 44 mol%. Phylogenetically, it was related to the phylogenetic clade comprising members of Bacillus rRNA group 2.

Family *Staphylococcaceae*

Another important family belonging to the class *Bacilli*, order *Bacillales,* is the family *Staphylococcaceae*. This family includes five genera (*Staphylococcus*, *Jeotgalicoccus*, *Macrococcus*, *Salinicoccus*, and *Gemella*) but only the genus *Salinicoccus* contains halophilic microorganisms. This genus was first proposed by Ventosa et al. (1990) based on a single species, *Salinicoccus roseus*. *Marinococcus hispanicus* was later transferred to the genus *Salinicoccus* as *Salinicoccus hispanicus* (Ventosa et al. 1992). To date, the genus *Salinicoccus* comprises 11 species with

validly published names: *Salinicoccus roseus* and *S. hispanicus* isolated from solar saltern (Ventosa et al. 1990, 1992), *S. alkaliphilus* from a soda lake (Zhang et al. 2002), *S. salsiraiae* from salted skate (Franca et al. 2006), *S. jeotgali* from jeotgal (Aslam et al. 2006), *S. luteus* from desert soil (Zhang et al. 2007), *S. siamensis* from fermented shrimp paste (Pakdeeto et al. 2007b), *S. iranensis* from textile industry wastewater (Amoozegar et al. 2008a), *S. halodurans* from saline soil (Wang et al. 2008b), and *S. albus* (Chen et al. 2009b) and *S. kunmingensis* (Chen et al. 2007) from salt mines. Members of this genus are moderately halophilic, aerobic Gram-positive cocci, which are chemotaxonomically characterized by having menaquinone-6 (MK-6) as the predominant isoprenoid quinone, a cell wall peptidoglycan type based on L-Lys–Gly_5, and a DNA G+C content of 46–51.2 mol% (Ventosa et al. 1992).

Class *Clostridia*

Within the class *Clostridia*, the order *Halanaerobiales* contains two families, *Halanaerobiaceae* and *Halobacteroidaceae*, both of them including halophilic species.

Family *Halanaerobiaceae*

This family is currently comprised of three genera, *Halanaerobium*, *Halocella*, and *Halothermothrix*. They are anaerobic, halophilic, fermentative bacteria that have attracted considerable attention by their functional diversity (Rainey et al. 1995). *Halanaerobium* is the genus that contains the largest number of described species in the family. It was created by Zeikus et al. (1983) to accommodate *H. praevalens*, the type species of the genus, isolated from surface sediments of the Great Salt Lake, Utah (Zeikus et al. 1983; Oren 2000). Since then, other nine obligately anaerobic, halophilic species isolated from different hypersaline habitats have been described within the genus *Halanaerobium*: *H. acetethylicum* (Rainey et al. 1995); *H. alcaliphilum* (Tsai et al. 1995); *H. congolense* (Ravot et al. 1997); *H. fermentans* (Kobayashi et al. 2000); *H. kushneri* (Bhupathiraju et al. 1999); *H. lacusrosei* (Cayol et al. 1995); *H. saccharolyticum* with two subspecies, *H. saccharolyticum* subsp. *saccharolyticum* (Rainey et al. 1995; Zhilina et al. 1992) and *H. saccharolyticum* subsp. *senegalensis* (Cayol et al. 1994a; Rainey et al. 1995); and *H. salsuginis* (Bhupathiraju et al. 1994). All these species are Gram-negative, non-spore-forming, chemoorganotrophic, moderately or extremely halophilic rods that are able to ferment a large number of compounds (Zeikus et al. 1983; Kobayashi et al. 2000).

The other two genera of this family, *Halocella* and *Halothermotrix*, include the species *Halocella cellulosilytica* and *Halothermotrix orenii*, respectively (Simankova et al. 1993; Cayol et al. 1994b). The descriptions of these two genera were simultaneous and based on phylogenetic, phenotypic, and chemotaxonomic features. *Halocella cellulosilytica* is an obligate anaerobic moderately halophilic bacterium that dissolves cellulose (Simankova et al. 1994). On the other hand, *Halothermotrix orenii*, isolated from the sediment of a Tunesian salted lake, was the first truly thermophilic halophilic anaerobic bacterium described, being able to grow optimally at 60°C in media containing 10% NaCl (Cayol et al. 1994b).

Family *Halobacteroidaceae*

Eight genera are currently classified within this family (Ludwig et al. 2008). The type genus of the family is *Halobacteroides*, with two species of halophilic anaerobic bacteria, *H. halobius* and *H. elegans*. *Halobacteroides halobius* is the type species of the genus and was found by dilution

counts to be abundant in the anaerobic sediments of the Dead Sea; it is a flexible moderately halophilic rod that produces ethanol as an endproduct of glucose fermentation (Oren et al. 1984b). On the other hand, the species *H. elegans* was described on the basis of 16S rRNA sequences analysis of three strains previously assigned by phenotypic evidence to *Halobacteroides halobius* (Zhilina et al. 1997).

The genus *Halanaerobacter* was firstly proposed by Liaw and Mah (1992) with the species *Halanaerobacter chitinivorans*. This species was able to grow at 50°C in a wide range of NaCl concentration (2–30%) and it could be differentiating from other anaerobic halophilic species for its chitinolytic activity (Liaw and Mah 1992, 1996). Other species of this genus is *H. lacunarum*, described by Zhilina et al. (1991) as the first fermentative halophilic anaerobe capable of growth under extremely halophilic conditions (30% NaCl). In 1999, Mouné and coworkers (Mouné et al. 1999) described the species *H. salinarius* as an extremely halophilic fermentative species capable of reducing glycine betaine to trimethylamine with hydrogen or serine as electron donors.

The other two genera belonging to the family *Halobacteroidaceae* are *Orenia* and *Sporohalobacter*. The genus *Orenia* was created by Rainey et al. (1995) and was based on the species *Orenia marismortui*, a Gram-negative, spore-forming, moderately halophilic rod that was isolated from the Dead Sea and produces H_2, CO_2, formate, acetate, butyrate, and ethanol as end products of glucose fermentation (Oren et al. 1987; Rainey et al. 1995). In 2000, the other two species names validly published were recognized in this genus: *O. salinaria* and *O. sivashensis*. They are moderately halophilic and anaerobic bacteria isolated from saltern and hypersaline lagoons of Lake Sivash, (the Crimea), respectively (Mouné et al. 2000; Zhilina et al. 2000).

The genus *Sporohalobacter* was created for the Gram-negative halophilic spore-forming *Clostridium lortetii* that was transferred to this genus as *Sporohalobacter lortetii* (Oren et al. 1987). Described by Oren (1983), this organism was the first obligately halophilic anaerobe. When transferring *Clostridium lortetii* to the genus *Sporohalobacter*, Oren et al. (1987) described an additional species, *Sporohalobacter marismortui*. The most significant difference between these two species was the ability of *S. marismortui* to utilize carbohydrates as the sole carbon and energy source and the production of ethanol as a fermentation endproduct.

Finally, the other four genera of this family include a single species. They are the genus *Acetohalobium*, which includes the extremely halophilic homoacetogen species *Acetohalobium arabaticum* (Zhilina and Zavarzin 1990); *Halonatronum* with the species *H. saccharophilum*, an alkaliphilic and moderately halophilic chemoorganotrophic anaerobic bacterium isolated from Lake Magadi, in Kenya (Zhilina et al. 2001); *Natroniella* with the species *N. acetigena*, an extremely haloalkaliphilic, chemoorganotrophic, homoacetogenic bacterium that was also isolated from Lake Magadi (Zhilina et al. 1996a); and *Selenihalanaerobacter* with the species *S. shriftii*, a halophilic anaerobe from Dead Sea sediments that respires selenate (Switzer Blum et al. 2001).

Phylum *Actinobacteria*

The class *Actinobacteria* constitutes one of the main phyla within the *Bacteria* (Ludwig and Klenk 2001) on the basis of its branching position in 16S rRNA phylogenetic trees. Besides the phylogeny of the 16S rRNA gene, some conserved indels in 23S rRNA and protein sequences (e.g., cytochrome-*c* oxidase I, CTP synthetase, and glutamyl-tRNA synthetase) support the distinctness of members of the class from all other bacteria (Gao and Gupta 2005).

To date, 219 genera (in 48 families) have been accommodated in the class *Actinobacteria,* this class is subdivided in four subclasses: *Acidimicrobidae*, *Actinobacteridae*, *Coriobacteridae*, and *Rubrobacteridae*, but only the *Actinobacteridae* includes halophilic bacteria. The subclass *Actinobacteridae* (Stackebrandt et al. 1997; Zhi et al. 2009) has the pattern of 16S rRNA signatures consisting of nucleotides at positions 242: 284 (U–G), 291: 309 (C–G), 316: 337 (U–G), 819 (G), 952: 1229 (C–G), and 1115: 1185 (U–G) (Zhi et al. 2009).

Of the three orders *Actinomycetales*, *Bifidobacteriales*, and *Nitriliruptorales* of the subclass *Actinobacteridae*, only the *Actinomycetales* comprises halophilic bacteria. Nowadays, the order *Actinomycetales* (Buchanan 1917; Stackebrandt et al. 1997; Zhi et al. 2009) is composed of thirteen suborders. The species of this order have a pattern of 16S rRNA signatures consisting of nucleotides at positions 688: 699 (G–C), 701 (C), 823: 877 (G–C), and 1060: 1197 (U–A) (Zhi et al. 2009). Halophilic bacteria are included in the following suborders of the order *Actinomycetales*: *Actinopolysporineae*, *Corynebacterineae*, *Glycomycineae, Micrococcineae*, *Pseudonocardineae*, and *Streptoporangineae.*

Suborder *Actinopolysporineae*

The suborder *Actinopolysporineae* includes a single family, *Actinopolyporaceae,* with a single genus, *Actinopolyspora*, which includes some halophilic species. The pattern of 16S rRNA signatures of species of this suborder consists of nucleotides at positions 127: 234 (A–U), 242: 284 (C–G), 657: 749 (G–C), 672: 734 (C–G), 828 (A), 829: 857 (G–C), 833: 853 (U–G), 840: 846 (C–G), 986: 1219 (U–A), 1100 (U), 1183 (C), 1117: 1183 (G–C), and 1309: 1328 (G–U) (Zhi et al. 2009).

The genus *Actinopolyspora* was described by Gochnauer and collaborators in 1975, and nowadays comprises three species, all of them halophilic actinomycetes. The first species was isolated as a contaminant of a culture medium containing 25% NaCl, and was classified as *Actinopolyspora halophila* (Gochnauer et al. 1975). It requires high NaCl concentrations for growth and can grow in saturated NaCl so it was classified as an extremely halophilic actinomycete. Years later, during the course of a screening for new antibiotics, a moderately halophilic actinomycete that produced nucleoside antibiotics was isolated from salty soil obtained from the Death Valley, California (USA). This isolate grew in media containing a wide range of NaCl concentrations (5–25% w/v). On the basis of its morphological and chemical properties, it was assigned to the genus *Actinopolyspora* and was proposed as a new specie, *Actinospolyspora mortivallis*, on the basis of physiological and biochemical characteristics and levels of DNA–DNA relatedness (Yoshida et al. 1991). The last species that has been included in this genus is *Actinopolyspora iraqiensis* isolated from a saline soil in Iraq. This microorganism is able to grow optimally between 10% and 15% NaCl, and so is classified as a moderately halophilic actinomycete species (Ruan et al. 1994).

Suborder *Corynebacterineae*

The suborder *Corynebacterineae* (Stackebrandt et al. 1997; Zhi et al. 2009) comprises several families, but only *Corynebacteriaceae* (Lehman and Neumann 1896; Stackebrandt et al. 1997; Zhi et al. 2009) with the single genus *Corynebacterium* includes a moderately halophilic actinomycete. The suborder has the pattern of 16S rRNA signatures consisting of nucleotides at positions 127: 234 (G–Y), 564 (C), 672: 734 (U–G), 833: 853 (U–G), 952: 1229 (U–A), and 986: 1219 (U–A) (Zhi et al. 2009).

The genus *Corynebacterium* was created by Lehman and Neumann (1896) and represents a large group of Gram-positive, asporogenous, rod-shaped bacteria with a high DNA G+C content (Liebl 1992). In recent years, many novel *Corynebacterium* species have been described, the majority of which were isolated from clinical samples or animals. *Corynebacterium halotolerans* was isolated from a saline soil in West China and is the only halophilic species of the genus, showing that optimal growth occurs at concentrations of NaCl or KCl of 10% (Chen et al. 2004).

Suborder *Glycomycineae*

The suborder *Glycomycineae* (Stackebrandt et al. 1997; Zhi et al. 2009) includes the single family *Glycomycetae* (Stackebrandt et al. 1997; Labeda and Kroppenstedt 2005; Zhi et al. 2009) with three genera, of which only the recently described genus *Haloglycomyces* comprises halophilic bacteria. The pattern of 16S rRNA signatures of species of this order consists of nucleotides at positions 657: 749 (G–U), 672: 734 (C–G), 681: 709 (A–U), 831: 855 (U–G), 832: 854 (G–U), 833: 853 (G–C), 840: 846 (C–U), 952: 1229 (C–G), 1064: 1192 (G–G), and 1117 : 1183 (A–U) (Zhi et al. 2009).

To date, *Haloglycomyces albus* (Guan et al. 2009) is the only moderately halophilic species in the family *Glycomycetae*. This species was isolated during an investigation of actinomycete diversity in hypersaline habitats, from a soil sample collected from Xinjiang Province, Northwest China. It grows in the presence of 3–18% NaCl with an optimal growth at 8–12% NaCl.

Suborder *Micrococcineae*

The pattern of 16S rRNA signatures of the suborder *Micrococcineae* (Stackebrandt et al. 1997; Zhi et al. 2009) consists of nucleotides at positions 127:234 (A–U), 598:640 (U–G), 657:749 (U–A), 953:1228 (G–C), 986:1219 (A–U), 987:1218 (A–U), and 1362 (A) (Zhi et al. 2009). Among all the families included in the suborder *Micrococcineae*, four of them comprise halophilic species: *Bogoriellaceae*, *Micrococcaceae*, *Ruaniaceae*, and *Promicromonosporaceae*.

Genus *Georgenia*

The genus *Georgenia*, belonging to the family *Bogoriellaceae* (Stackebrandt and Schumann 2000; Zhi et al. 2009; Hamada et al. 2009), was first proposed by Altenburger et al. (2002), with *Georgenia muralis* as the type species and was later on emended by Li et al. (2007). It currently comprises three species but only *Georgenia halophila* (Tang et al. 2010b) is a moderately halophilic actinomycete, showing optimal growth at 5–10% NaCl. This species was isolated from the saline Lake Quijiaojing in Xinjiang Province, Northwest China.

Genus *Nesterenkonia*

The family *Micrococcaceae* includes several genera but only the genus *Nesterenkonia* includes halophilic species. The genus *Nesterenkonia* was first proposed by Stackebrandt et al. (1995) with the reclassification of *Micrococcus halobius* (Onishi and Kamekura 1972) as *Nesterenkonia halobia*. The description of the genus was later emended by Collins et al. (2002) and Li et al. (2005). At the time of writing, the genus comprises eleven recognized species, six of them are halophilic.

Nesterenkonia halobia is a moderately halophilic Gram-positive nonmotile coccus that was isolated from unrefined solar salt of unknown origin obtained from Noda, Japan (Onishi and Kamekura 1972). Its optimum growth occurs on media containing 4–8% NaCl. *Nesterenkonia lacusekhoensis* (Collins et al. 2002) presents an optimal growth at 6–8% NaCl and was isolated from the hypersaline, meromictic, and heliothermal Lake Ekho in the ice-free Vestfold Hills (East Antarctica). *Nesterenkonia halotolerans*, *Nesterenkonia xinjiangensis*, and *Nesterenkonia halophila* are halophilic actinomycetes isolated by Li et al. (2004a, 2008b) from saline soils from Xinjiang Province, China. They are moderately halophilic, with optimal growth at 10% salt. Other moderately halophilic species belonging to this genus is *Nesterenkonia lutea* (Li et al. 2005) that was also isolated from a saline soil from China. This species is able to grow at 0–20% NaCl with an optimum at 5–10%.

Genus *Isoptericola*

Of all the genera included in the family *Promicromonosporaceae*, only the genus *Isoptericola* includes a halophilic species. The genus *Isoptericola* (Stackebrandt et al. 2004) comprises four species of which, only *Isoptericola halotolerans* is a moderately halophilic actinomycete. This species has optimal growth at 10% NaCl and was isolated from a saline soil from Qinghai Province, Northwest China (Zhang et al. 2005).

Genus *Haloactinobacterium*

The genus *Haloactinobacterium* has been recently described by Tang et al. (2010c). This genus is included in the newly described family *Ruaniaceae. Haloactinobacterium album* is the sole halophilic actinomycete representative in this family. This species is the type species of the genus and was isolated from a salt lake in Xinjiang Province, Northwest China. Its optimal growth occurs at 37°C, pH 7–8, and 7–10% NaCl.

Suborder *Pseudonocardianeae*

The suborder *Pseudonocardianeae* (Stackebrandt et al. 1997; Zhi et al. 2009) only includes two families of which one of them, *Pseudonocardiaceae*, includes halophilic species of actinomycetes.

The family *Pseudonocardiaceae* (Embley et al. 1988; Stackebrandt et al. 1997; Zhi et al. 2009), which has a pattern of 16S rRNA signatures consisting of nucleotides at positions 211 (G), 480 (U), and 142: 221 (C–G) (Zhi et al. 2009), includes a few genera of which those with halophilic species are the following: *Amycolaptosis, Praseurella*, *Saccharomonospora*, and *Saccharopolyspora.*

Genus *Amycolatopsis*

The genus *Amycolatopsis* was first established by Lechevalier et al. (1986) and currently comprises 41 species with validly published names, most of which have been described in the recent years. These species are non-halophilic actinomycetes, and have been isolated from geographically diverse soils, clinical material, vegetable matter, the wall of a hypogean cave, and ocean sediment. *Amycolatopsis halophila* (Tang et al. 2010a) is an aerobic, Gram-positive, moderately halophilic filamentous actinomycete with an optimal growth at 37°C, pH 7, and

5% NaCl. It was isolated from a salt lake in Xinjiang Province, Northwest China. It constitutes the only halophilic representative of this genus.

Genus *Prauserella*

The genus *Prauserella* (Kim and Goodfellow 1999) was proposed to reallocate the previously misclassified species *Amycolaptosis rugosa* (Lechevalier et al. 1986). Nowadays this genus comprises nine species and with the exception of the two recently described species *Prauserella muralis* (Schäfer et al. 2010) and *Prauserella marina* (Wang et al. 2010), all of them are moderately halophilic. These species are *Prauserella rugosa* (Kim and Goodfellow 1999), *Prauserella halophila* and *Prauserella alba* (Li et al. 2003c), *Prauserella salsuginis, Prauserella flava, Prauserella aidingensis*, and *Prauserella sedimina* (Li et al. 2009).

Genus *Saccharomonospora*

Nonomura and Ohara (1971) described the genus *Saccharomonospora* within the family *Pseudonocardiaceae* for organisms that produce predominantly single spores, and occasionally spores in pairs and short chains, on aerial hyphae. To date, eight species with validly published names are included in the genus. Of them, only three are moderately halophilic actinomycetes: *Saccharomonospora halophila* (Al-Zarban et al. 2002), *Saccharomonospora paurometabolica* (Li et al. 2003b), and *Saccharomonospora saliphila* (Syed et al. 2008). *Saccharomonospora halophila* was the first halophilic member of this genus and was isolated from marsh soil in Kuwait. *Saccharomonospora paurometabolica* was isolated from a soil from the Xinjiang Province, in the west of China, and the last one, *Saccharomonospora saliphila*, has been isolated from a muddy soil from Gulbarga, Karnataka, India. All three species present an optimal growth at 10% NaCl.

Genus *Saccharopolyspora*

The genus *Saccharopolyspora* was first described by Lacey et al. (1975) and it currently comprises 20 species with validly published names. Only two of them are halophilic actinomycetes: *Saccharopolyspora halophila* (Tang et al. 2009a) and *Saccharopolyspora qijiaojingensis* (Tang et al. 2009c). Both species were isolated from a saline lake in Xinjiang, Northwest China and present an optimal growth at 10–15% NaCl.

Suborder *Streptosporangineae*

The suborder *Streptosporangineae* (Stackebrandt et al. 1997; Zhi et al. 2009) includes three families but only the family *Nocardiopsaceae* includes halophilic bacteria. The pattern of 16S rRNA signatures of species of this suborder consists of nucleotides at positions 127: 234 (A–U), 829: 857 (G–C), 830: 856 (G–C), 953: 1228 (U–A), 950: 1231 (U–A), 955: 1225 (C–G), 986: 1219 (A–U), and 987: 1218 (A–U) (Zhi et al. 2009).

The family *Nocardiopsaceae* that was first described by Rainey et al. (1996) and emended several times (Stackebrandt et al. 1997; Zhang et al. 1998; Zhi et al. 2009), currently comprises five genera: *Nocardiopsis* (Meyer 1976), *Thermobifida* (Zhang et al. 1998), *Streptomonospora* (Cui et al. 2001), *Halactinospora* (Tang et al. 2008a), and *Marinactinospora* (Tian et al. 2009). With the exception of the genus *Thermobifida*, most species of the family *Nocardiopsaceae* have been isolated from saline soils, and some of them are strictly halophilic filamentous actinomycetes. At present, the genus *Nocardiopsis* contains 38 species with validly published names,

12 of them have been classified as moderately halophilic species (Al-Tai and Ruan 1994; Chun et al. 2000; Al-Zarban et al. 2002; Li et al. 2003a, b; Chen et al. 2009). The genus *Streptomonospora* contains five species (Cui et al. 2001; Li et al. 2003d; Cai et al. 2008, 2009) all of them are halophilic filamentous actinomycetes and the genus *Haloactinospora* only comprises a halophilic species (Tang et al. 2008a).

Genus *Haloactinospora*

During a study on isolation methods for halophilic filamentous actinomycetes from hypersaline soils, more than 500 non-*Nocardiopsis* strains were isolated from Xinjiang Province, Northwest China. One of these strains was subjected to a polyphasic study and was classified as a new genus and species, *Haloactinospora alba*. It is able to grow between 9% and 21% NaCl showing optimal growth at 15% NaCl, and so is classified as a moderately halophilic actinomycete (Tang et al. 2008a).

Genus *Nocardiopsis*

The genus *Nocardiopsis* was described by Meyer (1976) and, at present, it comprises 38 species with validly published names. Members of this genus have been reported to predominate in saline and/or alkaline soils. Only a few species have been described as halophilic actinomycetes: *Nocardiopsis halophila* (Al-Tai and Ruan 1994), *Nocardiopsis kunsanensis* (Chun et al. 2000), *Nocardiopsis halotolerans* (Al-Zarban et al. 2002), *Nocardiopsis xinjiangensis* (Li et al. 2003a), *Nocardiopsis salina* (Li et al. 2004b), *Nocardiopsis gilva* (Li et al. 2006), *Nocardiopsis rosea* (Li et al. 2006), *Nocardiopsis rhodophaea* (Li et al. 2006), *Nocardiopsis chromatogenes* (Li et al. 2006), *Nocardiopsis baichengensis* (Li et al. 2006), and *Nocardiopsis litoralis* (Chen et al. 2009e).

Genus *Streptomonospora*

The genus S*treptomonospora* was first established by Cui et al. (2001) with the type species *Streptomonospora salina*, and it represents a group of strictly halophilic filamentous actinomycetes. Subsequently, Li et al. (2003c) described another species, *Streptomonospora alba*, and emended the description of the genus *Streptomonospora*. During a biodiversity and taxonomic study on halophilic filamentous actinomycetes, the species *Streptomonospora halophila* has been recently described (Cai et al. 2008). The most recent species included in this genus are *Streptomonospora amylolytica* and *Streptomonospora flavalba,* both isolated from a salt lake in the northwest of China (Cai et al. 2009).

Phylum *Spirochaetes*

Four moderately halophilic species of spirochaetes have been described, all of them belonging to the genus *Spirochaeta*. This genus contains a group of free-living, saccharolytic, helix-shaped bacteria quite common on sediments, freshwater or marine muds, and microbial mats. The moderately halophilic species are *Spirochaeta halophila*, isolated from the hypersaline Solar Lake (Greenberg and Canale-Parola 1976) and the haloalkaliphilic species *Spirochaeta africana* and *Spirochaeta alkalica*, both isolated from brine sediments in the Lake Magadi, Kenya, and *Spirochaeta asiatica* isolated from sulfide-saturated mud sediments of alkaline Lake Khatyn in Tuva, Central Asia (Zhilina et al. 1996b). *S. asiatica* is strictly anaerobic while the other three species are facultative anaerobes. They are chemoorganotrophs with facultative type of

metabolism. The main products of glucose fermentation are acetate, ethanol, lactate, and H_2 (except for *S. asiatica*) (Greenberg and Canale-Parola 1976; Zhilina et al. 1996b).

Phylum *Bacteroidetes*

The phylum *Bacteroidetes* is composed of three classes "*Bacteroidia*," *Flavobacteria*, and *Shingobacteria* (Garrity and Holt 2001). All of them include some halophilic bacterial species.

Class "Bacteroidia"

The class "*Bacteroidia*" is composed of a single order, *Bacteroidales*, which includes several families, but only the family *Marinilabiaceae* comprises a halophilic bacterium: *Anaerophaga thermohalophila* (Denger et al. 2002). This organism is strictly anaerobic, chemoorganotrophic, with fermentative metabolism, non-phostosynthetic, not able to use external electron acceptors, and unable to form fruiting bodies. It is a moderately thermohalophilic bacterium isolated in a project to provide suitable organisms for microbially enhanced oil recovery. It requires media with enhanced CO_2/bicarbonate content and salt concentrations of at least 2% (w/v), showing optimal growth at 2–6% NaCl and 50°C (Denger et al. 2002).

Class *Flavobacteria*

The class *Flavobacteria* includes the order *Flavobacteriales,* with three families, but only the family *Flavobacteriaceae* comprises a halophilic representative.

The family *Flavobacteriaceae* is composed of a large number of genera and although some of them include halotolerant or marine species, such as the genera *Dokdonia* (Yoon et al. 2005a), *Maribacter* (Nedashkovskaya et al. 2004), *Polaribacter* (Gosink et al. 1998), or *Salimicrobium* (Lim et al. 2008), to cite a few examples, only the genera *Psychroflexus* (Bowman et al. 1998) and *Gramella* (Nedashkovskaya et al. 2005) include moderately halophilic species.

Psychroflexus tropicus is a moderately halophilic bacterium, with optimal growth at 7.5–10% NaCl and 30°C, isolated from water collected at a depth of 0.3 m in the hypersaline Lake Laysan on Laysan Island in Hawaii (Donachie et al. 2004).

Gramella echinicola is a halophilic bacterium isolated from the sea urchin *Strongylocentrotus interedius,* which is able to grow at 1–15% NaCl (Nedashkovskaya et al. 2005).

Class *Sphingobacteria*

The class *Sphingobacteria* includes the single order *Sphingobacteriales* composed of several families. Although some of these families included marine or slightly halophilic species, only the family *Rhodothermaceae* has halophilic representatives. This family is composed of three genera: *Rhodothermus*, *Salinibacter* (Antón et al. 2002), and *Salisaeta* (Vaisman and Oren 2009), the last two contain halophilic species.

Genus *Salinibacter*

The classification of *Salinibacter ruber* (Antón et al. 2002) as a novel species within the phylum *Bacteroidetes* extended the short list of extremely halophilic members of *Bacteria. Salinibacter*

ruber was isolated from a saltern crystallizer pond in Mallorca, Balearic Island, Spain. It is a rod-shaped or curved bacterium, Gram-negative, aerobic, heterotrophic, oxidase and catalase positive, and extremely halophilic, requiring at least 15% salt for growth and showing optimal growth at 15–30% salt. The G+C content of the type strain is 66.5 mol%.

Genus *Salisaeta*

A new genus, *Salisaeta*, with the single species *Salisaeta longa* has been recently described by Vaisman and Oren (2009) to include a red, halophilic member of the *Bacteroidetes*. This species grows optimally at 10% NaCl plus 5% $MgCl_2$ $6H_2O$ and tolerates 20% $MgCl_2$ $6H_2O$. It was isolated from an experimental mesocosm filled with a mixture of water from the Dead Sea and the Red Sea, Israel. It is a long rod-shaped bacterium, Gram-negative, aerobic, heterotrophic, and oxidase and catalase positive.

Phylum *Thermotogae*

The phylum *Thermotogae* is composed of the class *Thermotogae*, which has only one order, *Thermotogales*. Members of this order are anaerobic, rod-shaped microorganisms whose cells are surrounded by sheath-like structures. They are recognized as common inhabitants of deep, hot oil reservoirs located in marine or continental ecosystems (Magot et al. 2000; Olliver and Cayol 2005). Their DNA G+C content is from 34.0 to 36.1 mol%. The order *Thermotogales* is composed by a single family *Thermotogaceae*, which has several genera. Some of these genera comprise marine or slightly halophilic species such as *Kosmotoga* (Dipippo et al. 2009), *Marinitoga* (Wery et al. 2001), *Thermosipho* (Huber et al. 1989), or *Thermotoga* (Huber et al. 1986), but only the genus *Petrotoga* (Davey et al. 1993) has a moderately halophlic species, *Petrotoga halophila*. This species was isolated from an oil well in Congo, West Africa, and grows in the presence of 5–9% NaCl, with an optimum at 4–6% NaCl (Miranda-Tello et al. 2007).

Phylum *Cyanobacteria*

Cyanobacteria, also known as blue-green algae, blue-green bacteria, or *Cyanophyta*, have been found in various aquatic habitats including marine, freshwater, hot spring, hypersaline environments, and ice (Castenholz 2001), and play an ecological role as major contributors to photosynthesis in these environments (Whitton and Potts 2000). Some species can fix atmospheric nitrogen in a free-living state or in a symbiosis with plants (Becking 1978). The taxonomic classification of *Cyanobacteria* is confusing due to dual nomenclature systems: bacteriological and botanical (Oren 2004). Both codes are often used without coincidences with different prescriptions. The result is that only few cyanobacterial taxa can be considered as "validly described" (Oren 2004). Under the Bacteriological Code (1990 Revision) (Lapage et al. 1992), the names of only six genera (*Halospirulina, Planktothricoides, Prochlorococcus, Prochloron, Prochlorothrix, and Rubidibacter*) are validly published at present (Oren 2004).

Within these genera, the species *Rubidibacter lacunae* and *Prochlorococcus marinus* can be considered as marine *Cyanobacteria* isolated from seawater of Chuuk lagoon, Micronesia (Choi et al. 2008) and the Sargasso Sea (Chisholm et al. 1992), respectively, and *Halospirulina tapeticola* (Nübel et al. 2000) could be considered as slightly halophilic. This species belongs

to the order *Oscillatoriales* and was isolated from a microbial mat in a hypersaline evaporation pond of a saltern at the Pacific coast of Baja, California, Mexico. It is a euryhaline cyanobacterium with trichomes coiled into a tight, closed helix, able to grow at salinities between 3% and 20%, but not on freshwater (Nübel et al. 2000).

Besides, some examples of species considered as "not validly described species names" that can be classified as moderately halophilic species are: *Aphanothece halophytica* (Brock 1976), *Dactylococcopsis salina* (Walsby et al. 1983), *Halomicronema excentricum* (Abed et al. 2002), as well as species of the genera *Halothece* (Garcia-Pichel et al. 1998; Margheri et al. 2008; Oren 2009) or *Cyanothece* (Komarek 1976).

Phylum *Tenericutes*

This phylum includes the class *Mollicutes* that previously was classified within the phylum *Firmicutes* (Schleifer 2009). Only a moderately halophilic species has recently been described associated to the *Mollicutes*. Antunes et al. (2008a) described the features of a strictly anaerobic bacterium isolated from the hypersaline anoxic brine–sediment interface of the Shaban Deep, Red Sea. Phylogenetically, it represents a novel and distinct lineage within the domain *Bacteria*. However, it was equidistant to taxa belonging to the phyla *Firmicutes* and *Tenericutes*. Since the species shares several morphological characteristics with the wall-less species of the class *Mollicutes*, including the absence of a cell wall, typical colonies with a "fried-egg"-like appearance or the motility by cellular contraction of some "tentacle-like" protrusions. Its distant phylogenetic position might argue for the proposal of a high-level taxon to accommodate this bacterium, perhaps at the phylum level. However, since currently it is represented by only one isolate, it was placed on the new order *Haloplasmatales*, family *Haloplasmataceae*, as a new species designated *Haloplasma contractile* (Antunes et al. 2008a).

This bacterium is Gram-negative, non-spore forming, with pleomorphic cells, usually consisting of a central coccoid body with one or two "tentacle-like" protrusions. It is strictly anaerobic, denitrifying, and fermentative. It grows in media with neutral pH and at temperatures between 10°C and 44°C (optimum at 30–37°C). It is a moderately halophilic species; the NaCl range for growth is 1.5–18, showing optimal growth at 8% NaCl (Antunes et al. 2008a).

Acknowledgments

We thank Aharon Oren for the critical reading of the manuscript. We thank the financial support from the Spanish Ministerio de Educación y Ciencia (project BIO2009-10138) The National Science Foundation (DEB-0919290) and from the Junta de Andalucía (project P06-CVI-01829).

Cross-References

3.2 Diversity of Halophiles
3.4 Ecology of Halophiles

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3.2 Diversity of Halophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.2,

Introduction

Hypersaline environments are inhabited by a great variety of microorganisms, and these are often present in extremely high community densities. Thanks to the fact that some of the most prevalent types are colored by carotenoid and other pigments, no microscope is needed to see halophilic microorganisms in environments such as saltern crystallizer brines and other salt lakes with saturating or near-saturating salt concentrations. Thus, the red-purple color of halophilic microbes can be observed throughout the northern half of Great Salt Lake, Utah.

The world of the halophilic microorganisms is highly diverse. We find representatives of the three domains of life, Archaea, Bacteria, and Eucarya that are adapted to salt concentrations up to saturation. We know aerobic as well as anaerobic halophiles, heterotrophic, phototrophic, and chemoautotrophic types, able to transform a wide variety of substrates (Oren 2002a, b, 2006a, 2007, 2008). Among the halophiles we also encounter "polyextremophilic" types, adapted to grow at a combination of extremes, for example, of salt and alkaline conditions (a commonly found combination), salt and low pH (relatively rare), or salt and high temperatures; we even know some anaerobic halophilic thermoalkaliphilic bacteria.

This chapter explores the diverse world of the halophilic microorganisms, here defined as able to grow at salt concentrations above 100 g/l. Following overviews of the phylogenetic and metabolic diversity within the halophilic microbial world and a short description of the mechanisms used by these organisms to cope with salt stress, the question will be addressed why certain functions common in non-halophilic microorganisms are found up to salt saturation while other types of metabolism apparently cease to function above relatively low salt concentrations.

Phylogenetic Diversity in the Halophile World

Microorganisms able to grow at salt concentrations up to NaCl saturation (>300 g/l salt) are found all over the small subunit rRNA-based tree of life. Thus, three red-pigmented organisms are commonly found in salt-saturated saltern crystallizer ponds worldwide: the unicellular alga *Dunaliella salina* (Chlorophyceae, Eucarya), the square archaeon *Haloquadratum walsbyi* (Halobacteriales, Euryarchaeota), and *Salinibacter ruber* (Bacteroidetes, Bacteria) (Oren 2006a, 2008).

Among the extreme halophiles, the aerobic Archaea of the family Halobacteriaceae have been most studied. This family, the only family described in the order Halobacteriales, is a phylogenetically coherent group of salt-requiring prokaryotes (Oren 2006b). Most of the over a hundred species described grow optimally above 150 g/l salt, and no growth is obtained in its absence. Most species even lyse at salt concentrations below 100 g/l. The recent isolation of species that can survive or even grow at salt concentrations below 100 g/l (Fukushima et al. 2007; Savage et al. 2007, 2008) does not change our general concepts on the ecology and the physiology of the group. The family includes the above-mentioned square archaeon *Haloquadratum walsbyi*, which for long resisted all attempts toward its cultivation, but is now available for study (Burns et al. 2007).

No halophiles are yet known within the Crenarchaeota phylum of the Archaea, and all cultured types are classified within the Euryarchaeota. These also include truly halophilic methanogens within the family Methanosarcinaceae, *Methanohalobium evestigatum* being the most salt tolerant and salt requiring (Zhilina and Zavarzin 1987). The recent finding of

16S rRNA sequences related to the Nanoarchaeota in salt ponds of Inner Mongolia and South Africa (Casanueva et al. 2008) shows that our picture of the true diversity of halophiles among the Archaea is still incomplete.

Within the domain Bacteria we find halophiles in a number of phyla, notably in the Cyanobacteria, the Gammaproteobacteria, the Firmicutes, and the Bacteroidetes. Among the cyanobacteria, we find a variety of both unicellular and filamentous types that grow at high salt, albeit not at the highest concentrations found in saturated brines (Oren 2000). The best known group of halophilic or highly halotolerant types within the Bacteria is probably the family Halomonadaceae (Gammaproteobacteria), in which thus far about 70 species of versatile aerobic heterotrophs with a broad salinity range were described (Arahal and Ventosa 2006). In the aerobic Firmicutes and a number of other phyla, halophilic types are often closely related to organisms with little salt tolerance and requirement. Within the Firmicutes we also find the order Halanaerobiales with two families, the Halanaerobiaceae and the Halobacteroidaceae, consisting entirely of anaerobic halophilic, generally fermentative bacteria (Oren 2006c). A relatively recent addition to the list of true halophiles is the red aerobic *S. ruber* (Bacteroidetes) (Antón et al. 2000, 2002), now known as a major contributor to the biota of salt lakes and salterns close to salt saturation, dominated by Archaea of the Halobacteriaceae. Sometimes the exploration of new environments can lead to the recognition of an entirely new group of halophiles, as shown by the discovery of the anaerobic wall-less, pleomorphic, contractile bacterium *Haloplasma contractile* in the brine-filled Saban Deep in the Red Sea. Phylogenetically this organism falls between the Firmicutes and the Mollicutes. It grows between 15 and 180 g/l salt, and lives by fermentation or denitrification. It is thus far the only known representative of the order Haloplasmatales, family Haloplasmataceae (Antunes et al. 2009). How widespread this type is and whether it may belong to a larger group of yet-to-be characterized organisms is still unknown.

Eukarotic life at high salt concentrations is possible as well, even of some macroorganisms, as shown by the brine shrimp *Artemia*, found at salt concentrations up to 150–200 g/l and sometimes even higher. The best studied halophilic eukaryotes are undoubtedly the representatives of the green algal genus *Dunaliella*, some of which are orange rather than green as they accumulate massive amounts of β-carotene. *Dunaliella* is of great ecological importance in salt lakes as the sole or main primary producer at high salt concentrations, and thus it supports the life of the heterotrophic communities.

Only relatively recently was the existence and the importance of heterotrophic halophilic eukaryotic microorganisms recognized. Many types of flagellate and ciliate protozoa were discovered in salt lakes up to NaCl saturation (Park et al. 2003; Cho 2005; Hauer and Rogerson 2005), and culture-independent studies show that many more types are waiting to be discovered. Based on 18S rRNA studies, over 40 phylotypes of protists were found in a sample from the lower halocline in the hypersaline anoxic L'Atalante basin in the Eastern Mediterranean (depth 3,501 m, salinity 365‰) (Alexander et al. 2009). Fungi, especially black yeasts and other melanized types, including species of *Hortaea*, *Trimmatostroma*, *Candida*, *Pichia*, *Yarrowia*, *Debaryomyces*, and others, are an integral part of hypersaline ecosystems worldwide, including salterns and salt lakes such as the Dead Sea (Butinar et al. 2005a, b).

A survey of biodiversity in hypersaline environments should also include the viruses. Phages attacking halophilic Archaea and Bacteria are known, and such viruses abound in those (few) hypersaline systems where their distribution was studied. As in most freshwater and seawater environments, the number of virus particles may exceed the numbers of prokaryotes by one to two orders of magnitude. Different types of viruses were observed,

including head-and-tail, spherical, and spindle-shaped viruses of halophilic Archaea, lytic types as well as types propagated in a carrier state, and double-stranded DNA viruses as well as single-stranded DNA viruses with a membrane envelope (Porter et al. 2007; Jäälinoja et al. 2008; Pietilä et al. 2009).

Metabolic Diversity of Halophiles

With respect to the processes performed to obtain energy and carbon for growth, the halophile world is as diverse as expected in view of the large phylogenetic diversity of the organisms present at high salt concentrations. Still, some processes known from low-salt environments apparently do not function at salt concentrations above 100–150 g/l. Examples are autotrophic nitrification, methanogenesis from hydrogen and carbon dioxide or from acetate, and aerobic oxidation of methane.

The sections below present an overview of the types of microbial metabolism known to occur in halophilic microorganisms. Finally, the possible reasons for the apparent absence of certain types of microbial metabolism at high salt concentrations will be discussed.

Oxygenic and Anoxygenic Phototrophs

Oxygenic photosynthesis occurs up to salt saturation, but the diversity of phototrophs growing at high salt concentrations is rather small. Most phytoplankton groups common in seawater have not adjusted to life in concentrated brines. Diatoms are relatively well adapted, and some species are found up to 100–150 g/l salt, but only two major groups of phototrophs function at the highest salt concentrations: cyanobacteria and green unicellular algae of the genus *Dunaliella*. Cyanobacteria are commonly found both in the plankton and in shallow benthic microbial mats in salt lakes and saltern evaporation ponds. These include both unicellular (*Aphanothece halophytica* and related forms) and filamentous forms (e.g., *Microcoleus chthonoplastes*, *Halospirulina tapeticola*, *Phormidium* spp. and others) (Oren 2000). Heterocystous nitrogen-fixing species were never reported above 70 g/l salt. When the salt concentration increases above 200–250 g/l, also the cyanobacteria disappear, and the only types of oxygenic phototrophs remaining are species of the unicellular eukaryotic genus *Dunaliella*.

Anoxygenic photosynthesis with sulfide as electron donor likewise can function up to high salt concentrations. In hypersaline microbial mats dominated by cyanobacteria in the upper aerobic layers, purple layers of anoxygenic phototrophs are commonly found. The phototrophic sulfur bacteria include types that store elemental sulfur intracellularly (*Halochromatium*, *Thiohalocapsa*) and forms that excrete sulfur (*Ectothiorhodospira*, *Halorhodospira*). Some *Halorhodospira* species not only tolerate salt concentrations approaching saturation, but very high pH values as well (Ollivier et al. 1994). Halophilic photoheterotrophic purple bacteria were also described such as *Rhodovibrio salinarum*, *R. sodomense*, and *Rhodothalassium salexigens*, which thrive up to 200–240 g/l salt.

A completely different type of anoxygenic phototrophic life that functions at the highest salinities is based on the use of light energy absorbed by retinal-based pigments: bacteriorhodopsin and related pigments such as xanthorhodopsin. Bacteriorhodopsin, the major retinal pigment of *Halobacterium* and some related halophilic Archaea, can drive anaerobic growth of *Halobacterium salinarum* in the light (Hartmann et al. 1980). Organic substrates are used as

carbon source, and photoautotrophy does not occur in this group. Use of light as supplementary energy source is also possible in the phylogenetically unrelated but physiologically very similar *Salinibacter*. Its proton pumping retinal protein, xanthorhodopsin, uses the membrane carotenoid salinixanthin as light-harvesting pigment (Lanyi 2005; Mongodin et al. 2005). To what extent this interesting organism is also capable of photoheterotrophic growth remains to be ascertained.

Aerobic Heterotrophs

Most halophilic and halotolerant microorganisms described are aerobic heterotrophs, and we find these in the three domains of life. In environments with salt concentrations up to 200–250 g/l, representatives of the Bacteria are responsible for most of the heterotrophic activity, and some groups are very versatile both with respect to the range of salt concentrations tolerated and to the number of substrates used for growth (Ventosa et al. 1998; Arahal and Ventosa 2006). The list of organic substrates supporting growth at high salt concentrations is smaller than that known for freshwater environments, but it includes a number of hydrocarbons and aromatic compounds.

At the highest salinities, groups like the Halomonadaceae no longer function and aerobic degradation of organic compounds is taken over by two groups of prokaryotes: the Halobacteriaceae (Archaea) and *Salinibacter* (Bacteria). The halophilic Archaea grow up to saturating salt concentrations, but the range of substrates oxidized is rather limited: mainly amino acids and simple sugars. Hydrocarbons and aromatic compounds are poorly used, if at all (Oren 2006b). Until recently is was believed that the Halobacteriaceae are the only aerobic heterotrophs functioning above 200–250 g/l salt, but the discovery of *Salinibacter* as a major component of the biota of saltern crystallizer ponds (Antón et al. 2000, 2002) has changed our views. Growth experiments and analysis of the *Salinibacter* genome (Mongodin et al. 2005) showed that this organism is not very versatile with respect to its growth substrates.

Anaerobic Respiration

Many modes of respiration with electron acceptors other than molecular oxygen are operative at high salt concentrations. Electron acceptors used include not only nitrate and sulfate, but also more unusual compounds such as arsenate and selenate, and their reduction is of ecological importance in some hypersaline environments.

Nitrate is used as electron acceptor for respiration in a variety of halophilic and halotolerant prokaryotes. Many moderately halophilic members of the Halomonadaceae reduce nitrate to nitrite, and denitrification of nitrate to gaseous nitrogen is found in some halophilic Bacteria as well (Ventosa et al. 1998). Some halophilic Archaea (e.g., *Haloarcula marismortui*, *Haloarcula vallismortis*, *Haloferax mediterranei*) can grow anaerobically when nitrate is present as the electron acceptor, forming gaseous nitrogen and/or nitrous oxide (Mancinelli and Hochstein 1986). Some species also grow anaerobically by reduction of dimethylsulfoxide, trimethyl-*N*-oxide or fumarate.

Seawater is rich in sulfate, and therefore it is not surprising that in anaerobic marine and hypersaline, seawater-derived environments, dissimilatory sulfate reduction is the terminal process in the degradation of organic compounds (Oren 1988). However, compared to

freshwater and marine systems, certain types of sulfate reducing bacteria appear to be missing in high-salt environments. All halophilic and highly halotolerant dissimilatory sulfate reducers described thus far belong to the type of "incomplete oxidizers," which oxidize substrates such as lactate, pyruvate, and ethanol and excrete acetate rather than oxidize the substrates all the way to carbon dioxide. *Desulfohalobium retbaense*, isolated from Lake Retba in Senegal, with its growth optimum at 100 g/l salt while tolerating up to 240 g/l, is probably the most halophilic strain known thus far (Ollivier et al. 1991). More information about the species of halophilic sulfate reducers and their properties was given by Ollivier et al. (1994) and Oren (1999, 2002a). Sulfate reduction can proceed also under highly alkaline and hypersaline conditions. The haloalkaliphilic species *Desulfonatronospira thiodismutans* and *D. delicata*, isolated from the Kulunda Steppe, Altai, Russia, oxidize lactate and ethanol between 1 and 4 M total salt with an optimum at pH 9.5–10. They also grow chemoautotrophically by dismutation of sulfite or thiosulfate (Sorokin et al. 2008a). There are indications that sulfate reduction is possible at higher salt concentrations as well. For example, stable isotope data suggested that dissimilatory sulfate reduction may occur in the bottom sediments of the Dead Sea (>300 g/l total salts) (Nissenbaum and Kaplan 1976), but no information is yet available about the process and the organisms performing it. "Complete oxidizers," which oxidize substrates such as acetate to carbon dioxide, appear to be absent in hypersaline environments. The most salt-tolerant isolate known is *Desulfobacter halotolerans*, isolated from sediment of Great Salt Lake, Utah. It grows optimally at 10–20 g/l salt only, and slow growth is possible up to 130 g/l (Brandt and Ingvorsen 1997).

Arsenate and selenate are less common electron acceptors for microbial growth, but these can also support anaerobic growth of halophilic/haloalkaliphilic bacteria. The salt-saturated and highly alkaline Searles Lake, California, whose waters contain about 3.9 mM arsenic, supports a biogeochemical arsenic cycle in which As(III) is oxidized to As(V) by chemoauotrophs (see below) and As(V) is reduced to As(III) in anaerobic respiration processes (Oremland et al. 2005). An isolate described as *Haloarsenatibacter silvermanii* grows optimally at 350 g/l salt and pH 9.4, chemoautotrophically by oxidizing sulfide with arsenate as electron acceptor, or heterotrophically using a range of organic electron donors. It also uses Fe(III) or elemental sulfur as electron acceptors for respiration (Switzer Blum et al. 2009). An example of a halophilic selenate reducer, which converts Se(V) to a mixture of Se(III) and elemental selenium is *Selenihalanaerobacter shriftii*, isolated from the Dead Sea – an environment not known for a high content of selenium compounds (Switzer Blum et al. 2001).

Methanogens and Homoacetogens

In low-salinity environments most of the methane generated is derived from two reactions: reduction of carbon dioxide by hydrogen and splitting of acetate to yield methane and carbon dioxide. Neither of these reactions was shown to occur at high salt concentrations. Methanogenesis from hydrogen and carbon dioxide was demonstrated in the alkaline Mono Lake, California at 88 g/l salts (Oremland and King 1989), and the most halotolerant methanogen shown to grow on these substrates, *Methanocalculus halotolerans* isolated from an oil well, has its optimum at 50 g/l NaCl and does not grow above 120 g/l (Ollivier et al. 1998). Acetate-splitting methanogens are probably even less salt tolerant, but data are limited.

In spite of the above, anaerobic sediments of hypersaline lakes are often a rich source of methane, even at near-saturating salt concentrations. Methanogens growing at such salinities

have been isolated and characterized. They do not use the two common reactions mentioned above, but their substrates are methylated amines such as trimethylamine, and sometimes dimethylsulfide as well (Oremland and King 1989). Species such as *M. evestigatum* and *Methanohalophilus portucalensis* grow on such substrates even at 240–250 g/l salt (Zhilina and Zavarzin 1987).

As no methanogenesis is known to occur from hydrogen and carbon dioxide at high salt concentrations, it is somewhat surprising that the same substrates can be converted to acetate by homoacetogenic bacteria. *Acetohalobium arabaticum*, a representative of the Halanaerobiales (Firmicutes), isolated from a hypersaline lagoon of the Arabay spit, Crimea, grow up to 250 g/l salt (Zavarzin et al. 1994). A hypothesis to explain the observations is presented in section Why Are Not All Types of Microbial Metabolism Found at the Highest Salt Concentrations?.

Fermentation

Fermentation, that is, anaerobic growth by conversion of organic compounds in the absence of an external electron acceptor, is possible up to the highest salt concentrations (Oren 1988). Phylogenetically, we find fermentative halophiles in disparate groups. The Archaea of the family Halobacteriaceae are basically aerobes using oxygen as electron acceptor. However, some species can grow anaerobically by denitrification (see section Anaerobic Respiration) or using light energy absorbed by bacteriorhodopsin (see section Aerobic Heterotrophs), and species of the genus *Halobacterium* (and as far as ascertained only these) can grow anaerobically in the dark while obtaining energy by fermentation of arginine with the production of ornithine, ammonia, and carbon dioxide (Hartmann et al. 1980). Another representative of the Halobacteriaceae that probably grows by fermentation is *Halorhabdus tiamatea*, a non-pigmented isolate from a deep hypersaline anoxic basin near the bottom of the Red Sea. It only grows on complex substrates, and the mode of fermentation has not been ascertained (Antunes et al. 2008).

Specialized fermentative halophiles are found in a few groups in the domain Bacteria. The order Halanaerobiales with two families, the Halanaerobiaceae and the Halobacteroidaceae (Rainey et al. 1995), entirely consists of obligatory anaerobic halophiles, and nearly all obtain energy by fermentation (exceptions being *S. shriftii* discussed in section Anaerobic Respiration and the homoacetogenic halophiles mentioned in section Methanogens and Homoacetogens). Most species ferment simple sugars to products such as ethanol, acetate, hydrogen, and carbon dioxide, and most grow at salt concentrations between 100 and 200 g/l (Oren et al. 1984; Ollivier et al. 1994; Oren 2006c). In the domain Bacteria we also find a few fermentative prokaryotes phylogenetically not classified within the order Halanaerobiales. Examples are *Clostridium halophilum* (Firmicutes) and the unusual *Haloplasma contractile*, mentioned in section Phylogenetic Diversity in the Halophile World, which grows up to 180 g/l salt, forms lactate as fermentation product, and is unrelated to any other group within the Bacteria (Antunes et al. 2009).

Chemoautotrophs and Methanotrophs

Some aerobic chemoautotrophic processes function up to very high salt concentrations, while others appear to be restricted to low salinities. An example of a process commonly found in

freshwater and marine systems but absent in hypersaline environments is autotrophic nitrification in which ammonia is oxidized via nitrite to nitrate (Rubentschik 1929). Autotrophic ammonia oxidation is still found in the alkaline Mono Lake at total salt concentrations between 80 and 90 g/l (Joye et al. 1999), and 16S rRNA gene sequences related to *Nitrosomonas europaea* were recovered from the lake (Ward et al. 2000). However, no records exist of autotrophic ammonia oxidation above 150 g/l salt, and the most salt-tolerant isolate, described as "Nitrosococcus halophilus," has its salt optimum at 40 g/l and does not grow above 94 g/l (Koops et al. 1990). Information on autotrophic oxidation of nitrite to nitrate at high salt concentrations is lacking altogether.

Chemoautotrophic oxidation of reduced sulfur compounds proceeds at much higher salt concentrations. *Halothiobacillus halophilus*, isolated from a salt lake in Western Australia, oxidizes thiosulfate, elemental sulfur, and tetrathionate up to 240 g/l salt, with an optimum at 50–60 g/l (Wood and Kelly 1991). *Thiohalorhabdus denitrificans*, found in Siberian salt lakes and in Mediterranean salterns and representing a deep lineage within the Gammaproteobacteria, is even more halophilic: it grows between 11 and 290 g/l NaCl with an optimum at 175 g/l. It also grows anaerobically as a chemoautotroph using nitrate as electron acceptor (Sorokin et al. 2006, 2008c). A variety of chemoautotrophic sulfur bacteria have been isolated also from soda lakes, such as *Thiohalospira halophila*, *T. alkaliphila*, and *Thioalkalivibrio halophilus*, organisms able to grow at pH 8–9 and above and at Na^+ concentrations up to 5 M (Banicu et al. 2004; Sorokin and Kuenen 2005a, b; Sorokin et al. 2008b).

In the alkaline hypersaline Mono Lake (about 90 g/l salts) and Sears Lake, California (>300 g/l salts), where high concentrations of arsenic occur, a biogeochemical cycle of arsenic functions with anaerobic reduction of As(V) (arsenate) to As(III) (arsenite) (see section ❯ Anaerobic Respiration) and chemoautotrophic oxidation of As(III) to As(V) (Oremland et al. 2005). An isolate from Mono Lake, *Alkalilimnicola ehrlichii* (Gammaproteobacteria) grows as a chemoautotroph on arsenite using oxygen or nitrate as electron acceptor at salt concentrations up to 190 g/l (optimum 30 g/l), but can grow heterotrophically as well (Hoeft et al. 2007).

Although methane is produced in anaerobic sediments up to very high salt concentrations (see section ❯ Methanogens and Homoacetogens), attempts to show aerobic methane oxidation in hypersaline ecosystems either yielded negative results (Conrad et al. 1995) or very low rates of activity compared to those at lower salinities, as measured in hypersaline basins in the Crimea (Sokolov and Trotsenko 1995). A few methane-oxidizing bacteria were isolated from salt lakes such as "Methylomicrobium modestohalophilum" showing a moderate salt tolerance, but their optimum salt concentration for growth is only about 20 g/l (Khmelenina et al. 1996, 1997; Trotsenko and Khmelenina 2002).

Halophiles in Environments of Different Salinities and Ionic Composition

Many of the halophilic microorganisms discussed above grow in a broad range of salt concentrations. For example, most members of the Halomonadaceae are noted for their ability to grow from near-freshwater salinity to NaCl concentrations close to saturation. Other groups are much more restricted in their salt range. Many members of the red halophilic Archaea (family Halobacteriaceae) lyse when the salt concentration falls below 100–150 g/l. The family is therefore often considered as consisting of organisms that require extremely high salinities not only for growth, but also for structural stability and integrity.

However, in recent years it has become clear that at least some members of the Halobacteriaceae survive prolonged exposure to low salt, and they can be isolated from environments with salinities similar to that of seawater or even less. Example are *Halosarcina pallida* and *Haladaptatus paucihalophilus*, isolated from a low-salt, sulfide-rich spring in Oklahoma (Elshahed et al. 2004; Savage et al. 2007, 2008). An even more extreme case is the isolation of novel types of Halobacteriaceae from a traditional Japanese-style salt field (Fukushima et al. 2007).

Considerable diversity also exists among the halophiles with respect to the ionic composition of the media that support growth. NaCl is the dominant salt in seawater, and hypersaline environments derived from evaporated seawater (thalassohaline environments) are NaCl-dominated and neutral to slightly alkaline. Some environments are very rich in the divalent cations magnesium and calcium. These ions are toxic at high concentrations, as they are "chaotropic" ions that destabilize biological structures and not "kosmotropic ions" with a stabilizing function, such as sodium. The Dead Sea currently contains about 1.98 M Mg^{2+} and 0.47 M Ca^{2+}, in addition to 1.54 M Na^{+} and 0.21 M K^{+} (2007 values). Most halophiles that grow in saturated NaCl solutions do not tolerate brine with this ionic composition. In the past, when the divalent cation concentrations were somewhat lower than now, a few highly magnesium-tolerant species of Halobacteriaceae were isolated from the Dead Sea: *Halorubrum sodomense* and *Halobaculum gomorrense* (Oren 2002a, 2006b), but today's increased salinity and increased ratio of divalent to monovalent cations (due to massive precipitation of NaCl from the water column) has made the Dead Sea a hostile environement even for the best magnesium-adapted halophiles. The chaotropic action of magnesium and the presence or absence of sufficiently high concentrations of kosmotropic ions defines the limit of life in high-magnesium brines (Hallsworth et al. 2007).

Polyextremophilic Microorganisms

In many hypersaline environments the high salt concentration is not the only source of stress that requires adaptation of the microorganisms inhabiting them. Highly alkaline salt lakes and alkaline saline soils are common, acidic salt lakes are relatively rare, and hot and cold environments with high salt concentrations also exist. All these are inhabited by halophilic microorganisms adapted to the combination of extreme conditions. Such organisms can be called "polyextremophiles" (Rothschild and Mancinelli 2001).

Haloalkaliphilic microorganisms are found both within the Archaea and the Bacteria, and some examples were described in the sections above. The family Halobacteriaceae contains many alkaliphilic representatives that do not grow at salt concentrations below 150 g/l and require pH values above 8–9. Well-known examples are *Natrialba magadii* from a hypersaline soda lake in Kenya and *Natronomonas pharaonis* from the lakes of the Wadi An Natrun in Egypt (Oren 2006b). Related extremely halophilic alkaliphiles were isolated from hypersaline lakes in China and elsewhere. In the domain Bacteria we find a great diversity of haloalkaliphilic species with highly diverse types of physiology. The aerobic chemoheterotrophic family Halomonadaceae contains a number of alkaliphilic halophiles (Arahal and Ventosa 2006). Investigations of the microbiology of alkaline hypersaline lakes such as Mono Lake and Searles Lake, California, have in recent years yielded a large number of interesting novel genera of chemoautotrophic sulfur-oxidizing bacteria such as *Thioalkalimicrobium*, *Thioalkalivibrio*, and *Thioalkalispira* (Sorokin and Kuenen 2005a, b). The bacteria involved in the oxidation and

reduction processes of arsenic described in sections Chemoautotrophs and Methanotrophs and Anaerobic Respiration are also obligate haloalkaliphiles.

Hypersaline acidic environments are rare. The Dead Sea, with its extremely high concentrations of magnesium and calcium ions, has a pH around 6, but microorganisms isolated from the lake have their pH optimum in the neutral range. The recent isolation of truly acidophilic representatives of the Halobacteriaceae that only grow within the narrow range between pH 4.1 and 4.8 with an optimum at pH 4.4 and 200 g/l salt came as a surprise, as they were obtained from solar salt that showed an alkaline reaction (Minegishi et al. 2008). These intriguing strains deserve further study.

The isolation of the truly thermophilic *Halothermothrix orenii* from a salt lake in Tunisia shows that life is possible at high salt (optimum 100 g/l, tolerating up to 200 g/l), high temperature (up to 68°C; optimum 60°C), and in the absence of oxygen (Cayol et al. 1994). The genome sequence of this intriguing organism was recently published (Mavromatis et al. 2009). Possibly even more interesting is the combination of salt, pH, and temperature stress encountered in the shallow lakes of the Wadi An Natrun, Egypt. The lakes harbor a range of anaerobic halophilic alkalithermophiles, the first of which have now been characterized. *Natranaerobius thermophilus* grows optimally at 54°C (up to 56°C) at pH 9.5, and at a total Na^+ concentration between 3.3 and 3.9 M (range: 3.1–4.9 M). It is not closely releated phylogenetically with any of the recognized groups in the "Clostridia" class, with which it is affilated, and a new order, the Natranaerobiales and a new family, the Natranaerobiaceae, were established for its classification (Mesbah et al. 2007; Mesbah and Wiegel 2008).

Finally, the combination hypersaline and cold also occurs, but less research efforts have been devoted in the study of psychrophilic halophiles. The archaeon *Halorubrum lacusprofundi* from Deep Lake, Antarctica, can grow at near-saturating salt concentrations at temperatures as low as 4°C, although its optimum is 31–37°C.

The Diverse Strategies used to Cope with Salt Stress

For a microorganism to be able to live at high salt concentrations it is essential that the cytoplasmic contents are osmotically at least equivalent with the outside medium. A cell with an internal osmotic pressure lower than that of the surrounding salt solution will rapidly lose water through the water-permeable cell membrane. When a turgor is to be maintained (in all cells except possibly the extremely halophilic Archaea of the family Halobacteriaceae), the osmotic pressure inside the cell should even exceed that of its medium.

Nature has devised diverse ways to achieve osmotic stabilization of halophilic microorganisms. All halophiles have one feature in common: sodium is always excluded from the cytoplasm as much as possible by means of powerful energy-requiring sodium pumps (in prokaryotes generally based on Na^+/H^+ antiporter systems. However, what replaces sodium chloride to provide osmotic balance varies between the different types of microorganisms. One strategy is to accumulate potassium and chloride ions to concentrations similar to the ionic concentrations in the medium. The second strategy, which is much more widespread, is to exclude ions to a large extent and to accumulate small electrically neutral organic solutes ("compatible solutes") to prevent loss of water from the cell by osmosis.

The first strategy ("salt-in" to balance "salt-out") requires far-reaching adaptations of the entire intracellular machinery, as all enzymes and functions in the cytoplasm have to be functional in the presence of molar concentrations of KCl. This is not a trivial feat as

most enzymes from non-halophilic organisms function poorly, if at all, when suspended in saturated potassium chloride. Moreover, solubility of enzymes and other protein is often lowered at high salt. Therefore, modification of the entire proteome is necessary. A characteristic feature of halophilic proteins from microorganisms that accumulate KCl for osmotic balance is their highly acidic nature, with a great excess of acidic amino acids (glutamate, aspartate) over basic amino acids (lysine, arginine). Such proteins are highly negatively charged compared to their non-halophilic equivalents. In addition, halophilic proteins generally have a low content of hydrophobic amino acids (Lanyi 1974). The result of these protein modifications is that the constant presence of molar concentrations of salts is necessary to maintain the proteins in their native and active form; dilution of the salt leads to unfolding and denaturation. Therefore, those microorganisms that use the "salt-in" strategy are generally obligate halophiles with little flexibility with respect to the salt concentration range that allows growth, and they are thus adapted to life at an as much as possible constant and extremely high salinity. The advantage of using the "salt-in" strategy for osmotic adaptation is that it is energetically cheaper than biosynthesis of molar concentrations of organic osmotic solutes (see below), noting that those microorganisms that use the latter strategy also pump out sodium ions (Oren 1999).

The "salt-in" strategy is currently known to be used in three groups of prokaryotes only: the halophilic Archaea of the family *Halobacteriaceae*, the extremely halophilic red aerobic representative of the Bacteroidetes *S. ruber*, and members of the order Halanaerobiales (Firmicutes), which are anaerobic, mostly fermentative bacteria. In the Halobacteriaceae, intracellular KCl above 4.5 M was measured in cells growing in saturating NaCl concentrations. Potassium ions can enter the cell through special channels, but active transport of chloride has been documented as well, based on the light-driven primary chloride pump halorhodopsin and/or by cotransport with Na^+ ions. Analysis of the genome of *S. ruber*, phylogenetically unrelated with the Halobacteriaceae, showed a great similarity in properties with those of *Halobacterium* and relatives, including a highly acidic proteome. Extensive gene exchange may have occurred between *Salinibacter* and the halophilic Archaea, which share the same environment and have experienced the same environmental stress factors throughout their evolutionary history (Mongodin et al. 2005). Analysis of intracellular ionic concentrations in selected representatives of the anaerobic fermentative Halanaerobiales (*Halanaerobium praevalens*, *Halanaerobium acetethylicum*, *Halobacteroides halobius*) also showed presence of molar KCl concentrations, and their enzymes require high salt for optimal activity (Oren 1986, 2006c). However, analysis of the genome of the anaerobic thermophilic fermentative *Halothermothrix orenii*, which also belongs to this group, failed to show an acidic proteome. As also no genes were identified for the biosynthesis of any of the known organic osmotic solutes (see below), the mode of osmotic adaptation of this interesting polyextremophilic bacterium remains to be clarified (Mavromatis et al. 2009).

The second strategy of osmotic adaptation, that is, accumulation of organic osmotic solutes, is much more widespread in the microbial world. We find it in most halophilic and halotolerant members of the Bacteria (with the exceptions discussed above), in eukaryotic algae, in fungi, and also in methanogenic Archaea. The disadvantage of the strategy is the high energetic cost of the production of the solutes; the advantage is the large degree of flexibility and adaptability to a wide range of salt concentrations. As the solutes generally do not greatly affect protein structure and enzymatic activity, the cell can simply adjust the intracellular solute concentration according to the salinity of the environment. When suitable compatible solutes are found in the medium, these can often be taken up from the outside, thus saving much of the energy needed for de novo biosynthesis.

The diversity of organic osmotic solutes identified in the microbial world is large. These include glycerol and other polyols, simple sugars such as sucrose and trehalose, different amino acid derivatives such as glycine betaine and ectoine, and others (Galinski 1993, 1995; Roberts 2005). Most are uncharged or zwitterionic at the physiological pH. Glycerol is used by the unicellular algae *Dunaliella* (Ben-Amotz and Avron 1973) and by some yeasts and fungi. A prerequisite for the use of glycerol as osmotic solute is a very low permeability of the cytoplasmic membrane for the compound. Most membranes are highly permeable to glycerol, and it was never found as an osmotic solute in the prokaryote world. The prokaryotic oxygenic phototrophs – the cyanobacteria – use sugars (sucrose, trehalose), glucosylglycerol, or glycine betaine instead, the latter compound being the solute preferred by the most halotolerant types (Mackay et al. 1984). Glycine betaine can be accumulated from the medium by a wide range of bacteria and used for osmotic stabilization, but only a few prokaryotes synthesize the compound (Ventosa et al. 1998). More commonly encountered compatible solutes are ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) and its hydroxy derivative, synthesized from aspartate-β-semialdehyde – an intermediate of amino acid metabolism (Galinski 1995). Often cocktails of osmotic solutes are found in a single organism; thus, *Halorhodospira* (Gammaproteobacteria) cells may contain glycine betaine, ectoine, as well as trehalose.

As explained above, the Archaea of the family Halobacteriaceae use KCl for osmotic stabilization. However, an organic osmotic solute, 2-sulfotrehalose, was found in a number of haloalkaliphilic members, in which it functions together with KCl to provide osmotic balance (Desmarais et al. 1997). The methanogenic Archaea appear to rely on osmotic solutes: glycine betaine, β-glutamine, β-glutamate, and Nε-acetyl-β-lysine (Lai et al. 1991).

Why Are Not All Types of Microbial Metabolism Found at the Highest Salt Concentrations?

As discussed above, some microbial processes function in hypersaline environments up to the highest salt concentration defined by saturation of NaCl, typically at total salt concentrations around 340–350 g/l total salts in environments such as saltern crystallizer ponds, the north arm of Great Salt Lake, and also in the Dead Sea. Metabolic types found at such concentrations include oxygenic photosynthesis, anoxygenic photosynthesis, aerobic respiration, and denitrification. Fermentation, sulfate reduction with lactate as electron donor and chemoautotrophic sulfur oxidation have their salinity maximum at somewhat lower values. Finally, there are processes never shown to occur at salt concentrations above 150 g/l, examples being sulfate reduction with acetate as electron donor, methanogenesis by reduction of carbon dioxide with hydrogen or by splitting of acetate, and autotrophic nitrification.

Based on our understanding of the biology of halophiles, especially their modes of osmotic adaptation, and the bioenergetic aspects of the different types of dissimilatory metabolism in microorganisms, a general theory has been proposed to explain the differences in the upper salinity limits for these processes (Oren 1999, 2006a). The theory is based on the following assumptions:

1. Life at high salt concentrations is energetically costly.
2. Energetic constraints determine the upper salt concentration limit at which a dissimilatory process can occur.

3. Whether a certain type of metabolism can function at high salt depends therefore on (a) the amount of energy generated in its dissimilatory metabolism, and (b) the energy cost of osmotic adaptation. Organisms that use the "high-salt-in" strategy (see section The Diverse Strategies used to Cope with Salt Stress) have an advantage over those that have to synthesize organic osmotic solutes when the available energy is limited.

The following examples show that, with a single exception, the above hypothesis explains the observations well.

Phototrophs are not energy-limited when light is available. Therefore, energy limitation will not restraint the occurrence of oxygenic photosynthesis by *Dunaliella* (synthesizing glycerol as osmotic solute at a relatively low energy cost) or by cyanobacteria (producing glycine betaine and other osmotic solutes) and of anoxygenic photosynthesis, for example, by *Halorhodospira*, which produces glycine betaine and ectoine.

Aerobic respiration and anaerobic respiration with nitrate as electron acceptor yield relatively large amounts of ATP, and therefore these processes are possible at the highest salt concentrations both by microorganisms that accumulate KCl for osmotic stabilization (Halobacteriaceae, *Salinibacter*) and representatives of the Bacteria that produce organic osmotic solutes.

In the case of dissimilatory sulfate reduction there is a great difference in energy yield between the reactions performed by "incomplete oxidizers" that convert lactate to acetate and carbon dioxide and by "complete oxidizers" that use, for example, acetate as their substrate. The energy yield of oxidations using sulfate as electron acceptor is much lower than that of similar reactions with oxygen or nitrate. Moreover, the ATP-dependent activation of sulfate also costs energy. Relatively more energy is gained in reactions such as lactate or hydrogen oxidation by sulfate reducers than by oxidation of acetate: per 8 electrons transferred the $\Delta G^{\circ\prime}$ values are −160.1, −152.3, and −47.7 kJ, respectively. Little is known about the mode of osmotic adaptation of the sulfate-reducing bacteria, but they appear to synthesize organic solutes to provide osmotic balance. Therefore, the low energy yield of the acetate oxidation probably precludes the process from occurring at high salt concentrations.

Fermentation processes also yield small amounts of energy. However, occurrence of fermentative metabolism has been documented nearly up to the highest salt concentrations, and different types of fermentative halophiles have been isolated, notably the members of the Halanaerobiales. As far as known, all these use KCl rather than organic osmotic solutes (Oren 1986), so that the energy cost of osmotic adaptation is minimized.

Methanogenic Archaea growing at high salinities accumulate organic osmotic solutes, probably in addition to high potassium concentrations found also in non-halophilic methanogens. Accordingly, the upper salinity limit at which the different types of methanogenic metabolism are found agrees well with the amount of energy gained. The two most common reactions that produce methane, namely, the reduction of carbon dioxide with hydrogen and the aceticlastic split, were never shown to occur at salt concentrations above 100–120 g/l. These two reactions yield small amounts of energy only ($\Delta G^{\circ\prime} = -34$ and −31.1 kJ per mol of hydrogen and acetate, respectively). On the other hands, use of methanogenic precursors such as trimethylamine and other methylated compounds is possible up to salinities close to saturation, and the amount of energy gained during this type of metabolism is much larger (e.g., −92.1 kJ per mol of trimethylamine converted). The homoacetogenic reaction in which carbon dioxide is reduced by hydrogen to acetate yields even less energy ($\Delta G^{\circ\prime} = -26.1$ kJ per mol of H_2) than the formation of methane from the same substrates. Still, homoacetogens (*A. arabaticum*) function up to much higher salinities (Zavarzin et al. 1994).

No assays for the presence of organic osmotic solutes have yet been published, but according to their phylogenetic affiliation within the Halanaerobiales, it may be expected that these organisms use the energetically favorable "high-salt-in" strategy, which may explain their high halotolerance.

The correlation between the energy yield and the salinity range also exists for aerobic chemoautotrophic processes. These are typically performed by representatives of the Proteobacteria that accumulate organic osmotic solutes. Oxidation of sulfide and other reduced sulfur compounds is possible up to near-saturating salt concentrations, but the two reactions of autotrophic nitrification, namely, oxidation of ammonia to nitrite and of nitrite to nitrate were never convincingly shown above 100–150 g/l, and the limit may even be less. A comparison of the energy yield of the reactions may explain why: per 2 electrons transported, oxidation of sulfide to sulfate yields a $\Delta G^{\circ\prime}$ of −199.1 kJ, compared to −91.5 kJ for autotrophic ammonia oxidation and −74.1 kJ for the oxidation of nitrite.

There is, however, one case in which bioenergetic constraints alone cannot explain the apparent absence of a type of dissimilatory metabolism in high-salinity environments. Attempts to show aerobic oxidation of methane at salt concentrations above 100–150 g/l are generally unsuccessful (Conrad et al. 1995). Only seldom have methanotrophs from high-salt environments been isolated, and these are restricted in their salt tolerance (Khmelenina et al. 1996, 1997), in spite of the fact that methanogenesis proceeds up to very high salt concentrations. Aerobic methane oxidation yields much energy ($\Delta G^{\circ\prime}$ = −813.1 kJ per mol of methane converted to carbon dioxide). No explanation has yet been brought forward why aerobic oxidation of methane is not compatible with life at high salt concentrations.

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3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.3,

Introduction

Archaea are ubiquitous in nature and thus also inhabit saline environments or have to cope with changing salt concentrations in their environment (Martin et al. 1999; Achtman and Wagner 2008). Like for bacteria, the biggest challenge is to adjust the turgor and this feature may even be of more importance since a number of archaea do not have rigid outer cell surfaces such as the peptidoglycan in the Gram-positive bacteria that contributes intrinsically to salt resistance (Kandler and König 1998; Sleytr and Beveridge 1999). Most archaea also use the "compatible solute" strategy for turgor adjustment (Galinski and Trüper 1994) and have been in the focus of research since it was hoped to find new, biotechnologically interesting compatible solutes in archaea (Sowers et al. 1990; Empadinhas et al. 2001; Pflüger et al. 2003; Saum et al. 2009a). Indeed, the nature of the compatible solutes used by bacteria and archaea is different (Roeßler and Müller 2001). Generally, the necessity of compatibility with macromolecular and cellular functions in the cell is reflected by the limitation to a rather small number of compounds (Le Rudulier et al. 1984). These can be divided into two major groups: (1) sugars and polyols and (2) α- and β-amino acids and their derivatives, including methylamines. Most archaeal compatible solutes resemble, in structure, their bacterial counterpart, with the difference that the majority of them carry a negative charge (Roeßler and Müller 2001). By the addition of carboxylate, phosphate, or sulfate this anionic character is mediated. This preference of anionic solutes can be explained as a contribution to equilibrate the high intracellular concentration of inorganic cations, mostly K^+, found in most archaea.

Even though compatible solutes have been identified and characterized some 20 years ago, in contrast to eukarya and bacteria, the analyses of the molecular basis of salt adaptation in archaea has started comparatively late due to the lack of molecular tools (Roberts 2000, 2004; Pflüger et al. 2005). The establishment of these methods for some archaea has made new insights into the physiology of archaea possible. This chapter is intended to summarize the compatible solutes used by archaea and recent achievements regarding the processes involved in salt adaptation with a special focus on *Methanosarcina mazei* Gö1, a model methanogenic archaeon.

Compatible Solutes Found in Archaea

Archaea often use anionic derivatives of common bacterial carbohydrates as compatible solutes (Roeßler and Müller 2001). Examples are trehalose in bacteria and the negatively charged derivative 2-sulfotrehalose, which is found as an osmolyte in the halophilic *Natronococcus occultus* and *Natronobacterium* spp. (Desmarais et al. 1997). However, some thermophilic archaea like *Pyrobaculum aerophilum* and *Thermoproteus tenax* also use trehalose as a compatible solute (Martins et al. 1997; Kouril et al. 2008). A group of osmolytes relatively unique to archaea are polyol phosphodiesters. While glycerol is accumulated by cyanobacteria in response to external osmolality (Reed et al. 1986), a novel charged form of this compound, diglycerol phosphate (DGP) is found as an osmolyte in *Archaeoglobus fulgidus* (Martins et al. 1997). Another example for the correlation of uncharged compounds in bacteria and charged derivatives in archaea is glucosylglycerol, which is the predominant osmolyte accumulated by cyanobacteria (Erdmann et al. 1992). In *Methanohalophilus portucalensis* the charged counterpart, glucosylglycerate, was detected (Robertson et al. 1992b).

α-amino acids like proline and α-glutamate are widespread as compatible solutes in bacteria (Kempf and Bremer 1998; Saum and Müller 2007). In contrast, to date, there have

been no reports of proline being accumulated in response to osmotic stress in archaea. In addition, the "typical" bacterial solute ectoine has also not been detected in archaea. Reports on the function of α-glutamate in archaea are controversial. It is a compatible solute in *Methanococcus thermolithotrophicus* (Martin et al. 2001) and *M. mazei* (Sowers and Gunsalus 1995) and other archaea, while its internal concentration in several *Methanohalophilus* strains was shown to be independent of the external osmolality (Lai et al. 1991).

β-amino acids combine the extreme solubility of their β-isomers with the advantage that they are not metabolized significantly by the cells, making them ideal compatible solutes. β-glutamate (in several thermophilic halotolerant *Methanococcus* species as well as in the mesophile *M. cariaci* [Robertson et al. 1992a]), β-glutamine (in several *Methanohalophilus* species [Lai et al. 1991; Robinson and Roberts 1997]) and N^{ε}-acetyl-β-lysine (Sowers et al. 1990; Lai et al. 1991) are found as compatible solutes in methanogenic archaea. The zwitterionic N^{ε}-acetyl-β-lysine is the predominant compatible solute in various methanogenic archaea and is, so far, only found in archaea but not in bacteria.

Turgor Adjustment in *M. mazei*

M. mazei Gö1 is a model organism for studying the bioenergetics of methanogenesis for some 30 years (Deppenmeier et al. 1990a, b; Deppenmeier et al. 1996; Deppenmeier and Müller 2008). Its genome has been sequenced, it is genetically tractable and recently, genome-wide expression profiling studies have been performed with cells grown under different conditions to understand how *M. mazei* adapts to a changing environment (Hovey et al. 2005; Veit et al. 2006; Pflüger et al. 2007). A hyperosmotic shock by transferring cells of *M. mazei* from freshwater media to high salt media led to an increased lag phase, indicating a salinity-dependent re-programming of the cell physiology. This obvious change in cell physiology made the salt stress response a prime model system for the analyses of the molecular basis of signalling and responding to changing environments in *M. mazei* Gö1 (Spanheimer and Müller 2008). It should be emphasized that almost all studies used hypersalinity but not hyperosmolarity as stressor. Whether *M. mazei* Gö1 (and other methanogens) respond to hyperosmolarity the same way has not been addressed.

When cells of *Methanosarcina* species are adapted to low osmolarities (0.3 osmol/kg) potassium and α-glutamate are accumulated as predominant compatible solutes (Sowers and Gunsalus 1995). The amount of these compatible solutes in the cytoplasm increases with high osmolarities (1.0 osmol/kg). If the salt concentration is further increased to 2.0 osmol/kg, the level of intracellular potassium and α-glutamate is unaffected but a novel osmolyte, N^{ε}-acetyl-β-lysine, can be detected in the cells (Sowers et al. 1990). This switch of compatible solutes can also be observed in *M. mazei* Gö1. At moderate salt concentrations (400 mM NaCl), α-glutamate is the main compatible solute. At high salinities (800 mM NaCl) the glutamate concentration increased only slightly (1.4-fold), but N^{ε}-acetyl-β-lysine becomes the predominant compatible solute (Pflüger et al. 2003).

Biosynthesis of Compatible Solutes in *M. mazei*

Biosynthesis of N^{ε}-acetyl-β-lysine

As in other methanogenic archaea, α-lysine seems to be built via the diaminopimelate pathway, as four genes that are similar to α-lysine biosynthesis genes from the diaminopimelate pathway

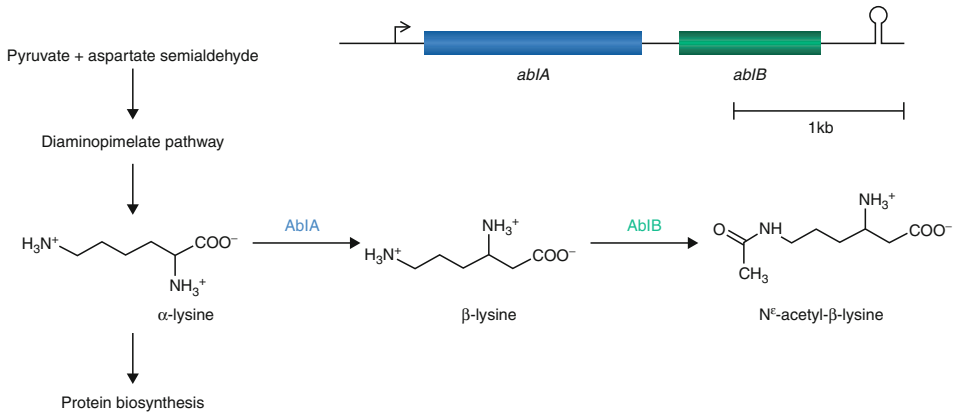

Fig. 3.3.1

Genetic organization and pathway for the biosynthesis of N^{ε}-acetyl-β-lysine in *M. mazei* Gö1. α-lysine is converted to β-lysine by the activity of a lysine-2,3-aminomutase (AblA). In the next step β-lysine is acetylated to N^{ε}-acetyl-β-lysine by AblB, a lysine acetyltransferase

were found in the genome of *M. mazei* Gö1 (Pflüger et al. 2003; Saum et al. 2009a). As shown in *Fig. 3.3.1*, α-lysine is then converted to β-lysine by the activity of a lysine-2,3-aminomutase, and then acetylated to N^{ε}-acetyl-β-lysine by a acetyltransferase (Roberts et al. 1992; Pflüger et al. 2003). These enzymes are encoded by the *abl* operon that consists of two genes, *ablA* and *ablB*, organized in the order 5′-*ablA*-*ablB*-3′ (Pflüger et al. 2003). They are separated by a short intergenic sequence of 204 bp. A promoter structure consisting of a TATA box and the factor B recognition element (BRE) was identified *in silico* upstream of the start codon of the first gene, *ablA*. Downstream of *ablB* is a potential rho-independent transcriptional terminator. This *abl* operon is also found in other methanogens, like *M. acetivorans*, *M. maripaludis*, and *M. barkeri* (Galagan et al. 2002; Hendrickson et al. 2004; Maeder et al. 2006).

To study the physiological role of N^{ε}-acetyl-β-lysine, knock-out strains were generated in two different methanogens. A Δ*abl* mutant of the metabolically restricted *M. maripaludis* did grow at low salt but growth was impaired at high salt indicating that N^{ε}-acetyl-β-lysine formation is essential for salt adaptation in this methanogen (Pflüger et al. 2003). In contrast, a Δ*abl* mutant of *M. mazei* Gö1 grew well at low and intermediate salinities. Even at high salinities (800 mM NaCl) the growth was only slowed down, not impaired (Saum et al. 2009a). This clearly shows that in *M. mazei* Gö1 N^{ε}-acetyl-β-lysine is not an essential compatible solute for growth. NMR analysis revealed that, to counteract this loss, *M. mazei* increases the internal glutamate pool and, in addition to glutamate, synthesizes a novel solute, alanine. The intracellular alanine concentration was as high as 0.36 ± 0.05 μmol · mg protein^{-1} representing up to 18% of the total solute pool at 800 mM NaCl. The cellular alanine concentration is salinity-dependent and also decreases in the presence of glycine betaine in the medium. This clearly indicates that *M. mazei* Gö1 uses alanine as compatible solute. The Δ*abl* mutant of *M. mazei* allowed to ask for genes upregulated in the absence of N^{ε}-acetyl-β-lysine. Transcriptional analyses did not reveal a candidate gene for alanine synthesis (Saum et al. 2009a).

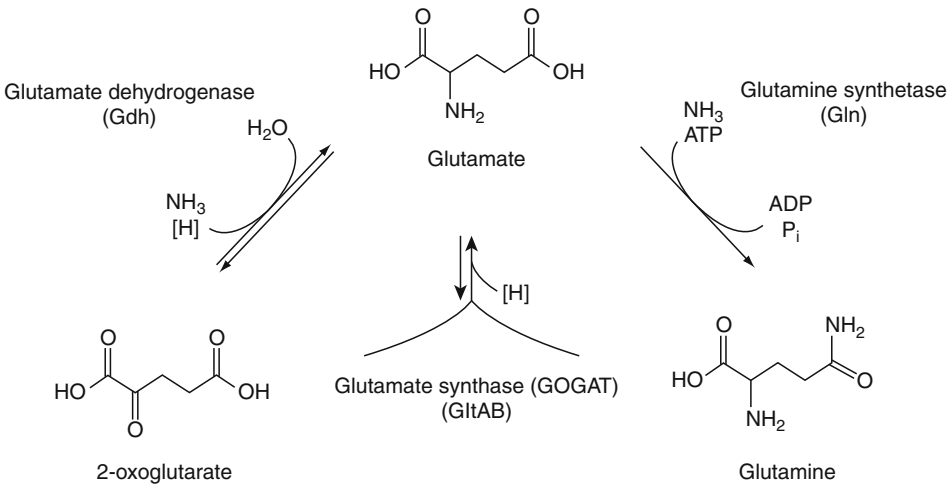

Fig. 3.3.2

Possible pathways for glutamate synthesis in *M. mazei* Gö1

Biosynthesis of Glutamate

The biosynthesis of glutamate and glutamine has not been addressed experimentally. *Figure 3.3.2* shows the different potential pathways for glutamate/glutamine synthesis.

The genome of *M. mazei* Gö1 encodes two potential glutamine synthetases, one glutamate synthase and three isoenzymes for the large subunit of the glutamate synthase (Deppenmeier et al. 2002). Therefore, it is well possible that the isogenes are regulated differently by osmolarity/nitrogen and one isoenzyme is involved in the synthesis of the compatible solutes glutamate/glutamine and regulated by salinity, whereas the other is regulated by the nitrogen regulon. It could be shown that one of the two glutamine synthetases plays a key role in ammonium assimilation, namely, glutamine synthetase 1 ($GlnA_1$) (Ehlers et al. 2005). Whether $GlnA_2$ is involved in osmoadaptation remains to be established. In addition, the genome of *M. mazei* Gö1 encodes three genes coding for potential glutamate dehydrogenases (Deppenmeier et al. 2002). Therefore, salinity-dependent accumulation of glutamate/glutamine might be via glutamate dehydrogenase or the glutamine synthetase/GOGAT pathways. Since the Δabl mutant increased the amount of glutamate in the cell, we checked for expression of genes potentially involved in glutamate production in this mutant versus the wild type. The transcription of the genes encoding glutamine synthetase 2 and glutamate synthase were slightly upregulated indicating that glutamate synthesis is via glutamin synthetase/GOGAT pathway. However, this has to be confirmed by mutational analyses. The elucidation of the pathways involved in biosynthesis of glutamate as compatible solute and their regulation by biochemical and genetic analyses is under way.

Uptake of Compatible Solutes

Uptake of compatible solutes is preferred over biosynthesis for energetic reasons (Oren 1999). A solute rather common in terrestric and marine ecosystems is glycine betaine and, therefore, all bacteria and archaea that have been looked at have at least one glycine betaine transporter

(Pflüger and Müller 2004). Glycine betaine was detected in methanogens already in 1990 (Robertson et al. 1990) but only *M. portucalensis* can synthesize it *de novo* (Roeßler and Müller 2001). Transport of glycine betaine has been studied in two methanogens. *Methanosarcina thermophila* TM-1 was the first methanogenic archaeon in which salinity-induced transport of glycine betaine was shown (Proctor et al. 1997). Kinetic analyses suggest a single, high affinity transporter, which seems to be a highly specific, secondary transporter for glycine betaine (Proctor et al. 1997). Unfortunately, up to date the genes encoding this transporter have not been identified.

Experiments with resting cells revealed that *M. mazei* Gö1 took up glycine betaine from the environment. Uptake was stimulated by the salinity of the growth medium (Roeßler et al. 2002). Interestingly, the genome of *M. mazei* Gö1 does not encode proteins with similarity to known secondary glycine betaine transporter but two primary, ATP-driven transporters with high similarity to glycine betaine transporters from bacteria, Ota and Otb. They are composed of three subunits, a cytoplasmic ATP hydrolyzing subunit (*otaA* and *otbA*), a transmembrane transporter subunit (*otaB* and *otbB1*/*otbB2*), and a substrate binding protein (*otaC* and *otbC*) (Spanheimer et al. 2008). This situation is rather unique since only *M. burtoni* has also Ota and Otb homologues, all other methanogens sequenced so far have only Ota.

Transcriptional analyses revealed that expression of *ota* but not *otb* was dependent on the salinity of the medium, indicating that Ota is an osmostress-induced glycine betaine transporter (Spanheimer et al. 2008). To analyze whether Ota is indeed a primary glycine betaine transporter, *ota* was cloned in an expression vector and transformed into the glycine betaine transport-negative mutant *Escherichia coli* MKH13 (Schmidt et al. 2007). Ota was produced as demonstrated by western blotting and could functionally complement *E. coli* MKH13. This mutant is defective in the transport of proline and glycine betaine due to the deletion of *putAB*, *proP*, and *proU* (Haardt et al. 1995; Kempf and Bremer 1995). Uptake studies revealed that Ota indeed catalyzed transport of glycine betaine in *E. coli* MKH13(pBAD-Ota) with a K_m of 10 ± 5 μM and a maximal velocity of 1.5 ± 0.5 nmol · min^{-1} · mg $protein^{-1}$. Transport was energy dependent and was activated by salinity gradients, but only marginally by osmolarity gradients across the membrane. Glycine betaine transport was inhibited to a small extent by an excess of dimethylglycin or proline betaine, but not by sarcosine or glycine. These data gave the final proof that Ota is indeed a glycine betaine transporter (Schmidt et al. 2007).

The physiological role of Ota and Otb was addressed by different experiments using the wild-type strain and Δ*ota* or Δ*otb* mutants. Deletion of either *ota* or *otb* had no growth phenotype, not at low or high salt (Saum et al. 2009c). This is reasonable as the cells will also grow at high salt in the absence of glycine betaine in the environment. Since it seems to be a general phenomenon that glycine betaine not only serves as an osmoprotectant but also as a chill protectant (Ko et al. 1994; Park et al. 1995; Gerhardt et al. 2000; Ozcan et al. 2005) we determined whether expression of *ota* or *otb* is influenced by the temperature. In the range tested (22–37°C), temperature had no effect on production of OtaC or OtbC in the wild type. Furthermore, at low temperature (22°C) and different salinities (38.5 and 400 mM NaCl) growth of the mutants was comparable to the wild type. These data demonstrate that the absence of Ota or Otb does not affect salt adaptation or cold adaptation.

To study the contribution of each glycine betaine transporter in salinity-dependent glycine betaine transport, phenotypes of the wild type and the mutants were compared. A deletion of *otb* had no effect on glycine betaine transport. In contrast, *M. mazei* Δ*ota::pac* was completely defective in glycine betaine transport, which is evidence that Ota is the only glycine betaine transporter in *M. mazei* Gö1 (Saum et al. 2009c).

Much to our surprise, *M. mazei* Δ*otb::pac* had an increased capacity for glycine betaine transport. Intracellular glycine betaine concentrations were increased, which is in line with the increased transport rates. Based on these findings, we asked whether the *otb* deletion also has an effect on *ota* transcription. Indeed, transcript levels of *ota* were enhanced by a factor of about 2 in *M. mazei* Δ*otb::pac* compared to the wild type. Vice versa, expression of *otb* was low and affected neither by salinity nor by the *ota* mutation.

What could be the role of Otb? Could it be a choline transporter? Neither the wild type nor the mutants did take up radioactively labeled [^{14}C]choline, and even by NMR no osmolyte accumulated in cells other than the well-known glutamate, N$^{\varepsilon}$-acetyl-β-lysine and glycine betaine. It can also be speculated that Otb is a constitutively expressed glycine betaine transporter that acts as a fast response after an osmotic upshock. However, this could be ruled out by several lines of evidence (Schmidt et al. 2007; Saum et al. 2009c). Therefore, the function of Otb remains obscure. However, it should be kept in mind that methanogens have a high degree of genetic redundancies (Bonacker et al. 1993; Pihl et al. 1994; Deppenmeier et al. 2002). Some enzymes of the methanogenic pathway are encoded by isogenes as well as other, for example, the mentioned glutamine synthetases genes. Some of these genes might have been acquired rather late in evolution and their products might not be integrated into the cells metabolism. This is apparently the case for the F_1F_O-like ATP synthase genes present in *M. acetivorans* and *M. barkeri* that are not expressed under the laboratory conditions used. Deletion of the F_1F_O-like ATP synthase genes had no effect on growth (Saum et al. 2009b). The apparent absence of an Otb homologue in the close relatives *M. acetivorans* and *M. barkeri* would support a rather late acquisition of Otb in *M. mazei* from bacteria. The same could have happened in *M. burtonii*.

Regulation of Solute Accumulation

This is by far the least established part of the salt adaptation network in methanogens and the analyses of regulatory events are still in its infancy. Transcriptional analyses revealed a salt-dependent transcription of *otaC* and cellular levels of Ota increased with increasing salt concentrations (Roeßler et al. 2002; Spanheimer et al. 2008). A maximum was reached at 300–500 mM NaCl. Ota concentrations reached a maximum 4 h after the upshock. Ota production was not only observed in response to hypersalinity but also hyperosmolarity. In addition to osmolarity, *ota* expression was also regulated by the growth phase. However, OtbC levels were close to detection limit, indicating low expression rates compared to OtaC. The cellular level of OtbC did not change with the growth phase and did not increase after an osmotic upshock (Spanheimer et al. 2008). Expression of *ota* as well as transport of glycine betaine was downregulated in the presence of glycine betaine. Production of Ota is not regulated by the solute transported, as externally supplied solutes such as glycine betaine or glutamate had no effect on cellular Ota levels (Spanheimer et al. 2008).

Transcriptional studies of the *abl* operon in *M. mazei* Gö1 showed high expression of the *abl* operon at 400 and 800 mM NaCl, while expression was apparently impaired at 38.5 mM NaCl (Pflüger et al. 2003). These data clearly show that the regulation of N$^{\varepsilon}$-acetyl-β-lysine synthesis is via the expression of the *abl* operon. An additional regulation on the level of enzymatic activity has been reported for *M. thermolithotrophicus.* There, the lysine-2,3-aminomutase activity in cells shifted from 0.68 to 1.4 M NaCl was induced about eightfold (Martin et al. 2001).

It is known that several processes are affected by glycine betaine when present in the medium. Uptake of glycine betaine is preferred over de novo synthesis of compatible solutes and, therefore, these processes should be shut down. Uptake of glycine betaine in *M. mazei* Gö1 is induced by hyperosmolarity and does not require the presence of glycine betaine. Regulation is at least on two levels: induction of expression (Spanheimer et al. 2008) and activity of the transporter (Schmidt et al. 2007). Again, nothing is known about the mechanisms involved. Glycine betaine accumulation led, as expected, to a reduction in pool sizes of glutamate and N^{ε}-acetyl-β-lysine in the wild type. This was not observed in *M. mazei* Δ*ota*::*pac* indicating that transport is required for glycine betaine repression (Saum et al. 2009c).

Interestingly, there is a cross talk between *ota* and *otb*. The deletion of *otb* led to an increased transcription and production of Ota and, concomitantly, to increased transport rates for glycine betaine. This effect probably involves regulation of *ota* expression by or via Otb by a yet not understood mechanism. Upregulation of gene expression in response to a disruption of another system was also observed in *Bacillus subtilis* (Holtmann et al. 2003). *B. subtilis* has two types of Ktr systems, KtrAB and KtrCD, to accumulate K^+. To analyze the roles of the two Ktr transport systems, mutants were constructed with chromosomal disruption of the corresponding genes. Levels of *ktr* transcripts were analyzed in different genetic backgrounds by Northern Blot analysis. It could be observed that in comparison to the wild type, *ktrAB* transcription was upregulated in the *ktrCD* mutant. Vice versa, an increase of *ktrC* and *ktrD* expression was observed in the *ktrAB* mutant. There, the authors speculated that the observed changes in transcript levels have a compensatory effect, to ensure sufficient K^+ acquisition. However, a compensatory effect of Otb if Ota is out of function could be excluded in this study.

The most important question how "salt" is sensed is completely open as is the signal transduction chain that leads as far as to gene expression and protein activation. This is where future work has to start. However, a clear difference to bacteria is signal perception. Whereas bacteria have a multitude of two-component systems to record changes in environmental parameters, archaea have only very few classical (Ashby 2006). There is no Kdp or EnvZ-OmpR system in *M. mazei* Gö1 and other archaea. However, *M. mazei* Gö1 has about 28 predicted histidine kinases (Ashby 2006). How methanogens perceive signals from the environment is still an open and very challenging question. Mostly, the archaeal promoters are similar to the eukaryotic counterparts, even though they are much less complex (Soppa 2001; Huber and Soppa 2008; Geiduschek and Ouhammouch 2005; Thomm 1996). Interestingly, the regulatory factors are primarily of the bacterial type. Still, a few regulatory systems are known until now.

Another good investigated example is the nitrogen regulon of *M. mazei* Gö1. *M. mazei* can use molecular nitrogen as a sole nitrogen source under nitrogen-limiting condition via a molybdenum nitrogenase encoded by a *nif* operon (Deppenmeier et al. 2002; Ehlers et al. 2002). Under nitrogen-rich conditions a repressor, NrpRI, binds at a palindromic DNA motif, inhibiting the transcription of the *nif* genes (Weidenbach et al. 2008). Furthermore, the activity of the glutamine synthetase is regulated by an archaeal PII-like protein called $GlnK_1$ (Ehlers et al. 2005). Such a regulatory system is yet unknown for the salt stress regulon. Nevertheless, several putative regulators of the bacterial type could be identified with microarray analyses. Some of them are directly upstream of likewise salt stress-induced genes. Two of these regulators are of the Mar family, one of the ArsA-family, and two show similarity to TrmB regulators (Pflüger et al. 2007). Whether one of these putative regulators is involved in osmoadaptation has to be investigated in future.

Unlike in other archaea (Torarinsson et al. 2005; Brenneis et al. 2007), in methanogens many mRNAs carry long 5′ untranslated region (UTR) sequences as shown via deep

sequencing transcriptome analyses (Jäger et al. 2009). The same studies also revealed many sRNA in the genome of *M. mazei*, which could bind these 5′UTRs and thereby regulate the protein synthesis on the translational level.

The Unravelling of Genes Involved in Salt Adaptation

Genome-wide expression profiling suggests that *M. mazei* Gö1 is able to adapt to high salinities by multiple upregulation of many different cellular functions including protective pathways such as solute transport and biosynthesis, import of phosphate, export of Na^+, and upregulation of pathways for modification of DNA and cell surface architecture (Pflüger et al. 2007). A few of these adaptations are discussed below.

Export of Na^+

Na^+ is cytotoxic and, therefore, every living cell tends to expel Na^+ from its cytoplasm (Lanyi 1979). Na^+ enters the cell by a multitude of secondary transporter. At low salinity, Na^+ is expelled by *M. mazei* Gö1 by action of the primary Na^+ pump methyltetrahydrosarcinapterin: coenzyme M methyltransferase (Müller et al. 1987, 1988; Becher et al. 1992). This enzyme creates a sodium ion gradient across the cytoplasmic membrane ($Na_i^+ < Na_o^+$) that is essential for methanogenesis. At elevated external Na^+ concentrations additional mechanisms have to apply. These should not be coupled directly to the process of methanogenesis and have a high turnover. Potential candidates for such exporters are Na^+/H^+ antiporter or ATP-driven efflux pumps. Inspection of the genome sequence of *M. mazei* Gö1 revealed three genes coding for potential Na^+/H^+ antiporters and a primary, ATP-driven Na^+ pump (MM1056) (Deppenmeier et al. 2002). The expression of only one of the Na^+/H^+ antiporter genes and MM1056 was (slightly) upregulated at high salt, which suggests their involvement in Na^+ homeostasis (Pflüger et al. 2007).

K^+ Uptake?

After an osmotic upshock, most bacteria respond in two phases. In the first phase, they will take up K^+ followed by the synthesis of glutamate to ensure electroneutrality. In the second phase, potassium glutamate is exchanged against another compound such as trehalose in *E. coli* or proline in *B. subtilis* (Kempf and Bremer 1998; Roeßler and Müller 2001). Whether there is a first phase with K^+ uptake in *M. mazei* remains to be established. In general, methanogens (like other microbes) accumulate K^+ (Sprott and Jarrell 1981). K^+ is often enriched to high intracellular levels in hyperthermophiles and, therefore, it is difficult to decide whether K^+ uptake is also a response to osmostress, and only few data on this topic are available. A salinity-dependent change in cytoplasmic K^+ concentrations has been found in *Methanosarcina* species adapted to different salinity (Sowers and Gunsalus 1995). There is only one study that followed the time dependence of cytoplasmic K^+ after an osmotic upshock (Martin et al. 2001). These studies revealed in *M. thermolithotrophicus* a rather high intracellular K^+ concentration that increased 1.5-fold compared to non-shocked cells when the salinity was raised from 0.68 to

1.02 M NaCl. Again, the interpretation of the data is complicated by the thermophilic nature of the organisms and its high K^+ content in the absence of external salt.

What can be learned from the genome data? Most bacteria accumulate potassium via the primary, ATP-driven potassium uptake system Kdp (Bakker 1992) that is activated by osmolarity. In addition, expression of the genes encoding the Kdp ATPase is induced by an osmotic upshock. Signal transduction is mediated via the two-component system KdpDE. KdpD is a membrane-bound sensor that responds to changes in osmolarity as well as changes in intracellular potassium. This signal is then transmitted to the response regulator KdpE resulting in a phosphorylation of KdpE. In its phosphorylated form it activates expression of the *kdpABC* operon (Sugiura et al. 1994; Jung and Altendorf 2002). So far, Kdp-like transport activities have not been detected in methanogens and, in addition, the genomes of *M. mazei* Gö1, *M. barkeri*, or the marine *M. acetivorans* do not encode Kdp-like ATPases (Deppenmeier et al. 2002; Galagan et al. 2002; Maeder et al. 2006). Therefore, the question is how methanogens accumulate potassium. The genome of *M. mazei* Gö1 encodes two homologues of the *trkA* and *trkG* genes of *E. coli*. The gene products could form the key complex of the Trk transport system that catalyzes K^+ transport in *E. coli* with low affinity but high rate. Furthermore, there are four genes in the *M. mazei* Gö1 genome that code for putative K^+ channels. None of these genes is differently regulated in salt-adapted (800 mM NaCl) versus freshwater cells (Pflüger et al. 2007). Mutational inactivation is required to identify, if any, the role of these proteins in potassium uptake and salt adaptation. The nature of the anion accumulated along with potassium is unknown, but glutamate might be involved.

If there is a first phase of salt adaptation by a fast accumulation of potassium in *Methanosarcina*, potassium would be exported in the second phase concomittant with the synthesis of compatible solutes. The genome of *M. mazei* Gö1 encodes a couple of different, potential K^+ export systems. There are three glutathione-regulated potassium-efflux system proteins that have similarities to KefC and are located upstream of the *abl* operon. The KefC protein is a member of the CPA2 family of monovalent cation/proton antiporters and plays a role in responding to changes in osmotic pressure and in protecting the cell from electrophile toxicity in *E. coli*. It is interesting to note that the *abl* operon is preceded by genes encoding potential potassium efflux systems along with regulators. The potential potassium transporters are divergently transcribed from the *abl* operon, which would be consistent with a co-regulation of both clusters.

Restructuring of the Cell Surface

The cell surface is challenged by drastic changes in the salinity of the environment and has to adapt to higher salt concentrations. *M. mazei* Gö1 has a proteinaceous cell wall and carries 12 genes on the chromosome that are annotated as putative surface layer proteins (Kandler and König 1998; Deppenmeier et al. 2002). Expression of 2 of them is induced about fivefold in salt-adapted cells (Pflüger et al. 2007). Closer inspection of these genes showed that MM2587 might have an eco-paralogue in MM0043, as they have 70% sequence identity along the complete sequence. Thus, in this case, expression of MM2587 and also of MM1589 probably leads to a surface layer that provides the cell with higher osmostability. Modification of the cell surface in response to elevated salt concentrations is a known phenomenon in methanogens. *M. mazei* strain S-6 and strain LYC have an S-layer and are surrounded by an amorphous outer layer when grown in medium with "normal" salt concentrations (i.e., 0.05 M NaCl),

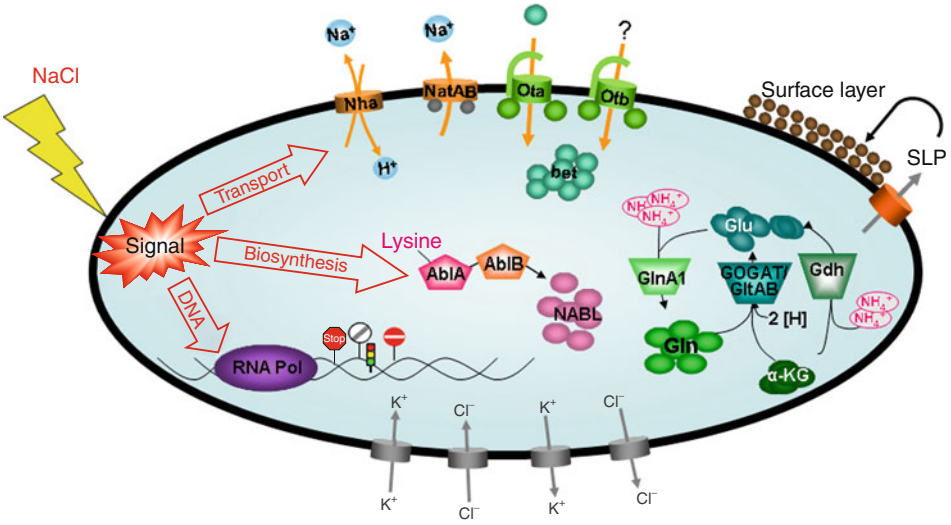

Fig. 3.3.3

Cellular response of *M. mazei* Gö1 to hyperosmotic stress. The export of Na^+ may be mediated by Na^+/H^+ antiporter (Nha1, Nha2, Nha3) and an ATP-driven Na^+ efflux pump (NatAB). The functions of K^+ and Cl- channels are yet unknown. The synthesis of N^{ε}-acetyl-β-lysine (NABL) from lysine ia catalyzed the lysine-2,3-aminomutase (AblA) and the acetyltransferase (AblB). Glycine betaine (bet) is taken up by the ABC-type transporter Ota, the function of Otb is unknown. The pathway for glutamate (Glu) synthesis is unclear. Glu can be produced from α-ketoglutarat (α-KG) via the glutamat dehydrogenase (Gdh) or the glutamat synthetase (GlnA1) together with the GOGAT/ GltAB (glutamine oxoglutarat amidtransferase /glutamat synthase) from glutamine (Gln) and α-KG. The adaptation of the surface layer via SLP (surface layer proteins) has to be investigated further

which makes them grow as aggregates (Sowers et al. 1984; Sowers et al. 1993). However, when the cells were transferred to high salt media (>0.4 M NaCl), they disaggregated and grew as single cells.

Conclusive Remarks

M. mazei has an extended regulatory network to adapt to changing salinities in its environment. Salt adaptation (*Fig. 3.3.3*) is achieved by uptake and biosynthesis of compatible solutes. Glycine betaine is taken up by Ota, whereas the function of Otb still remains unclear. N^{ε}-acetyl-β-lysine is synthesized de novo by the activity of a lysine-2,3-aminomutase (AblA) and a β-acetyltransferase (AblB). The pathway for the biosynthesis of glutamate is unknown. Additionally, changes in the surface layer by differential expression of Slp play an important role in salt adaptation. The proteins involved in Na^+ export and K^+ uptake (if any) remain to be identified.

However, it should be noted that in contrast to the uniform pathway of methanogenesis, methanogens are phylogenetically very divers (Jones et al. 1987). In addition, they have a very different ecophysiology and are ubiquitous in nature: they inhabit cold (arctic), hot

(hyperthermal vents), saline, or freshwater habitats. Clearly, the different lifestyle has evolved different mechanisms to cope with stress. For example, hyperthermophilic methanogens accumulate different solutes than mesophiles that protect against heat as well as salt (Müller et al. 2005; Empadinhas and da Costa 2006). K^+ is often accumulated to high intracellular levels in hyperthermophiles and, therefore, it is difficult to decide whether K^+ uptake is also a response to osmostress. In addition, organisms like *Methanocaldococcus jannaschii*, *Methanothermobacter thermoautotrophicus* or other methanococci have a restricted metabolism. This is also reflected by the genome size that is roughly half the size in *Methanococcus* and *Methanothermobacter* compared to *Methanosarcina* species (Deppenmeier et al. 2002; Galagan et al. 2002; Hendrickson et al. 2004; Maeder et al. 2006). A restricted metabolism may restrict also the ability to form different compatible solutes, or the other way around, metabolically versatile methanogens repond to a deletion of the pathway for one solute with the the production of an alternative compound (Saum et al. 2009a). In addition, the enzymatic equipment may be different, which is best exemplified in the energy metabolism of methanogens. *Methanosarcina* species have cytochromes and methanophenanzine, whereas others are devoid of these electron carriers (Kühn et al. 1979; Kühn and Gottschalk 1983; Abken et al. 1998; Thauer et al. 2008). Clearly, signalling and responding to environmental changes is as different between methanogens as it is between different bacteria, such as, for example, *E. coli*, *Halomonas elongata*, *B. subtilis* and *Halobacillus halophilus* (Cánovas et al. 1996, 1998; Kempf and Bremer 1998; Cánovas et al. 2000; Saum et al. 2006; Saum and Müller 2007, 2008). Generalizations cannot be made and data obtained for a given organism cannot be transferred in general to another, only distantly related one. *Methanococcus* and *Methanosarcina* respond differently to changing environmental conditions such as salinity (Martin et al. 1999; Roberts 2004; Müller et al. 2005).

A major challenge is the identification of the signal transduction chain(s) involved in perceiving and responding to hypersalinity. Mutational inactivation of potential signal perceivers is under way. Output modules will be found the same way. In addition, the promoters of salt-regulated genes will be mapped and used to fish the regulators that interact with the DNA. These are challenging questions that need to be addressed in the near future.

Acknowledgments

Generous support of the project by the Deutsche Forschungsgemeinschaft (Priority programme 1112) and the "Biodiversity and Climate Research Center" (Bik-F), Frankfurt, is gratefully acknowledged.

Cross-References

- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 3.1 Taxonomy of Halophiles
- 3.2 Diversity of Halophiles
- 3.4 Ecology of Halophiles

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3.4 Ecology of Halophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.4,

Introduction

"Thriving in salt" – this was the title of a recent article published in *Science* featuring the world of microbial life in saline and hypersaline environments (Boetius and Joye 2009). Halophilic microorganisms are present everywhere where high salt concentrations are found, in hypersaline lakes, saline soils, salted food products, and in some unexpected places as well such as brines deep in the sea, on plants that excrete salts from their leaves, and on ancient wall paintings.

This chapter explores the ecology of salt-loving microorganisms and the approaches used to answer the basic question in microbial ecology: who are the organisms present, in what numbers do they occur, how do they make a living, and how do they interact with their environment and with each other. The chapter thus complements Chaps. 3.1 Taxonomy of Halophiles and 3.2 Diversity of Halophiles.

Rather than providing in-depth descriptions of specific environments and surveying all information available on their biota, I here attempt to highlight selected aspects of the studies performed. Techniques commonly used in microbial ecology studies in freshwater and marine environments have also been applied to the hypersaline world, and the results of such studies are discussed below. Special emphasis will be placed on those approaches specific to the study of high-salt environments and on special problems often encountered when attempting to apply methods in common use in microbial ecology to the study of the world of halophiles.

It is of course impossible to cover all aspects of the ecology of halophiles in this chapter. More information is found in books and review articles dedicated to the topic (Oren 1994b; Javor 1989; Oren 2002).

Diversity of Hypersaline Environments

Salt lakes and other hypersaline water bodies are often classified into thalassohaline and athassohaline environments. Thalassohaline environments are derived from the evaporation of seawater, and such brines reflect in their ionic composition that of the sea. Most halophilic microorganisms known prefer media with NaCl as the main salt at near-neutral pH, and these are thus adapted to life in thalassohaline environments. When seawater is left to evaporate in closed lagoons or in man-made salterns, there is a sequential precipitation of calcium carbonate and calcium sulfate (gypsum) until finally NaCl saturation is achieved at salt concentrations above 300 g/l and salt precipitates as halite. The pH of the brines remains in the range 7–8. Great Salt Lake, Utah, a remnant of the ice-age saline Lake Bonneville that has largely dried out, has no connection with the sea, but the composition of its waters is similar to that of seawater, and therefore it can be considered thalassohaline. Great Salt Lake is divided by a railway causeway into two parts; the north arm is currently saturated with respect to NaCl, while the south arm has a salinity of about 120 g/l. In comparison to many other hypersaline lakes and to solar salterns, the microbiology of the lake has been little studied (Post 1977; Stephens and Gillespie 1976), but recently the interest in the biological properties of this interesting environment has increased (Baxter et al. 2005).

Coastal solar salterns are man-made ponds constructed for the production of salt by evaporation of seawater. Salterns are operated as a series of ponds in which the salinity gradually increases until halite precipitates in the crystallizer ponds. The salinity of each

individual pond is maintained approximately constant, and thus the saltern ecosystem presents the microbiologist with a gradient of salinities, from seawater (35 g/l) to halite saturation (>300 g/l) (Javor 1989; Oren 2002). The development of dense and stable microbial communities in saltern ponds has made the salterns popular environments for the study of halophilic microorganisms. Much of our understanding of halophile microbiology is based on studies of saltern ponds and the microorganisms isolated from them.

A prime example of an athalassohaline hypersaline lake is the Dead Sea on the border between Israel and Jordan. The ionic concentration of its brines (total salt concentration currently 347 g/l) is dominated by the divalent ions magnesium (nearly 2 M) and calcium (0.47 M) rather than by sodium (1.54 M) (2007 values). Chloride and bromide are the main anions, and there is very little sulfate. The pH is about 6. As the lake is currently supersaturated with respect to Na^+ ions, halite precipitates from the water column, and the relative concentrations of the divalent cations are continuously increasing. Although the present-day Dead Sea is somewhat too extreme for even the best salt-adapted microorganisms, in the near past it harbored microbial communities with a high level of tolerance to magnesium and a relatively low sodium requirement. Dense microbial communities only develop in the lake when massive winter rain floods cause a dilution of the upper water layers; this has happened only twice in the last 3 decades (Oren 1988; Oren and Gurevich 1995; Oren et al. 1995).

Examples of extremely alkaline athassohaline salt lakes are Mono Lake, California (total salt concentration around 90 g/l, pH 9.7–10) and some of the soda lakes in the East African Rift Valley such as Lake Magadi, Kenya, and also the lakes of Wadi An Natrun, Egypt (Javor 1989; Grant et al. 1999; Oren 2002). There also are industrial evaporation ponds for the production of valuable minerals from such alkaline brines, and a few studies have been devoted to their microbiology, a recent example being the assessment of the diversity of haloalkaliphilic Archaea in evaporation ponds in Botswana (Gareeb and Setati 2009).

Hypersaline brines have been discovered near the bottom of the Red Sea, the Mediterranean Sea, and the Gulf of Mexico. These brines are anoxic, and sometimes the ionic composition differs greatly from that of seawater. Thus, Discovery Basin is a $MgCl_2$-saturated lake on the Mediterranean Sea floor at a depth of 3.5 km. The lake itself does not appear to support life, but the gradient between the seawater and the brines below provides an excellent opportunity for the study of life at low water activities and the specific effects of chaotropic ions such as magnesium on extremophilic microorganisms. A concentration of 2.3 M $MgCl_2$ appears to be the upper limit of life when no kosmotropic ions such as Na^+ are also present (Hallsworth et al. 2007). The microbiology of the Mediterranean and Red Sea brines, located between 1.5 and 3.5 km below the sea surface, has been intensively studied (Eder et al. 1999, 2001; Daffonchio et al. 2005; van der Wielen et al. 2005), and some aspects are given below.

Many salt-tolerant and salt-requiring bacteria have been isolated from saline soils, found in arid areas worldwide. Except for the taxonomic description of many new species, little is known about their distribution, and the microbial ecology of saline soils has been poorly studied thus far. Halophiles have also been isolated from salted fish and food products processed by fermentation in brine. The production of certain traditionally fermented foods in the Far East depends on the activity of halophilic bacteria.

There also are more unusual environments where the microbial ecology of halophiles can be studied. Certain plants that grow on saline soils in arid areas excrete salts from glands on their leaves. The nature of the microbial communities on the phylloplane of *Atriplex halimus* and on the leaves of the tamarisk tree (*Tamarix* spp.) has been documented. The environment on such leaves is highly dynamic: the salinity increases as a result of evaporation during

daytime, and the pH of the excreted brine can be high as well (Simon et al. 1994; Qvit-Raz et al. 2008). The nostrils of some animals may also harbor interesting communities of halophilic microorganisms, as shown by the finding of *Halococcus* in the nostril salt glands of the seabird *Calonextris diomedea* (Brito-Echeverría et al. 2009) and of the salt-tolerant *Bacillus dipsosauri* in the nasal cavities of desert iguanas (Deutch 1994).

Archaeological monuments and historical wall paintings are an intriguing environment to search for halophiles. Locally, the salinity can be high, and it is therefore not surprising that diverse communities of halophilic and halotolerant microorganisms have been found on paintings in prehistoric caves, deteriorating marble in Greek temples and Roman monuments, and in medieval castles (Saiz-Jimenez and Laiz 2000; Piñar et al. 2001; Saiz-Jimenez et al. 2001).

An unexpected source from which halophilic microorganisms were isolated is the steam that emerges from geothermal vents. Halophilic Archaea related to the genus *Haloarcula* were recovered from steam waters in Kamchatka, Russia, Hawaii, New Mexico, California, and Wyoming. Up to 1.6×10^9 cells may be discharged each year from a single fumarole. The growth of halophiles in the fumarole area may have been enabled by the leaching of salts and minerals through the porous volcanic rock. The isolates survived exposure to 75°C for 5–30 min (Ellis et al. 2008).

Finally, different types of halophiles, Archaea as well as Bacteria, have been isolated from rock salt that had been buried for millions of years. Such organisms survived within fluid inclusions entrapped in the salt crystals during their formation (Norton and Grant 1988; Vreeland et al. 2007).

Community Densities of Halophilic Microorganisms

The presence of dense communities of halophilic Archaea in environments such as saltern crystallizer ponds, the north arm of Great Salt Lake, and some soda lakes in Egypt and Africa can often be recognized because the pink-red color of the water. Numbers of prokaryotes in such brines are generally between 10^7 and 10^8/ml, but higher numbers have been reported as well (Javor 1983, 1989; Oren 2002).

For the quantification of the total community density of prokaryotes in salt lakes, different techniques have been used, including microscopic enumeration in counting chambers (Oren and Gurevich 1995) and counting fluorescent cells on filters after staining with DAPI (3′,6-diamidino-2-phenylindole) (Pedrós-Alió et al. 2000a). Chlorophyll-containing phytoplankton cells from saltern ponds of different salinities were quantified by flow cytometry, enabling high throughput counting (Estrada et al. 2004). For the estimation of the numbers of organisms belonging to specific phylogenetic groups, fluorescence in situ hybridization (FISH) with 16S rRNA targeted probes has been adapted to use in highly saline solutions (Antón et al. 1999). The technique was successfully applied to the enumeration of specific types of prokaryotes in salterns in Spain (Antón et al. 2000) and Peru (Maturrano et al. 2006).

With certain modifications, the LIVE/DEAD *Bac*Light kit can be used to estimate the relative numbers of live and dead cells in hypersaline environments, and the methods work not only for Bacteria but for halophilic Archaea as well (Leuko et al. 2004). In such studies, it is important to include proper controls to ensure that the protocols indeed function at high salt concentrations and that cultures of known properties give the expected results before the methods are applied to samples collected from the natural environment.

Biomarkers Useful in the Study of Hypersaline Environments

Many types of halophiles contain specific cellular components that can be used as biomarkers to detect the presence of those organisms and to obtain quantitative information about their abundance. Some of these biomarkers can be detected using simple methods; others require more advanced analytical facilities. Below are a number of examples:

Halophilic Archaea of the family Halobacteriaceae contain diphytanyl diether lipids that can easily be detected by thin-layer chromatography. All known genera contain the diphytanyl diether derivatives of phosphatidylglycerol and the methyl derivative of phosphatidylglycerol phosphate. Some also contain phosphatidylglycerol sulfate but others lack this lipid. Some genera lack glycolipids, others have different diglycosyl, triglycosyl and/or tetraglycosyl lipids, which in some cases carry one or more sulfate groups. Presence or absence of certain polar lipids in extracts of biomass collected from the environment can therefore yield information about the presence of halophilic Archaea and the relative abundance of different genera within the group. This approach has been exploited in studies of microbial community in the Dead Sea (Oren and Gurevich 1993) and in saltern evaporation and crystallizer ponds (Oren 1994a; Oren et al. 1996; Litchfield et al. 2000; Litchfield and Oren 2001).

A technically more demanding method to obtain information on the polar lipid composition of halophilic communities is electrospray mass spectrometry. This technique led to the discovery of a unique sulfonolipid in the extremely halophilic bacterium *Salinibacter ruber* (Bacteroidetes). This 660 Da lipid could also be directly quantified in lipid extracts of saltern crystallizer biomass; simultaneously, information was obtained on the archaeal diether lipids, including different types of glycolipids, in the community (Corcelli et al. 2004).

Certain fatty acids in the lipids of Bacteria and Eucarya are useful biomarkers for certain groups of organisms in hypersaline environments, as shown in a study of the fatty acid patterns in the microbial communities at different depths within a gypsum crust in a saltern evaporation pond. Presence or absence of certain monounsaturated and polyunsaturated fatty acids could be correlated with the presence of certain types of cyanobacteria, enabling conclusions about their mode of life in situ (Ionescu et al. 2007).

Pigments are convenient biomarkers for certain types of halophiles. Spectrophotometric analysis and high-performance liquid chromatography (HPLC) can be used to detect and quantify the bacterioruberin pigments of halophilic Archaea (Oren and Gurevich 1995), as well as other pigments unique to halophiles such as the C_{40}-carotenoid acyl glycoside salinixanthin of *Salinibacter* (Oren and Rodríguez-Valera 2001) and other common pigments such as different types of chlorophyll present in algae and cyanobacteria in the brines (Estrada et al. 2004). Spectrophotometric methods can also be used to assess the presence of different types of chlorophyll, bacteriochlorophyll, and carotenoids in benthic microbial mats in salterns, including microbial communities embedded within gypsum crusts found in ponds at 150–200 g/l salinity (Caumette et al. 1994; Oren et al. 2009). Application of emission spectroscopy and kinetic fluorometry to the study of benthic microbial mats in saltern evaporation ponds led to the recognition of the presence of chlorosomes-containing anoxygenic phototrophs in mats at high salinity; their nature is still to be elucidated (Prášil et al. 2009).

Few attempts have yet been made to assess the presence of the light-driven proton pump bacteriorhodopsin found in some members of the Halobacteriaceae and other retinal proteins in the prokaryotic communities in hypersaline brines. Such measurements are based on the absorption spectra of the pigments (Oren and Shilo 1981; Javor 1983) or on spectroscopic measurements (Stoeckenius et al. 1985).

Cultivation-Dependent Techniques

It is generally assumed that at best only a few percent of the prokaryotes living on Earth have yet been cultivated. The numbers of colony-forming organisms on agar plates are generally several orders of magnitude below the total microscopic or flow cytometric counts. Examples are also found in studies of hypersaline environments. Thus, in a study of the microbial communities in the chemocline of Urania Basin, a hypersaline deep-sea basin in the Mediterranean Sea, bacterial numbers estimated by most probably number methods using dilutions in 11 different growth media were only between 0.006% and 4.3% of the total cell count (Sass et al. 2001).

This does not mean that culture-dependent techniques cannot be applied to obtain quantitative information on the communities of halophiles in their natural environment. Using appropriate growth media (preferentially low in nutrients) and incubation times as long as 8–12 weeks, Burns et al. (2004b) showed that the majority of prokaryotes detected in the saltern crystallizer ponds using 16S rRNA gene sequence-based, culture-independent techniques, can be cultured. Long incubation times appear to be more important than the exact medium composition. The saltern crystallizer environment is therefore probably the first ecosystem for which the "great plate count anomaly," as the phenomenon is often called, no longer exists. Such cultivation-dependent approaches with long incubation times resulted in the isolation of *Haloquadratum walsbyi*, the square flat archaeon that had resisted cultivation for nearly a quarter of a century since it was first observed (Bolhuis et al. 2004; Burns et al. 2004a).

Selective media can be applied to cultivate and enumerate specific groups of halophilic prokaryotes. The antibiotics anisomycin and bacitracin were used for the selective isolation of *Salinibacter* and related extremely halophilic Bacteria from hypersaline environments. Members of the Halobacteriaceae that inhabit the same ecosystems are inhibited by these compounds (Elevi Bardavid et al. 2007).

Different media should be tested to determine the conditions that give the highest colony yield, and sometimes media with unusual ionic compositions give the best results. In a study of halotolerant and halophilic bacterial communities on monuments such as the cathedral of Jerez, Spain, the chapel of Herberstein castle in Austria, and the prehistoric rock paintings of Altamira, Spain, the highest counts were obtained in media with 100 g/l salt, with optimal results when $MgSO_4$ was the main component (Saiz-Jimenez and Laiz 2000).

A modification of the most probable number approach was used to enumerate sulfate-reducing bacteria in sediments of Great Salt Lake. Here, instead of checking the tubes for visible growth (commonly done by adding iron ions to the medium and looking for black iron sulfide), the medium was supplemented with $^{35}SO_4^{2-}$ and the tubes were tested for formation of ^{35}S-labeled sulfide. This method proved more sensitive and reliable and gave higher counts. Depending on the composition of the medium, counts were up to $4.8 \times 10^8/cm^3$ for a sediment sample at 11.5% salt, $2.2 \times 10^7/cm^3$ at 12.5% salt, and 2.2×10^7 for a sediment whose overlaying water had 27% salt (Brandt et al. 2001).

Cultivation-Independent Approaches Based on Small Subunit rRNA Gene Sequences

Techniques based on phylogenetic analysis of the components of microbial communities based on sequences the genes for 16S (Bacteria, Archaea) and 18S rRNA (Eucarya) are extensively used in microbial ecology, and they have extensively been applied to hypersaline environments as well.

Common methods used in such studies are generally applicable also to high salt samples, and only few special modifications are needed of the standard protocols. Especially the non-coccoid halophilic Archaea lyse easily and recovery of their DNA is simple. However, when Archaea of the genus *Halococcus* are present, organisms with a thick and rigid cell wall, special lysis methods are required. Different methods were tested, based on physical disruption, chemical lysis, and enzymatic lysis, in an attempt to develop a method that efficiently releases the DNA while keeping it relatively intact. The best protocol used incubation for 2 h at 63°C in buffer containing potassium ethyl xanthogenate (Leuko et al. 2008).

16S rRNA sequence-based studies of saltern pond communities were first introduced by Benlloch et al. (1995, 1996), examining the crystallizer ponds of Spanish salterns. These studies led to the recognition of the 16S rRNA sequence of *Haloquadratum*, the dominant archaeon in the ponds, which was first isolated in 2004 (see above). Since that time, the technique was employed in many studies, generally using cloning or denaturing gradient gel electrophoresis to separate the PCR-amplified gene fragments. Such studies have been reported from the brines in the salterns in Spain (Benlloch et al. 1995, 1996, 2002; Casamayor et al. 2002; Ochsenreiter et al. 2002), Tunisia (Baati et al. 2008), Slovenia and Kroatia (Pašić et al. 2005, 2007), India (Manikandan et al. 2009), Taiwan (Wang et al. 2007), and the Peruvian Andes (Maturrano et al. 2006). Similar approaches were used to study the biota of the waters of hypersaline Lake Tebenquiche (Salar de Atacama, Argentina) (Demergasso et al. 2008), the alkaline hypersaline Mono Lake, California (Humayoun et al. 2003), the alkaline hypersaline lake Nevada Lake, Nevada, (Ochsenreiter et al. 2002), mountain lakes on the Tibetan plateau (Wu et al. 2006), Baer Lake, a soda lake in Inner Mongolia (Ma et al. 2004), Lake Magadi and other hypersaline soda lakes in Kenya (Rees et al. 2004) and East African alkaline saltern ponds (Grant et al. 1999), the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah et al. 2007), the alkaline evaporation ponds in Sua Pan, Botswana, used for production of sodium carbonate (Gareeb and Setati 2009), a small pond near a slag heap of a potassium mine in Germany (Ochsenreiter et al. 2002), and the brines of Kebrit Deep and Shaban Deep in the Red Sea (Eder et al. 1999, 2001, 2002). Similar studies were made of the microbial communities in the sediments of Mediterranean salterns (Mouné et al. 2002), the archaeal communities inhabiting the benthic microbial mats at Guerrero Negro, Baja California Sur, Mexico (Robertson et al. 2009), the alkaline-saline soil of the former lake Texcoco (Mexico) (Valenzuela-Encinas et al. 2008), and the Wadi An Natrun, Egypt, lakes (Mesbah et al. 2007). Finally, 16S rRNA sequence-based methods were used to obtain information on the nature of the community of prokaryotes on the salt-excreting leaves of the tamarisk tree (Qvit-Raz et al. 2008) and on the types of Archaea that had colonized deteriorated wall paintings and archaeological monuments (Piñar et al. 2001; Saiz-Jimenez et al. 2001). Fingerprinting methods such as DGGE, ribosomal internal spacer analysis (RISA), and terminal restriction fragments length polymorphism (T-RFLP) have been applied to monitor changes in the archaeal, bacterial, and eukaryal assemblages along a salinity gradient in a multipond solar saltern in Spain (Casamayor et al. 2002). Our knowledge of the 16S rRNA sequences of the different prokaryotes present in hypersaline environments also enabled the direct microsopical detection of those organisms by fluorescence in situ hybridization (FISH). The successful adaptation of the FISH method to be used at high salt concentrations (Antón et al. 1999) led to the recognition of the presence of Bacteria in salt-saturated saltern ponds and finally to the isolation of *Salinibacter ruber*.

In addition to the sequences of 16S rRNA genes, the intergenic spacer region between the 16S and the 23S genes in the rRNA operon can be used as a phylogenetic marker. Intergenic spacer region length polymorphism enabled the estimation of the halophilic archaeal diversity

within stromatolites and microbial mats of Hamelin pool, Western Australia, from which *Haloferax* and *Halococcus* species were recovered (Leuko et al. 2007).

An in-depth discussion of the results of all these studies and information about the phylogenetic affiliation of the large numbers of sequences retrieved and analyzed in each of them is outside the scope of this chapter. A few generalizations can be made: (1) There is considerable variation in the community composition in salterns and other hypersaline environments of similar salinity and similar ionic composition worldwide. (2) Most sequences recovered do not match those of the organisms known in culture, and there remain many species and genera to be isolated and described from such environments. In this respect, hypersaline environments are no different from other natural environments in which the technique was used. (3) At salinities up to about 200–250 g/l salt Bacteria dominate, at the higher salinities archaeal sequences are most frequently found. (4) In aerobic thalassohaline brines, *Haloquadratum* and *Halorubrum* spp. often abound at the highest salinities. (5) *Salinibacter*-like sequences are often recovered from the highest salinity ponds in marine salterns when using PCR primers targeted to amplify bacterial 16S rRNA gene sequences.

Cultivation-Independent Approaches Based on the Analysis of Functional Genes

To obtain information about the types of organisms present with emphasis of special physiological groups, genes specific for the requested organisms can be targeted. The number of studies in which functional genes were sequenced from DNA extracted from hypersaline environments is relatively small. The following genes have been used:

- The *bop* gene codes for the protein moiety of bacteriorhodopsin, the light-driven proton pump of certain members of the Halobacteriaceae. Ten different bacteriorhodopsin phylotypes were recovered from a solar saltern on the Adriatic coast; the diversity of Archaea was studied in parallel by 16S rRNA phylogeny (Pašić et al. 2005).
- Distribution of the genes *cbbL* and *cbbM*, coding for form 1 and form 2 of ribulose bisphosphate carboxylase/oxygenase (RuBisCO), was investigated along the redox gradient in the water column of Mono Lake, California in different seasons. Only form 1 sequences were obtained. Except for a single case (the *cbbL* gene of the cyanobacterium *Cyanobium*), it was not possible to attribute the sequences retrieved to known groups of photoautotrophic or chemoautotrophic microorganisms (Giri et al. 2004).
- To characterize the nature of the sulfate-reducing anaerobic prokaryotes in sediments and anaerobic brines, the *drsAB* genes, coding for the dissimilatory sulfite reductase, are a suitable marker. A study of the diversity of sulfate-reducing bacteria in the sediments of Great Salt Lake showed that in the north arm (270 g/l salt), the major lineage was affiliated with the genus *Desulfohalobium*; others lineages detected clustered with the Desulfobacteriaceae and the sulfate reducers within the Firmicutes (Kjeldsen et al. 2006). The alkaline Mono Lake yielded *dsr* gene sequences of Desulfovibrionales, Desulfobacterales, putative *Desulfotomaculum* species, and others (Scholten et al. 2005). Analysis of *dsrA* genes retrieved from L'Atalante and Urania Basins, two deep hypersaline anoxic basins in the Eastern Mediterranean Sea, showed presence of diverse communities of sulfate-reducing bacteria, most of them affiliated with the Deltaproteobacteria (van der Wielen and Heijs 2007). The *dsrAB* genes were also used to probe the sulfate-reducing community in a brine pool (salinity up to 140 g/l) at a depth of 650 m in the Gulf of Mexico (Joye et al. 2009).

- Another gene specific for dissimilatory sulfate reducers is *apsA*, coding for adenosine-5′-phosphosulfate reductase. This gene was used in addition to *dsrAB* as a probe to study the presence of sulfate reducers in the water column of Mono Lake (Scholten et al. 2005).
- To obtain information on the presence and diversity of methanogenic Archaea in anaerobic hypersaline environments, the *mcrA* gene, coding for methyl coenzyme A reductase, was used in studies of Mono Lake, California (Scholten et al. 2005) and in a brine pool in the Gulf of Mexico (Joye et al. 2009).

Metagenomic and Metaproteomic Approaches Applied to the Study of Hypersaline Ecosystems

Øvreås et al. (2003) measured melting profiles and reassociation kinetics of DNA isolated from saltern evaporation and crystallizer ponds near Alicante, Spain. Based on the thermal behavior of the DNA, the complexity of the microbial communities was estimated. The metagenome of a pond at 220 g/l salinity had an apparent complexity 7 times that of the *Escherichia coli* genome, a 320 g/l pond 13 times that of *E. coli*, and the crystallizer pond at 370 g/l salt had a simpler ecosystem with 4 times the complexity of the *E. coli* genome. The abundance of *Haloquadratum* cells in the crystallizer sample was indicated by shape of the melting curve of the community DNA, as *Haloquadratum* has a lower content of guanine + cytosine in its DNA (46.9 mol%) than all other described genera within the Halobacteriaceae (generally within the range of 59–71 mol%).

Following the earlier elucidation of the *Haloquadratum walsbyi* genome sequence, a metagenomic study was made in the same saltern ponds based on fosmid clone sequencing to assess the extent of variability between the genomes of *Haloquadratum* cells within a single population. A large pool of accessory genes was discovered within an otherwise coherent species. The genomic areas where heterogeneity was most pronounced contained many transposition and phage-related genes (Legault et al. 2006).

In a study of the metaproteome of the layered microbial mats in the Guerrero Negro, Baja California lagoons (about 90 g/l salt), the acidic nature of the proteins of the different layers on a millimeter scale was assessed by their isoelectric point. In all layers, the metaproteome showed a marked acid-shifted isoelectric point profile (Kunin et al. 2008). To some extent, this was unexpected as only the extremely halophilic prokaryotes that accumulate KCl for osmotic adaptation, such as extremely halophilic Archaea of the family Halobacteriaceae and *Salinibacter* (Bacteroidetes), are known for their acidic proteome, while moderate halophiles that use organic solutes for osmotic stabilization require little adaptation of their proteome.

Dispersal of Halophilic Microorganisms in Nature

Many halophiles, especially the species of the archaeal family Halobacteriaceae, require the constant presence of salt for structural integrity. At salt concentrations below 100 g/l, such cells rapidly lyse. Therefore, the question should be asked how such halophiles are dispersed in nature to colonize new hypersaline environments.

The longevity of cells entrapped within salt crystals may help in the dispersal of halophilic microorganisms. When halite crystallizes, fluid inclusions are formed within the crystals and microorganisms are entrapped within these inclusions. Laboratory experiments showed that

such bacteria can remain viable for long times (Norton and Grant 1988). Evidence is accumulating that live prokaryotes can even be recovered from underground salt deposits formed millions of years ago. These include not only endospore-forming Bacteria of the Bacillaceae (Vreeland et al. 2000), but also Archaea of the family *Halobacteriaceae* may survive under those conditions (Vreeland et al. 2007).

Not all "extremely" halophilic Archaea do rapidly lyse when exposed to low salt concentrations. Thus, halophilic Archaea related to the genus *Haloferax* and other types were isolated from estuary sediments in Essex, UK, with pore waters close to seawater salinity. Two isolates even grew slowly at salt concentrations as low as 25 g/l (Purdy et al. 2004). Additional low-salt adapted representatives of the Halobacteriaceae were recovered from a low-salt sulfur spring in Oklahoma (for additional information see Chap. 3.2 Diversity of Halophiles).

Extremely halophilic Archaea could also be isolated from seawater. *Halococcus* isolates have been obtained from Mediterranean Sea waters far from coastal areas (Rodriguez-Valera et al. 1979). In contrast to most other members of the Halobacteriaceae that lyse at low salt, cells of the genus *Halococcus* possess thick rigid cell wall that resist suspension in distilled water. A possible explanation for the finding of *Halococcus* in seawater was recently brought forward when it was discovered that the gull-like seabird *Calonextris diomedea* carries a stable community of *Halococcus morrhuae* and *Halococcus dombrowskii* within the salt-excreting glands in its nostrils (Brito-Echeverría et al. 2009). It is tempting to speculate that also other halophiles may be carried from place to place by migrating animals. The isolation of the highly salt-tolerant *Bacillus dipsosauri* from the nasal cavities of desert iguanas is another example of animals carrying halophilic microorganisms (Deutch 1994).

In Situ Activities of Halophilic Microbial Communities

When attempting to understand the ecology of halophilic microorganisms, knowledge of the types of organisms present in the ecosystem provides partial information only: it is much more important to understand the processes that those organisms perform in their habitat, the rates at which they perform those processes, and the interactions between the different groups of organisms. Here, our understanding lacks behind the wealth of information that has accumulated on the components of the microbial communities, especially thanks to the application of molecular phylogenetics and metagenomics.

A survey of the halophilic microorganisms available in culture (see Chap. 3.2 Diversity of Halophiles) shows that not all types of functional groups of microbes occurring in low-salt environments can also be found in high-salt ecosystems. Still, there are halophiles that degrade hydrocarbons (Bertrand et al. 1990) and other difficult-to-degrade substrates (Peyton et al. 2004).

A method to obtain information on the potential of a natural community to metabolize a large number of substrates is provided by the Biolog® system, originally developed for the characterization of bacteria in pure culture. Utilization of substrates in wells of microtiter plates results in the reduction of an indicator dye. The Biolog® system can be used with samples from saltern ponds and other hypersaline environments up to around 150 g/l salt; above that salinity, the salt interferes with reduction of the dye (Litchfield and Gillivet 2002). The method was used to compare heterotrophic communities in oligotrophic (Eilat, Israel) and more eutrophic saltern ponds (Newark, California) (Litchfield et al. 2001).

To assess the rates at which different compounds are transformed in hypersaline ecosystems, microelectrodes or radiolabeled substrates can often be used. Photosynthesis rates in

benthic microbial mats can be measured with oxygen microelectrodes, as shown, for example, in a study of the biogeochemical processes in a gypsum crust on the bottom of a saltern pond (salinity 200 g/l) (Canfield et al. 2004). Similar methods have been employed in the study of microbial mats at lower salinities.

Uptake rates of radiolabeled compounds are often measured to obtain information on the activities of microbial communities in their natural environment, and such techniques have also been applied to studies of hypersaline environments. Uptake of ^{14}C-labeled bicarbonate was used to assess primary production rates in Great Salt Lake (1976), in the Dead Sea (Oren et al. 1995), and in Spanish saltern ponds (Joint et al. 2002). This study of the primary production along the salinity gradient in the saltens near Alicante showed maximum production rates at 80 g/l salt; in the crystallizer ponds rates were very low, in spite of the presence of a dense *Dunaliella* population and high chlorophyll concentrations. Thus, the algae appeared to be heavily stressed by the too high salinity and showed little activity. Inorganic carbon uptake measurements during the 1992 *Dunaliella* bloom in the Dead Sea were supplemented by stable isotope studies in which changes in the ^{13}C content of the dissolved inorganic carbon were related to biological phenomena in the lake (Oren et al. 1995).

Measurement of thymidine uptake is often used to assess the growth rate of the heterotrophic prokaryotes. This method was used in salterns in Israel (Oren 1990d) and in Spain (Gasol et al. 2004). To estimate rates of dissimilatory sulfate reduction in the sediments of Great Salt Lake, Utah $^{35}SO_4^{2-}$ was used as a tracer, measuring the formation of $^{35}S^{2-}$. In the hypersaline north arm (270 g/l salt), rates were only about 1/10 of those measured in the south arm at 115–125 g/l. Manipulation of the salinity of sediment slurries from the hypersaline north arm showed optimal sulfate reduction rates at 100–150 g/l salt; at 250 g/l the rate was 30% of the optimal value (Brandt et al. 2001). Radiolabeled sulfate was also used to measure sulfate reduction rates in hypersaline coastal pans in South Africa (Porter et al. 2007). Information on the possible occurrence of dissimilatory sulfate reduction in sediments of the Dead Sea in the past was inferred from the stable isotope composition of the sulfide and the sulfate in the sediments and in the water: the isotopic fractionation with enrichment of ^{32}S in the sulfide was considered evidence for the biological nature of the sulfide (Nissenbaum and Kaplan 1976).

Microautoradiography combined with fluorescence in situ hybridization ("MAR-FISH") is a technique that enables the establishment of the phylogenetic affiliation of cells that had taken up specific radiolabeled substrates. Using this method, adapted to use in hypersaline media, it was found that both major components of the heterotrophic community in Spanish saltern crystallizer ponds, the square archaeon *Haloquadratum walsbyi* and the extremely halophilic bacterium *Salinibacter ruber*, readily take up acetate and amino acids, but under the conditions of the experiment none incorporated labeled glycerol (Rosselló-Mora et al. 2003). This finding was unexpected, as glycerol, produced as osmotic solute by the alga *Dunaliella* in the ponds, is expected to be available as carbon source (Elevi Bardavid et al. 2008). Such osmotic solutes can contribute much to the carbon cycle in hypersaline ecosystems (Welsh 2000). Studies of *Salinibacter* in culture showed that glycerol is used by the organism (Sher et al. 2004). Measurements of glycerol uptake in saltern crystallizer ponds in Eilat, Israel showed high rates and short turnover times (Oren 1993, 1995). When micromolar concentrations of ^{14}C-labeled glycerol were added to the brine, about 10% of the added label was converted to partial oxidation products: D-lactate and acetate (Oren and Gurevich 1994). *Salinibacter* converts part of the glycerol added to its growth medium to dihydroxyacetone, another partial oxidation product; dihydroxyacetone is efficiently taken up and used by *Haloquadratum* (Elevi Bardavid

and Oren 2008a), showing that glycerol may be the basis of a food chain in which several organisms jointly degrade the compound. Also, under anaerobic conditions glycerol metabolism by halophilic microorganisms shows interesting features. *Halanaerobium* spp. in pure culture convert glycerol to acetate, hydrogen, and CO_2, but rates of glycerol consumption were low. In mixed culture with *Desulfohalobium retbaense*, glycerol consumption increased, a phenomenon probably based on interspecies hydrogen transfer (Cayol et al. 2002).

Another osmotic solute of interest in studies of the community dynamics in hypersaline ecosystems is glycine betaine, produced, for example, by halophilic cyanobacteria and other photosynthetic prokaryotes (Welsh 2000). We know different pathways for the degradation of glycine betaine under aerobic and under anaerobic conditions, and some of these yield trimethylamine as one of the products (Oren 1990a). Trimethylamine is the preferred substrate of halophilic methanogenic Archaea, and most of the methane evolved in hypersaline anaerobic sediments is probably derived from this compound (Oremland and King 1989).

Interrelationships Between Halophilic Archaea, Bacteria, and Fungi

An overview of the microbial diversity in hypersaline ecosystems (see Chap. 3.2 in this volume) shows that in the higher salinity range and up to salt saturation, halophilic Archaea of the family Halobacteriaceae generally dominate the community of heterotrophs, but many halophilic and halotolerant members of the Bacteria can also grow up to very high salt concentrations. In the domain Bacteria we even find extreme halophiles, as the example of *Salinibacter ruber* shows (Antón et al. 2002).

The question should therefore be asked what the relative contribution of Archaea and Bacteria is to the heterotrophic activities in environments of different salt concentrations. To obtain such information, inhibitors were used that specifically inhibit either group. The non-coccoid Halobacteriaceae are lysed by low concentrations of bile acids such as taurocholic acid, and this has been exploited in attempts to assess the contribution of such Archaea to the uptake of amino acids or glycerol in saltern evaporation and crystallizer ponds of different salinities (Oren 1990b). The effect of different antibiotics targeting protein synthesis was also tested to differentiate between archaeal and bacterial amino acids incorporation. Anisomycin can be used to selectively inhibit archaeal protein synthesis, while erythromycin gave the best results when inhibition of bacterial activities was the goal (Oren 1990c; Gasol et al. 2004). The conclusion of such studies was that at salt concentrations above 250 g/l essentially all activity can be attributed to the archaeal component of the community.

Following the recognition of *Salinibacter* as a quantitatively significant component of the heterotrophic community in saltern crystallizer ponds (Antón et al. 1999, 2000), a reevaluation of the contribution of Bacteria to the heterotrophic activities in crystallizer ponds was required. The rates of amino acids uptake per cell were about two orders lower for *Salinibacter* than for *Haloquadratum* when suspended in crystallizer brine. Chloramphenicol, earlier used in attempts to differentiate between bacterial and archaeal activity (Oren 1990b), inhibited *Salinibacter*, but significant inhibition of *Haloquadratum* was also found, especially at the highest salt concentrations. Erythromycin gave satisfactory results as it inhibited *Salinibacter* without affecting amino acids incorporation by *Haloquadratum* (Elevi Bardavid and Oren 2008b).

In recent years, presence of fungi was recognized in saltern brines up to the highest salinities. Certain species of black yeasts (*Trimmatostroma salinum*, *Hortaea werneckii*) were

found in marine salterns (Gunde-Cimerman et al. 2000; Zalar et al. 2005). To what extent such fungi may contribute to the heterotrophic activity in salterns and in other hypersaline ecosystems remains to be assessed.

Factors Responsible for the Decline of Microbial Communities in Hypersaline Ecosystems

Studies on the factors that lead to decline and death of halophilic microorganisms in their natural environment are relatively rare. Among the factors assessed in such studies are grazing by eukaryotic protists, lysis by phages, and action of halocins.

A number of flagellate, ciliate, and amoeboid protozoa have been observed in hypersaline brines, in some cases even up to salt saturation (see Chap. 3.2 in this volume). However, their activity at the highest salt concentrations is probably minor. Analysis of the microbial food web along the salinity gradient in Spanish saltern ponds showed that in the ponds of intermediate salinity the eukaryotic predators were the most important factor controlling the abundance of heterotrophic prokaryotic plankton, but they contributed little in the crystallizer ponds (Pedrós-Alió et al. 2000a, 2000b; Gasol et al. 2004).

At the highest salinities, viral lysis is probably the most important loss factor to the prokaryotic communities. The ecology of halophilic viruses was first studied by Wais and Daniels (1985) in their investigation of the populations of phages infecting *Halobacterium* in a transient brine pool in Jamaica. An abundance of virus-like particles was found in an electron microscopic examination of Spanish saltern brines (Guixa-Boixareu et al. 1996). Collection of virus particles from saltern brines by tangential flow filtration and ultracentrifugation, followed by DNA extraction and pulsed-field gel electrophoresis showed the viral community to be less diverse than in the marine environment (Diez et al. 2000). The viral diversity increased from 40 to 150 g/l salt, then decreased with increasing salinity (Sandaa et al. 2003). Application of a metagenomics approach led to the elucidation of the sequence of EHP-1, a halophilic phage from the crystallizer brine of the Alicante, Spain saltern (Santos et al. 2007).

Similar techniques of phage concentration and analysis were applied to samples from the alkaline, hypersaline Mono Lake, where at least 27 different viruses could be recognized. Enumeration of virus-like particles collected on membrane filters in the fluorescence microscope yielded numbers of $0.1–1 \times 10^9$/ml, compared to $0.3–4.4 \times 10^7$ prokaryotic cells/ml (Jiang et al. 2004). Also in the Dead Sea did the number of virus-like particles exceed that of the prokaryotic cells by 1–2 orders of magnitude (Oren et al. 1997).

Some halophilic Archaea of the family Halobacteriaceae excrete halocins, bacteriocin-like protein antibiotics that inhibit other members of the same family (Rodriguez-Valera et al. 1982). Therefore, attempts were made to assess whether such inhibitors may be present in halophilic Archaea-dominated brines at concentrations sufficiently high to cause inhibition of related organisms. No evidence was found that halocins may be important ecological factors in the salterns in Israel, Spain, and California (Kis-Papo and Oren 2000).

Final Comments

The above survey shows that the parameters investigated in studies of the ecology of halophilic microorganisms are generally similar to those measured in freshwater environments. In many

cases, modification of the commonly used methods is necessary to overcome problems caused by the presence of high salt concentrations.

In the past decade, most studies published on the ecology of hypersaline environments dealt involved sequencing of 16S rRNA genes recovered from environmental DNA. Here, the situation is probably no different from that in other types of microbial ecosystems. While such studies yield valuable information on the diversity within the community present, the amount of knowledge gained is limited as the organisms harboring the 16S rRNA genes have not been isolated and their properties remain unknown. Methods of metagenomics are now also applied to high-salt environments, shedding more light on the true diversity, not only phylogenetically but also with respect to the presence of functional genes.

Hypersaline environments pose a number of specific questions to the investigator, and overall relatively little research effort has been devoted to obtain the answers. A few studies have tried to assess the relative contribution of the different groups of halophiles (Archaea, Bacteria, and others) to the heterotrophic activity at high salinities. Specific inhibitors have been employed to differentiate between the activities, and attempts have been made to assess the possible role of a few low-molecular-weight substrates (glycerol, dihydroxyacetone, lactate, amino acids, etc.) in the interactions between the groups. However, our understanding of the true interactions between the community components remains limited.

Not all microbial processes known from low-salt environments function also at the highest salinities. A survey of the upper limit at which different dissimilatory processes are active and the microorganisms responsible for those processes led to a coherent model explaining why some types of metabolism can function up to salt saturation while others cannot (Oren 1999, 2001). Little effort has yet been made to assess the fate of those compounds that are easily metabolized in freshwater and marine environment but for which no degradation process is yet known at high salt concentrations.

Much of our understanding of the microbial ecology of halophiles has resulted from in-depth studies of a few model ecosystems. Thus, we have learned much from studies performed in the saltern ponds of Alicante, Spain, and Eilat, Israel, many of which were cited in this chapter. Conditions in saltern ponds are kept as much as possible constant over time, and therefore the results of the individual studies can at least to a certain extent be related to each other, even if they were not performed at the same time. It is even better when a large number of different approaches can be applied at the same time to the study of a single ecosystem. Many of the papers discussed above featuring the saltern ponds of Alicante, Spain, and published in the first years of this century were performed by a multidisciplinary team of scientists in May 1999, each applying different techniques to the same samples. Such studies contribute much to our understanding of the ecology of halophilic microorganisms.

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3.5 Genetics and Genomics of Triangular Disc-Shaped Halophilic Archaeon *Haloarcula japonica* Strain TR-1

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.5,

The extremely halophilic archaea often exhibit unusual morphologies. Some cells may take a ribbon shape, a disk shape, or occasionally a square or triangular shape (Grant and Larsen 1989a; Grant and Larsen 1989b; Grant and Larsen 1989c). Square halophilic microbes have been observed in naturally occurring brines (Walsby 1980), and box-shaped halophilic microbes have also been isolated from Californian salterns (Javor et al. 1982). The square morphology, however, had not been convincingly demonstrated in the laboratory. The extremely halophilic archaea are now divided into 27 genera. The genus *Haloarcula* contains a number of pleomorphic isolates (Grant and Larsen 1989b). Thus, the square microbes described so far are likely to be *Haloarcula* spp. Recently, two square halophilic archaea were cultured, isolated, and taxonomically identified (Oren et al. 1999; Bolhuis et al. 2004). One of them actually belongs to the genus *Haloarcula*.

In the course of screening a large number of samples from hypersaline environments for new isolates of halophilic microbes, a predominantly triangular disk-shaped halophilic microbe, strain TR-1, was isolated (Takashina et al. 1990; Otozai et al. 1991; Horikoshi et al. 1993). In this contribution, isolation, taxonomy, morphology and cell division, and genetics and biochemistry of strain TR-1 are described. Genome analysis and comparative genomics are also mentioned.

Isolation, Taxonomy, Cell Morphology, and Cell Division

Isolation

Strain TR-1 was isolated from a saltern soil located at Noto peninsula in Japan. The microbe was motile by flagella, non-spore forming, and had red flat cells that are predominantly triangular in shape although rhomboidal cells were also observed (see Fig. 3.5.4) (Takashina et al. 1990; Otozai et al. 1991; Nakamura et al. 1992). The cells were typically 1–2 × 0.2–0.3 μm in size in liquid media. Strain TR-1 was extremely halophilic, and requires high concentrations of NaCl (1.7–4.3 M) and Mg^{2+} ions (41–650 mM) for growth. The concentrations of NaCl and Mg^{2+} were critical for the formation of the triangular shape, and optimal concentrations of NaCl and Mg^{2+} were 3.4 M and 160 mM, respectively. The temperature range for growth was 24–45°C, with an optimal temperature of 42°C. Growth of the microbe occurred within a pH range of 6.0–8.0, with an optimal range of 7.0–7.5.

Taxonomy

Biochemical characteristics of strain TR-1 were as follows (Takashina et al. 1990; Otozai et al. 1991): mono- and di-carbohydrates including galactose, arabinose, xylose, rhamnose, sucrose, glycerol, maltose, trehalose and cellobiose, and sugar alcohols such as mannitol and sorbitol were utilized as sole carbon sources with acid production. No hydrolysis of casein, gelatin, or starch was observed. Reduction of nitrates with gas and production of indole and H_2S were positive. No amino acid requirement was observed. Growth was inhibited by bacitracin, novobiocin, and anisomycin.

Chemical composition of the cell envelope of strain TR-1 (Otozai et al. 1991; Nishiyama et al. 1992) was almost the same as that of *Haloarcula hispanica* (Juez et al. 1986). The bulk proteins of strain TR-1 (Otozai et al. 1991) contained relatively higher amount of acidic amino acids such as aspartic acid and glutamic acid as compared with *Ha. hispanica* (Juez et al. 1986).

Strain TR-1 had C_{20}, C_{20} diether core lipids (Takashina et al. 1990; Otozai et al. 1991). C_{25}, C_{20} (sesterterpanyl) diethers were not detected. Strain TR-1 contained C_{20}, C_{20} derivatives of phosphatidyl glycerophosphate, phosphatidyl glycerosulphate, phosphatidyl glycerol, and triglycosyl diether (TGD-2) as the main polar lipids. When compared with *Haloarcula vallismortis*, the presence of three unknown minor lipids was characteristic for strain TR-1.

The G+C content of chromosomal DNA of strain TR-1 was 63.3% (Takashina et al. 1990; Otozai et al. 1991). DNA–DNA hybridization experiments revealed that the DNA homology between strain TR-1 and *Haloarcula marismortui* was 32%, that between strain TR-1 and *Haloarcula californiae* was 30%, that between strain TR-1 and *Haloarcula sinaiiensis* was 24%, and that between strain TR-1 and *Ha. hispanica* was 35%, although that between strain TR-1 and *Halobacterium salinarum* was 7%.

The 16S ribosomal DNA sequence of strain TR-1 was compared with those of reference strains. Strain TR-1 exhibited very high homology with *Ha. varismortis* (99.3%) or *Ha. marismortui* (99.4%) (Otozai et al. 1991). Other haloarchaea *Hb. salinarum*, *Haloferax volcanii*, and *Halococcus morrhuae* exhibited lower homology with strain TR-1 (90.4%, 89.8%, and 90.1%, respectively).

According to the results shown above, strain TR-1 should clearly be a member of the genus *Haloarcula* and was proposed to represent a new species as *Haloarcula japonica* (Takashina et al. 1990; Otozai et al. 1991; Horikoshi et al. 1993). *Ha. japonica* is deposited as strain TR-1 in Japan Collection of Microorganisms (JCM, RIKEN BioResource Center, Japan).

Cell Morphology and Cell Division

Electron micrographs of thin sections of *Ha. japonica* cells were prepared using the rapid freezing and substitution fixation method (Nishiyama et al. 1992; Nishiyama et al. 1995). The cells had predominantly triangular shape, and a thick surface (S) layer was clearly seen outside of the cells (*Fig. 3.5.1*). Atomic force microscopic images of typical triangular disk-shaped cells of *Ha. japonica* were also observed using rapid membrane filtration method (*Fig. 3.5.2*) (Umemura et al. 1998).

The course of cell division of *Ha. japonica* was analyzed by time-lapse microscopic cinematography. The cell plate formed between the apex of a triangle and the middle of the opposite side, or between the middle of any two sides. In the former case, septation occurred roughly symmetrically to form two triangular cells. Rhomboid-shaped cells were generated by cell division in the latter, asymmetric manner. The second cell plate formed perpendicular to the first cell plate, approximately at the middle of the other two sides. In most of the rhomboid cells, cell plates formed between the middle of two opposite sides and two rhomboid-shaped daughter cells were thus generated after this division. Some of these became triangular through cultivation. *Figure 3.5.3* shows the photographs of typical asymmetric cell division processed by a color image analyzer (Hamamoto et al. 1988; Otozai et al. 1991; Horikoshi et al. 1993).

Genetics and Biochemistry

Cell Surface Glycoprotein

The cell surfaces of many extremely halophilic archaea are covered by a surface (S) layer (Sleytr and Messner 1983; Sumper 1987). *Halobacterium salinarum* and *Haloferax volcanii* have

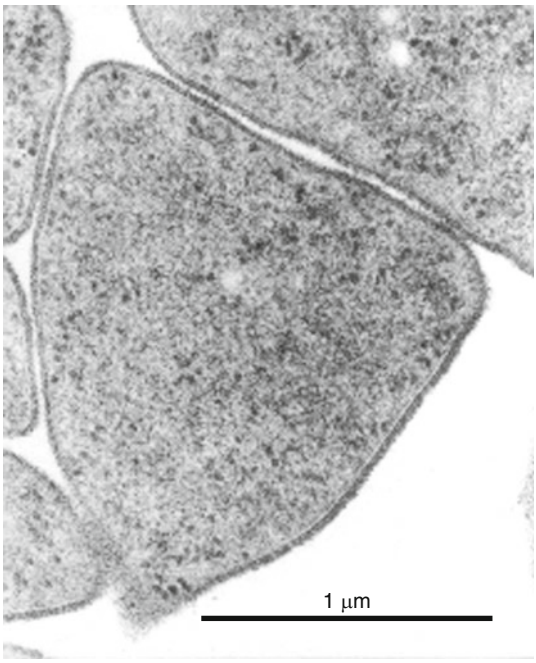

Fig. 3.5.1
Electron micrograph of thin section of *Ha. japonica* cells prepared by the freeze-substitution fixation method

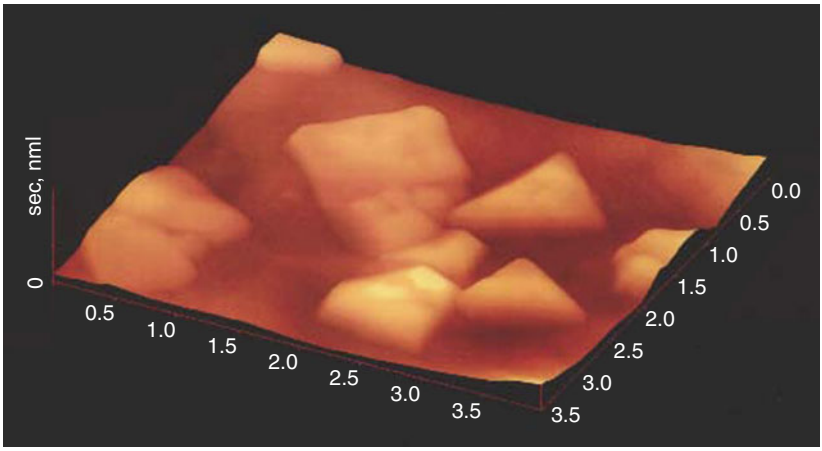

Fig. 3.5.2
Atomic force microscopic image of *Ha. japonica* cells on a membrane filter (contact mode in air)

glycoprotein on the cell surface (Mescher et al. 1974; Wieland et al. 1980; Kessel et al. 1988). The cell surface glycoprotein (CSG) is the major constituent of the S layer of *Hb. salinarum* (Mescher et al. 1974; Wieland et al. 1980). Photolytic degradation of the CSG on the cell surface and the shedding of the CSG by removal of magnesium ions from the culture medium

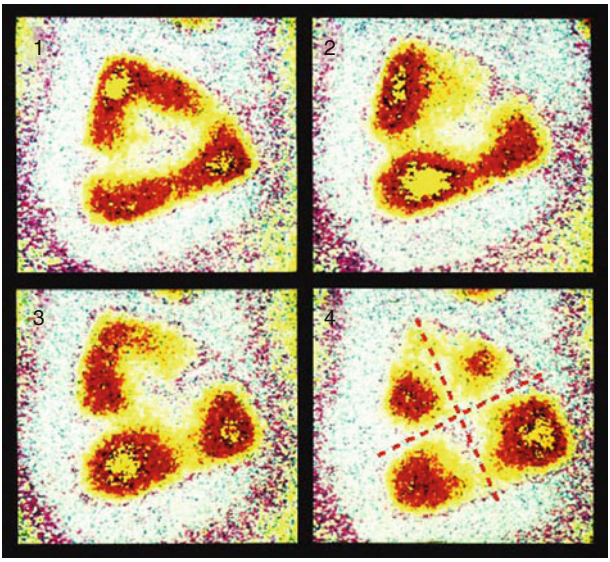

Fig. 3.5.3
Photographs of the cell division of *Ha. japonica*cell division was recorded with a time-lapse cinematomicroscope and then processed by a color image analyzer

caused morphological changes in *Hb. salinarum* and *Hf. volcanii* (Mescher and Strominger 1976; Sumper et al. 1990). Thus, it seems that the CSG is the structural component responsible for maintenance of the cell morphology in each strain. The genes encoding the CSG have been cloned from *Hb. salinarum* and *Hf. volcanii* (Lechner and Sumper 1987; Sumper et al. 1990). The analyses of the CSG, however, are confined to examples from the genera *Halobacterium* and *Haloferax*, and no study had been done with the genus *Haloarcula*.

The cell envelope fraction of *Haloarcula japonica* was prepared and examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A high molecular mass protein was detected as a major band at 170 kDa by staining with Coomasie brilliant blue (CBB) (Nakamura et al. 1992). This protein was also visualized by the periodate-Schiff (PAS) method. A similar phenomenon had been observed with the CSG of *Hb. salinarum* (Mescher et al. 1974). These results suggested that the cell envelope of *Ha. japonica* had a high molecular mass, carbohydrate-containing protein as the major envelope protein.

Haloarcula japonica has a predominantly triangular disk shape in liquid medium (*Fig. 3.5.4a*). However, chelation of Mg^{2+} ions by adding an equimolar amount of ethylenediaminetetraacetic acid (EDTA) to the culture medium converted the cells to spherical forms (*Fig. 3.5.4b*) (Nakamura et al. 1992). The resulting spheroplasts were removed by centrifugation and the supernatant was analyzed by SDS-PAGE. A 170-kDa band, presumed to be the CSG, was detected by CBB and PAS staining. Thus, EDTA treatment simultaneously caused the release of CSG and the morphological change in *Ha. japonica* cells. The same morphological change and release of the CSG were also observed when the triangular cells were suspended in a medium without magnesium. So it is likely that the CSG is important for the maintenance of the characteristic shape of *Ha. japonica* (Nakamura et al. 1992).

The CSG was purified (Nakamura et al. 1995) and the gene encoding the CSG was cloned from *Ha. japonica* (Wakai et al. 1997). The CSG gene encoded a 34-amino acid (34-aa)

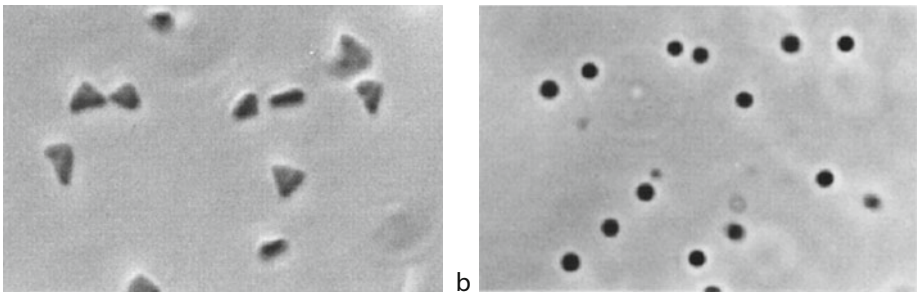

Fig. 3.5.4
Phase contrast photomicrographs of *Ha. japonica* cells before (a) and after (b) conversion to spheroplasts

Sec-type signal peptide followed by an 828-aa mature sequence. It contained a large amount of acidic amino acids such as aspartic acid and glutamic acid. Five potential N-glycosylation sites were found in the mature CSG sequence. The amino acid sequence of the *Ha. japonica* CSG showed 51.4% and 41.6% identities to those from *Hb. salinarum* and *Hf. volcanii*, respectively. The hydropathy profiles of these CSGs were almost similar to one another. Further studies are necessary to clarify the contribution of the CSG to maintenance of the characteristic triangular cell morphology of *Ha. japonica*.

Light-Driven Energy-Converting System

Bacteriorhodopsin (bR) was found in the purple membrane of *Hb. salinarum*. It was demonstrated to function as a light-driven proton pump creating an electrochemical proton gradient that can be used for ATP synthesis (*Fig. 3.5.5a*) (Oesterhelt and Stoeckenius 1973). Several new members of the bR-like protein family (light-driven proton pump family) have been reported, for example, archaerhodopsins (aRs) of *Halorubrum* spp. (Sugiyama et al. 1989; Uegaki et al. 1991; Mukohata et al. 1999) and cruxrhodopsins (cRs) of *Haloarcula* spp. (Tateno et al. 1994; Sugiyama et al. 1994; Kitajima et al. 1996). The amino acid sequence of these proteins also indicated that all essential residues for proton pumping were conserved.

By using flash-induced fluorescence spectroscopic analysis (Ohtani et al. 1992), a bR-like retinal protein was identified on the cell envelope vesicles of *Ha. japonica* (Yatsunami et al. 1997). The bR-like protein gene, encoding a polypeptide of 250 aa, was cloned from *Ha. japonica*. The deduced amino acid sequence of the *Ha. japonica* bR-like protein was 52%, 47–50%, and 75–96% identical to those of bR, aRs, and cRs, respectively (Yatsunami et al. 1997, 2000a). This result suggested that the bR-like protein of *Ha. japonica* belonged to the cR subfamily (*Fig. 3.5.5b*). Northern analysis revealed that transcription of the *Ha. japonica* cR gene was induced by high light intensity and low oxygen tension (Yatsunami et al. 1999a).

Halorhodopsin (hR), another retinal-containing protein found in the membrane of *Hb. salinarum*, is a light-driven anion pump that utilizes light energy to actively transport chloride ions (*Fig. 3.5.5a*) (Schobert and Lanyi 1982). The deduced amino acid sequence of hR showed 32% overall identity to bR. Novel hR-like proteins have been isolated in other halophilic archaea *Halorubrum* spp. and *Haloarcula* spp., and named archaehalorhodopsins (ahRs) and cruxhalorhodopsins (chRs), respectively (Mukohata et al. 1999).

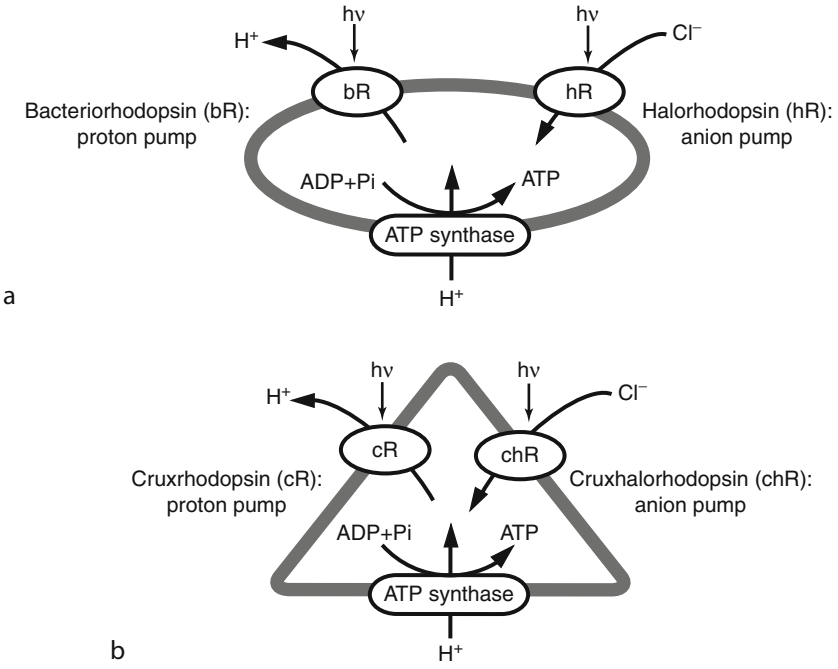

Fig. 3.5.5
Light-driven energy-converting system of *Hb. salinarum* (a) and *Ha. japonica* (b)

The hR-like protein gene, encoding a polypeptide of 276 aa, was cloned from *Ha. japonica*. The deduced amino acid sequence of the *Ha. japonica* hR-like protein was 62%, 68–69%, and 91–99% identical to those of hR, ahRs, and chRs, respectively, suggesting that the hR-like protein of *Ha. japonica* belonged to the chR subfamily (*Fig. 3.5.5b*) (Yatsunami et al. 2000b). Transcription of the *Ha. japonica* chR gene was inducible by high light.

Archaeal ATP synthases, called A_0A_1-ATP synthases, synthesize ATP at the expense of a transmembrane electrochemical proton gradient (*Fig. 3.5.5a*). Previously, the genes encoding A- and B-subunits of A_1-part, corresponding to the catalytic headpieces of A_0A_1-ATP synthases, were cloned from *Hb. salinarum* (Ihara and Mukohata 1991) and *Hf. volcanii* (Steinert et al. 1997).

When sequencing the *Ha. japonica* genomic DNA fragment containing the cR gene, the reading frames encoding the possible A_1-ATPase were found upstream and downstream of the cR gene (Yatsunami et al. 1999b). The neighboring DNA fragment was cloned by gene walking and then sequenced. The genes encoding AtpI (741 aa), AtpK (86 aa), AtpE (194 aa), AtpC (356 aa), AtpF (107 aa), AtpA (586 aa), and AtpB (486 aa) were found upstream of the cR gene (*Fig. 3.5.6*) (Yatsunami et al. 2001). On the other hand, the gene encoding AtpD (230 aa) and another open reading frame (OrfX, 131 aa) were located downstream of the cR gene. The genes encoding the *Ha. japonica* A_0A_1-ATP synthase subunits proved to be split into two clusters. RT-PCR analyses revealed that genes encoding AtpI and AtpK, genes encoding AtpE to AtpB, and genes encoding AtpD and OrfX were cotranscribed as a single mRNA, respectively. Therefore, OrfX gene might also be a component of the *Ha. japonica* A_0A_1-ATP synthase.

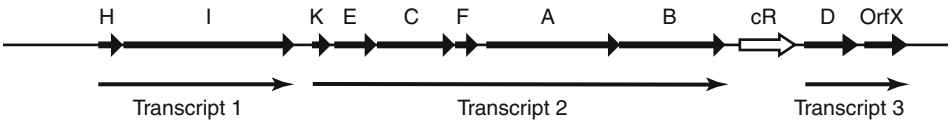

Fig. 3.5.6
Organization of the *atp* gene cluster of *Ha. japonica*

Recently, the complete genome sequence was reported for *Hb. salinarum* NRC-1 (Ng et al. 2000) and some other halophilic archaea. In these strains, the genes encoding A_0A_1-ATP synthases were also split into two clusters. However, the existence of an additional component, OrfX, had not been pointed out in other halophilic archaea.

Ferredoxin

Ferredoxins (Fds) are ubiquitous iron-sulfur (Fe-S) proteins involved in electron transfer reactions in a variety of metabolic pathways (Hall and Evans 1969). As Fe-S clusters, [2Fe-2S] and [4Fe-4S] ([3Fe-4S]) clusters have been found in Fds from many organisms. To date, [2Fe-2S] Fds have been isolated from plants, algae and cyanobacteria; and others mainly from bacteria (Hall et al. 1974; Thomson 1985). [2Fe-2S] Fds have been also purified from *Hb. salinarum* (Kersher et al. 1976) and *Haloarcula marismortui* (Werber and Mevarech 1978). The Fd genes have been cloned from *Hb. salinarum* (Pfeifer et al. 1993) and *Hf. volcanii* (Mevarech et al. 2000). The crystal structure of *Ha. marismortui* Fd has been solved (Frolow et al. 1996).

The Fd of *Ha. japonica* was purified and characterized (Sugimori et al. 2000). It also contained a [2Fe-2S] cluster. The Fd gene was cloned from *Ha. japonica* (Matsuo et al. 2001). The *Ha. japonica* Fd, as well as other haloarchaeal Fds, was fairly homologous to plant Fds, but it was 30 aa longer. Amino acid sequence alignment of haloarchaeal and plant Fds suggested that haloarchaeal Fds consisted of two domains: an N-terminal additional domain and a common core domain (Matsuo et al. 2001). Cysteine residues that serve as ligands to iron atoms are located in the core domain. Acidic amino acid contents in the N-terminal domains are about 40%, while contents in the core domains are about 20%. The crystal structure was also solved with the *Ha. japonica* Fd (*Fig. 3.5.7*) (Nitanai et al. manuscript in preparation). In the *Ha. japonica* and *Ha. marismortui* Fds, many carboxyl groups covered the protein surface except for the vicinity of the [2Fe-2S] cluster. Furthermore, the N-terminal domains of these Fds, located far from the [2Fe-2S] clusters, contributed to increase the solvent-accessible surface areas by providing more surface carboxylates. Thus, it was suggested that the N-terminal domains might be important for haloadaptation in the *Ha. japonica* and *Ha. marismortui* Fds.

The modified *Ha. japonica* Fd, in which the N-terminal domain was replaced by the corresponding region of the spinach Fd, was prepared and characterized (Hirota et al. 2005). The chimeric Fd was successfully produced as a holoprotein in *Escherichia coli*. The recombinant *Ha. japonica* Fd produced as a holoprotein in *Ha. japonica*, while it produced as an apoprotein in *E. coli*. These results suggested that the N-terminal domain of the *Ha. japonica* Fd would contribute to the polypeptide folding and successive cluster incorporation under high salt conditions. Both the chimeric Fd and the *Ha. japonica* Fd were stable under high salt conditions, suggesting that the core domain would mainly contribute to the stability

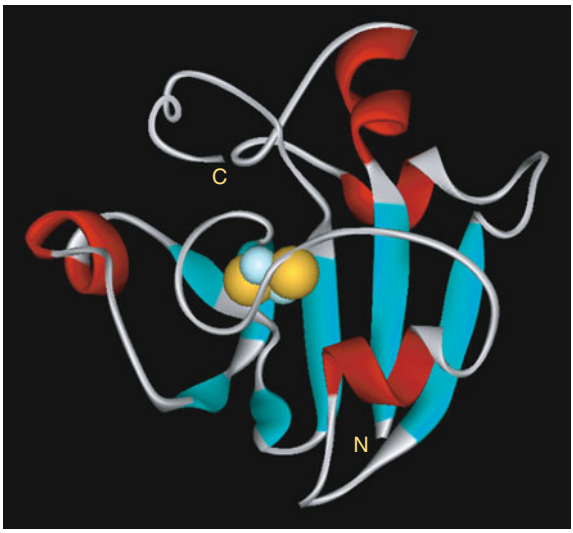

Fig. 3.5.7
Three-dimensional structure of the *Ha. japonica* Fd

at high salt concentrations. On the other hand, substitution of the N-terminal domain of the *Ha. japonica* Fd to that of the spinach Fd injured the solubility under high salt conditions. Thus, it was concluded that the N-terminal domain of the *Ha. japonica* Fd should perform the essential functions for haloadaptation from the folding process through the folded state.

FtsZ

FtsZ is a key protein in the cell division processes of *E. coli* and other bacteria (Erickson 1997; Lutkenhaus and Addinall 1997). It self-assembles to form a circumferential ring at the inner face of cytoplasmic membrane at the division site. During cell cycle, the ring designated an FtsZ ring directs a process of septation. FtsZ is thought to be the prokaryotic homolog of eukaryotic tubulins based on its biochemical properties. Like tubulins, the *E. coli* FtsZ is a GTP/GDP binding protein with GTPase activity and polymerization activity, which forms filaments in the presence of GTP (de Boer et al. 1992; RayChaudhuri and Park 1992; Bramhill and Thompson 1994; Mukherjee and Lutkenhaus 1994).

The *ftsZ* genes were found not only in bacteria but also in archaea. Several *ftsZ* genes were cloned from *Hb. salinarum* (Margolin et al. 1996), *Hf. volcanii* (Wang and Lutkenhaus 1996), and *Haloferax mediterranei* (Poplawski et al. 2000). Recent genome analyses indicated that *Hb. salinarum* NRC-1 and *Ha. marismortui* had five *ftsZ* paralogs (Ng et al. 2000; Baliga et al. 2004).

The *ftsZ1* gene was cloned from *Ha. japonica* (Ozawa et al. 2005). The *ftsZ1* gene contained an open reading frame encoding 386 amino acids. The *Ha. japonica* FtsZ1 indicated 68% and 33% identities to the *Hb. salinarum* FtsZ1 and the *E. coli* FtsZ, respectively. The *Ha. japonica* FtsZ1 had the same amino acid sequence as of the *Ha. marismortui* FtsZ1 deduced from the genome sequence except only one amino acid. The GTP-binding motif, GGGTGTG (in single letter codes), was also conserved in the *Ha. japonica* FtsZ1.

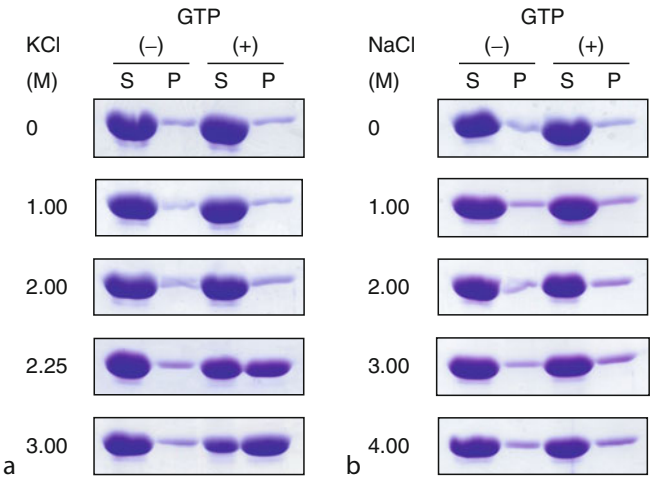

Fig. 3.5.8

Effects of KCl (a) and NaCl (b) concentrations on polymerization of the *Ha. japonica* FtsZ1 in the presence (+) or absence (–) of GTP.S, supernatant; P, pellet

A recombinant FtsZ1 with hexahistidine-tag was successfully expressed in *Ha. japonica* (Ozawa et al. 2005). The recombinant FtsZ1 exhibited GTP-dependent polymerization activity and GTPase activity. Polymerization of the recombinant FtsZ1 was observed at KCl concentrations of 2.25–3.00 M, while it showed no polymerization activity at KCl concentrations below 2.00 M (*Fig. 3.5.8a*). The FtsZ1 polymer was not detected when using the reaction mixtures containing several concentrations of NaCl instead of KCl (*Fig. 3.5.8b*). Similar to polymerization activity, GTPase activity of the recombinant FtsZ1 also required high concentrations of KCl. This is the first report about in vitro polymerization of a haloarchaeal FtsZ.

Foreign Gene Expression in *Ha. japonica*

Chitin is a polysaccharide that made up of N-acetylglucosamine by β-1,4-linkages, and chitinase is an enzyme that can degrade chitin into small molecules. Chitinases occur widely in bacteria, archaea, and fungi, while chitinases from extremely halophilic archaea have not been reported to date. Recently, the complete genome sequence of *Hb. salinarum* NRC-1 was reported (Ng et al. 2000), and a chitinase-homolog (named ChiN1) was found in the genome.

The *chiN1* gene encoded a Tat (twin-arginine translocation)-type signal peptide of 28 aa, followed by a 518-aa mature sequence. The majority of proteins in prokaryotes are translocated *via* the ubiquitous Sec system (Mori and Ito 2001). In extremely halophilic archaea, however, most proteins are predicted to translocate *via* the Tat system (Bolhuis 2002; Rose et al. 2002). The Tat system is unique in its ability to translocate folded proteins across membranes. Halophilic archaea accumulate high levels of salt intracellularly, and newly synthesized haloarchaeal secretory proteins have to fold very rapidly to prevent aggregation before reaching the membrane.

The deduced amino acid sequence revealed that the mature ChiN1 was composed of three domains: a chitin-binding domain of carbohydrate-binding module (CBM) family 5 (http://afmb.cnrs-mrs.fr/CAZY/), a functionally unknown polycystic kidney disease domain (PKD),

and a catalytic domain (CatD) of glycoside hydrolase (GH) family 18 (http://afmb.cnrs-mrs.fr/CAZY/). The sequence F-D-G-L-D-I-D-W-E (in single letter codes) found in ChiN1 matched the consensus sequence pattern [LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-X-E of GH family 18 chitinases (http://www.expasy.ch/sprot/prosite.html). The last residue E (glutamic acid) is the potential catalytic residue that is conserved in all of GH family 18 chitinases. The mature sequence of ChiN1 proved to be rich in acidic amino acids with a 17% aspartic-acid/glutamic-acid content, corresponding to the nature of proteins from extremely halophilic archaea.

The *chiN1* gene was successfully expressed in *Ha. japonica* by employing the *E. coli*-haloarchaea shuttle vector pWL102 (Lam and Doolittle 1989) and the promoter sequence of *Ha. japonica* cell surface glycoprotein (CSG) gene (Wakai et al. 1997). A large amount of recombinant ChiN1 was secreted into the culture medium of *Ha. japonica* (Yatsunami et al. 2010). The recombinant ChiN1 was purified and characterized. Optimal pH and temperature of the recombinant ChiN1 were pH 4.5 and 55°C, respectively. The enzyme was most active at 1.0 M NaCl and stable over a wide range of NaCl concentration from 1.0 to 4.5 M (*Fig. 3.5.9*). This is the first report on a chitinase from extremely halophilic archaeon.

Genome Analysis and Postgenomics

Whole Genome Analysis

Until now, genomes of several categories of halophilic archaea have been sequenced (Ng et al. 2000; Baliga et al. 2004; Falbet et al. 2005; Bolhuis et al. 2006; Pfeiffer et al. 2008; Anderson et al. 2009; Malfatti et al. 2009; Saunders et al. 2009; Tindall et al. 2009). The genome of *Haloarcula japonica* has also been sequenced (Nakasone et al. manuscript in preparation). The 4,294,979-base pairs (bp) genome of *Ha. japonica* is divided into relatively high and low G+C content replicons, including the largest chromosome I, a 3,028,012-bp replicon with a G+C content of 62.7%, and four smaller replicons ranging from 51,166 to 613,700 bp with G+C contents ranging

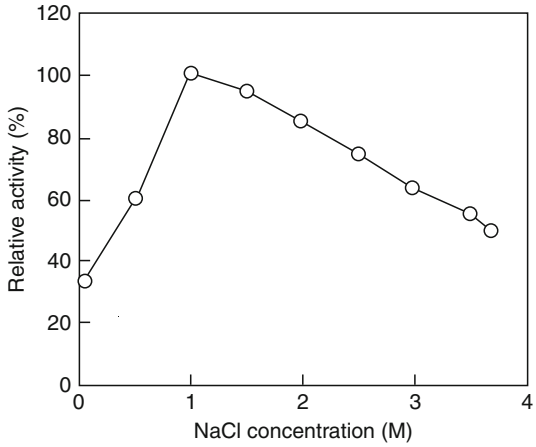

Fig. 3.5.9
Effect of NaCl concentration on activity of the recombinant ChiN1

from 54.7% to 58.7% (Table 3.5.1). The total number of predicted protein coding sequences (CDSs) was 4,057, and 2 *rrn* operons were found only in chromosome I (Fig. 3.5.10).

Figure 3.5.11 shows the phylogenetic tree for genome-completed halophilic archaea, as well as genome sizes and numbers of replicons. Several halophilic archaea vary in genome size and number of replicons. The genome of *Ha. japonica* was compared with that of closely related *Haloarcula marismortui* (Baliga et al. 2004). The genome size of *Ha. japonica* is

Table 3.5.1

General feature of the genome of *Ha. japonica*

Replicon	Size (bp)	No. CDSs	No. rRNA	No. tRNA	G+C content (%)
Chromosome I	3,028,012	2,928	2	45	62.73
Chromosome II	613,700	562	0	1	56.79
Plasmid pHJ1	506,725	429	1	0	58.74
Plasmid pHJ2	95,376	90	0	0	54.77
Plasmid pHJ3	51,166	48	0	0	56.63
(Total)	4,294,979	4,047	3	46	57.93

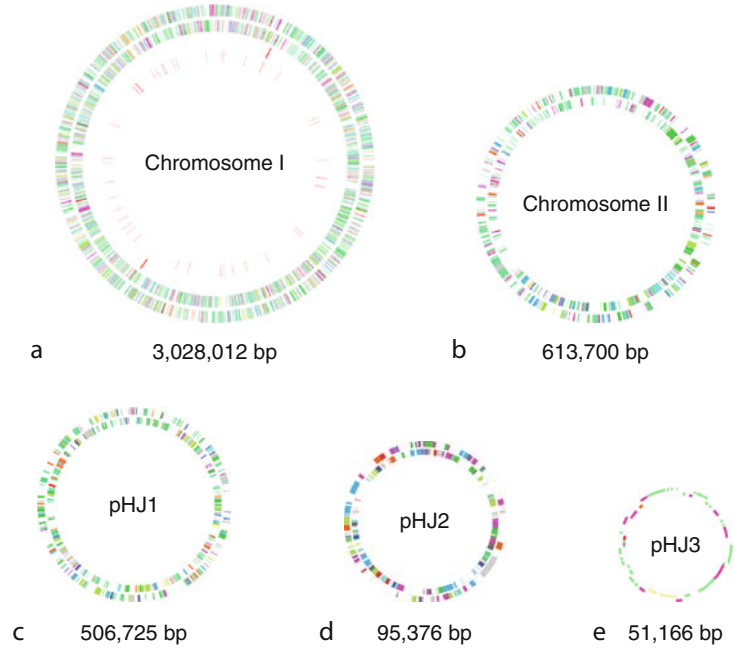

Fig. 3.5.10

Graphical circular maps of the genome of *Ha. japonica*(a) chromosome I; (b) chromosome II; (c) plasmid pHJ1; (d) plasmid pHJ2; (e) plasmid pHJ3. From outside to the center: genes on forward strand [color by Clusters of Orthologous Groups of proteins (COG) categories], genes on reverse strand (color by COG categories). rRNA and tRNA genes were shown in chromosome I

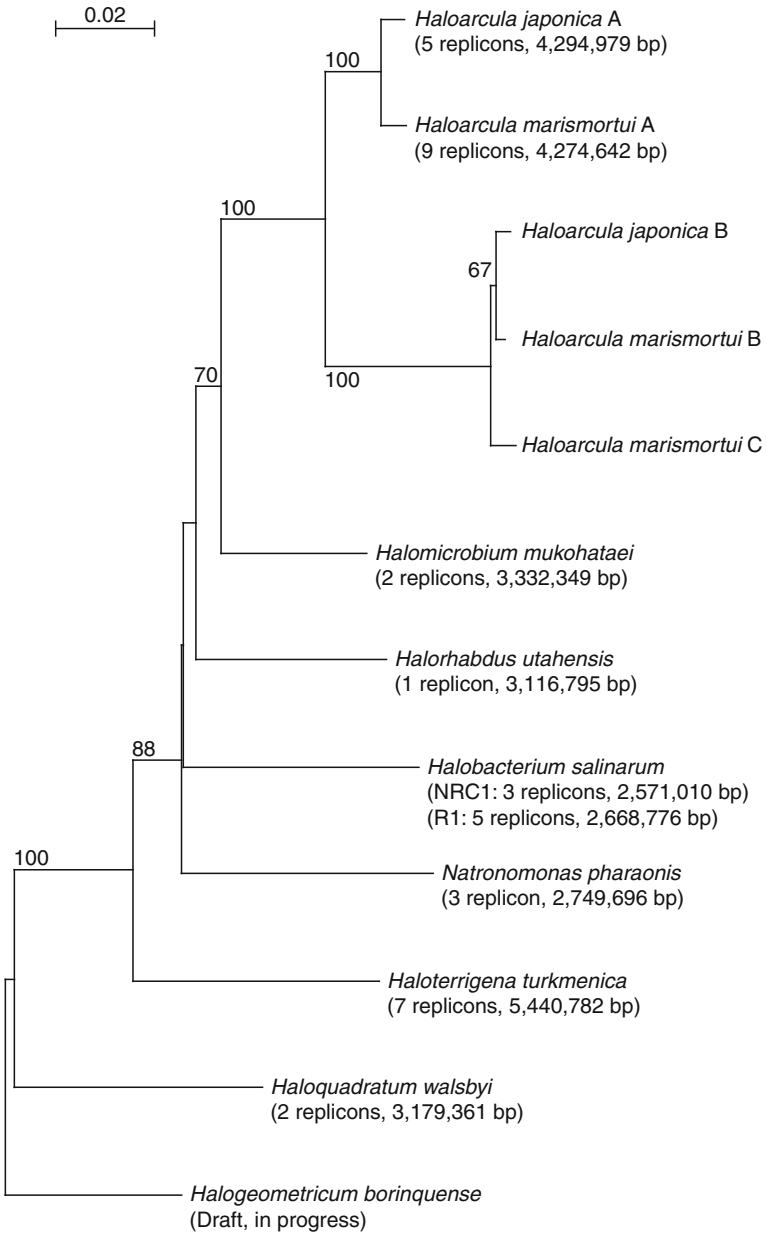

Fig. 3.5.11

Phylogenetic tree for genome-completed halophilic archaea.The genome sizes and numbers of replicons were shown below the species names

relatively similar to that of *Ha. marismortui*, whereas the number of replicons in *Ha. japonica* is fewer than that in *Ha. marismortui*. The chromosomes I of both strains are very similar, in terms of size and genomic organization. For the chromosomes II, the sizes are 613,700 bp in *Ha. japonica* and 288,050 bp in *Ha. marismortui*. In addition, gene contents and genomic

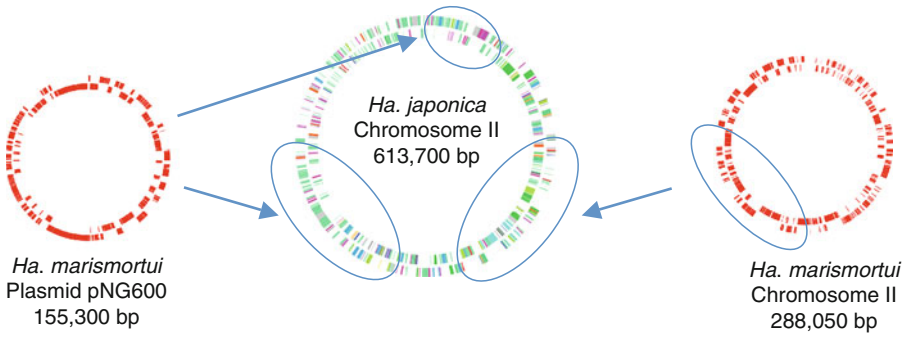

Fig. 3.5.12

Possible rearrangement of *Ha. japonica* chromosome II from *Ha. marismortui* chromosome II and plasmid pNG600

organizations between both strains seem to be different. Thus, further comparative analysis of the chromosomes was carried out to make genome rearrangement maps. The analysis revealed that the chromosome II of *Ha. japonica* is fusion or mosaic, rearranged with the chromosome II, plasmid pNG600, and partially plasmid pNG700 of *Ha. marismortui*. Possible rearrangement from these replicons was summarized in *Fig. 3.5.12*. The generation mechanism of chromosome II in *Ha. japonica* is very interesting, and future comparative genomics and postgenomics will be required to understand it.

Diversity of TATA Binding Proteins

Whereas many researches have been directed toward determining the mechanism and regulation of transcription in eukarya and bacteria (eubacteria), relatively few works have been conducted on archaeal transcription systems. Over the past decades, however, several lines of evidence have suggested that the transcription systems of eukarya and archaea are fundamentally homologous, including RNA polymerase, and basal transcription factors TATA binding protein (TBP) and transcription factor B (TFB). TBP is a basal transcription factor involved in transcription initiation in eukarya and archaea. Through exhaustive analysis of the whole genome of extremely halophilic archaeon *Ha. japonica*, six *tbp* genes were found (Nakasone et al. manuscript in preparation). The *Ha. japonica tbp* genes, designated as *tbpA*, *tbpB*, *tbpC*, *tbpD*, *tbpE*, and *tbpF*, were thought to diverge from other archaeal *tbp* genes that had been known. The *tbpA* was found to encode a polypeptide consisting of 182 aa, showing 35.0% identity to that of *Ha. marismortui*. The *tbpB* was found to encode a polypeptide consisting of 182 aa, showing 36.5% identity to that of *Ha. marismortui*. The *tbpC* was found to encode a polypeptide consisting of 186 aa, showing 100% identity to that of *Ha. marismortui*. The *tbpD* was found to encode a polypeptide consisting of 185 aa, showing 42.9% identity to that of *Ha. marismortui*. The *tbpE* was found to encode a polypeptide consisting of 182 aa, showing 35.4% identity to that of *Ha. marismortui*. The *tbpF* was found to encode a polypeptide consisting of 182 aa, showing 46.1% identity to that of *Ha. marismortui*. The TBPC is conserved in amino acid sequence with other archaeal strains including methanogens and thermophiles. It may suggest that the TBPC is the core TBP that functions in transcriptional initiation such as

housekeeping genes. It has been already reported that there are some variations in the multiple *tbp* genes and their expression during growth in different *Halobacterium salinarum* strains (Katharina et al. 2008). This study suggests environmental adaption in the stationary phase under aerobic and anaerobic growth conditions.

Studies on disruption mutants of these *tbp* genes were also carried out. Several plasmids were constructed to disrupt the *tbp* genes with single crossover recombination. Protoplast transformation of *Ha. japonica* with these plasmids allowed obtaining several independent disruption mutants of the *tbp* genes. Growth studies on the disruptants showed that delayed growth was observed with the *tbpC* disruptant and no effects with other mutants (Fig. 3.5.13). The result suggests that the TBPC has a crucial role in *Ha. japonica* and other TBPs are not essential. Future postgenomics such as microarray analysis and proteomics using these mutants will definitely be useful to understand the global regulation by these TBPs in *Ha. japonica*.

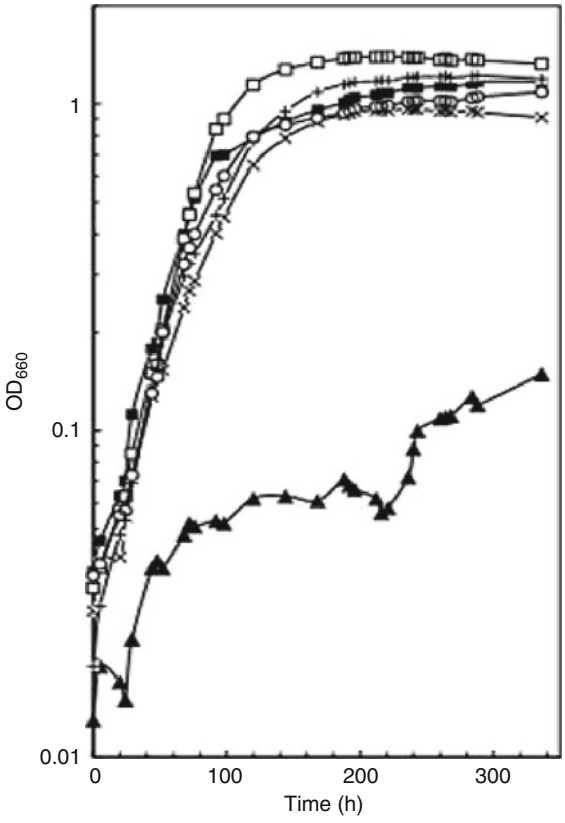

Fig. 3.5.13

Growth of *tbp* gene disruption mutants.○, wild-type (control); ■, *tbpB* disruption mutant; ▲, *tbpC* disruption mutant; ×, *tbpD* disruption mutant; □, *tbpE* disruption mutant; +, *tbpF* disruption mutant

Uracil Auxotrophy for Reverse Genetics

Reverse genetics is crucial technology for postgenomics of *Ha. japonica*. The gene disruption system by homologous recombination has already been developed in *Hb. salinarum* (Peck et al. 2000). The *pyrF* gene, encoding orotidine-5′-phosphate decarboxylase, was used for a counterselectable marker in this system. As a prerequisite for future establishing the *Ha. japonica* gene manipulation system, the single copy *pyrF* gene was cloned from *Ha. japonica* (Nakasone et al. manuscript in preparation). A plasmid for *pyrF* gene disruption was constructed using the shuttle vector pWL102 (Lam and Doolittle 1989) and was introduced into *Ha. japonica*. Transformants were screened with 5-fluoroorotic acid (5-FOA) to generate a uracil-auxotrophic mutant through homologous recombination. The isolated mutant was designated as *Ha. japonica* strain KUH01. Establishment for an efficient new genetic strategy toward the systematic disruption of several genes in *Ha. japonica* is now being proceeded.

Cross-References

- 3.1 Taxonomy of Halophiles
- 3.2 Diversity of Halophiles
- 3.4 Ecology of Halophiles

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3.6 Adapting to Changing Salinities: Biochemistry, Genetics, and Regulation in the Moderately Halophilic Bacterium *Halobacillus halophilus*

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_3.6,

Introduction

The availability of water is the most important prerequisite for life of any living cell and exposure of cells to hypersaline conditions always threatens the cells with a drastic loss of water followed by shrinkage and cell death if no counter measures are taken. To re-establish the essential turgor pressure, cells increase the water activity of their cytoplasm using one of two principal strategies: the "salt-in-cytoplasm" and the "compatible solute" strategy. The former describes the accumulation of inorganic ions, mainly K^+ and Cl^-, in the cytoplasm, until the internal salt concentration is similar to the extracellular one. This strategy can be found in extremely halophilic *Halobacteria* (*Archaea*) and halophilic, anaerobic *Haloanaerobiales* (*Bacteria*) and *Salinibacter ruber*. This strategy is not covered here but the reader is referred to excellent reviews (Galinski and Trüper 1994; Ventosa et al. 1998). The vast majority of bacteria cope with increasing osmolality by uptake or synthesis of compatible solutes, which are defined as small, highly soluble, organic molecules that do not interfere with the central metabolism, even if they are accumulated to high concentrations (Brown 1976). This strategy is widespread and evolutionarily well conserved in all three domains of life (Bohnert 1995; Kempf and Bremer 1998; Roberts 2000; Roeßler and Müller 2001b). Cell turgor adjustement is an obvious challenge for a halophilic organism and we shall concentrate in this review on recent progress in the Gram-positive model bacterium *Halobacillus halophilus*, a moderate halophile isolated from the salt marsh. This organism is not only a model to study adaptation of moderate halophiles to changing salinities in their environment, but also for the role of the halide chloride since *H. halophilus* is the first prokaryote known for its strict chloride dependence (Saum and Müller 2008a).

Adjusting Cell Turgor to Different External Salinities

H. halophilus copes with the effect of external salt by accumulating different compatible solutes, mainly amino acids such as glutamine, glutamate, proline, and alanine and also amino acid derivatives such as N^{ε}-acetyl lysine, N^{δ}-acetyl ornithine, glycine betaine, and ectoine (Severin 1993). Interestingly, *H. halophilus* switches its osmolyte strategy with the salinity of the environment. At moderate salinities (1.5 M NaCl), glutamine and glutamate are the predominant solutes, whereas proline dominates at salinities around 3 M NaCl (Saum and Müller 2007). In addition, there is a growth phase-dependent regulation at high salt: proline is dominant in the exponential phase but replaced to some extent by ectoine in the stationary phase (Saum and Müller 2008b). The biosynthetic routes for the solutes and their regulation have been identified (Saum et al. 2006; Saum and Müller 2007, 2008b) and are described in the next chapters.

Biosynthesis of Glutamate and Glutamine

The biosynthesis of glutamate can either be catalyzed by a glutamate synthase (GOGAT) or by a glutamate dehydrogenase (GDH) while glutamine is synthesized by the action of a glutamine synthetase (GS). Based on the genome sequence, the situation in *H. halophilus* resembles that of *Bacillus subtilis* (Kunst et al. 1997). *H. halophilus* has two isogenes for glutamate dehydrogenase (*gdh1* and *gdh2*), one gene encoding a large subunit of a glutamate synthase (*gltA*), and two

genes encoding the small subunit of a glutamate synthase (*gltB1* and *gltB2*) (Saum et al. 2006). A clue to the origin of glutamate in *H. halophilus* came up when cells were subjected to an osmotic upshock from 0.8 to 2 M NaCl rather than using preadapted cells. While the genes encoding the subunits for the glutamate synthase were not affected by the sudden increase of salinity, one of the putative glutamate dehydrogenase genes (*gdh1*) was induced and the mRNA level increased within 1.5 h to about fourfold compared to the level before the upshock. It was, therefore, suggested that the compatible solute glutamate is produced by the Gdh 1 that converts one molecule of 2-oxoglutarate and one molecule of NH_4^+ reductively to one molecule of glutamate (Csonka and Epstein 1996). Transcripts of the second glutamate dehydrogenase gene (*gdh2*) were close to the detection limit and therefore no final conclusion on the regulation could be made. However, the presence of transcripts points to a physiological functionality. It is likely that the Gdh 2 is involved in nitrogen metabolism rather than osmoregulation. In contrast to the upshock experiments, transcriptional analyses did not reveal a salinity dependence of expression of *gdh1*, *gdh2*, or *gltA* although the glutamate concentration increased with increasing salinity. Furthermore, glutamate dehydrogenase or glutamate synthase activity was not detected in cell-free extracts.

Glutamine is synthesized by the action of a glutamine synthetase in *H. halophilus* (Saum et al. 2006) (Fig. 3.6.1). The genome encodes two isoenzymes. While the first one (*glnA1*) is clustered with a gene (*glnR*) encoding a regulatory protein with very high similarity to the regulator GlnR of *B. subtilis* that is known to be essential in nitrogen metabolism, the second one (*glnA2*) lies solitary and is preceded by a putative promoter that shares very high similarity to the promoter of *B. subtilis* that is recognized by σ^B, the general stress σ factor. Expression of *glnA2* is salinity dependent with a maximal increase of transcripts of about fourfold (compared to the value at 0.4 M NaCl) at 1.5 M NaCl or higher. Expression of *glnA1*, however, was not altered by different salinities. The existence of two isoenzymes can be regarded as a simple means for regulation that makes it feasible to separate and process completely different physiological demands: nitrogen-metabolism and osmoregulation.

Glutamine synthetase activity in cell-free extracts of *H. halophilus* increased with increasing salinity of the growth medium (Saum et al. 2006). Interestingly, maximal activity was not found at intermediate salt concentrations as expected from the gene expression data and the glutamate concentration measured in the cells, but was found at 2.5 M NaCl or higher. This observation does not necessarily reflect a contradiction, but rather underlines the role of glutamine, and also glutamate, that can easily be made from glutamine, as precursor molecules for other building blocks and finally compatible solutes such as proline or ectoine at higher salinities, as discussed later.

The Biosynthesis of Proline

The inspection of the genome revealed a cluster of three genes – *proH*, *proJ*, and *proA*, which are organized in an operon – that encode for putative proline biosynthesis enzymes (Fig. 3.6.2). The enzymes are a putative pyrroline-5-carboxylate reductase (ProH), a putative glutamate 5-kinase (ProJ), and a putative glutamate 5-semialdehyde dehydrogenase (ProA). The amount of transcript of the *pro* operon increased with increasing salinity of the medium and was maximal at 2.5 M NaCl. At the same time, proline concentrations were maximal at 3 M NaCl.

Similar to *B. subtilis* (Belitsky et al. 2001), the complete genome of *H. halophilus* contains two more genes with the potential to encode pyrroline-5-carboxylate reductase (*proC and comER*).

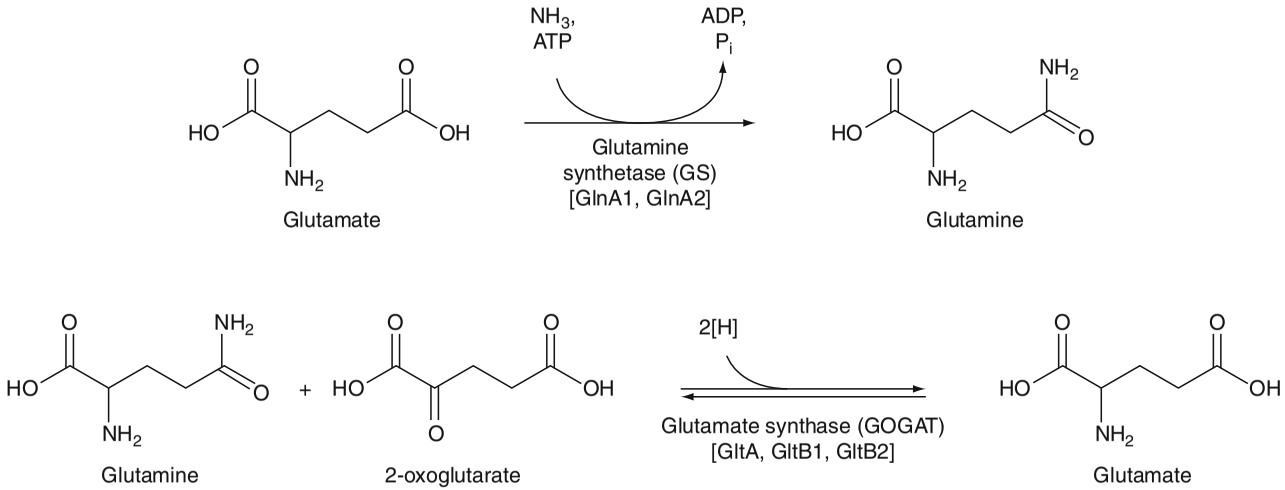

Fig. 3.6.1

Pathways for the synthesis of the compatible solutes glutamine and glutamate in *H. halophilus*

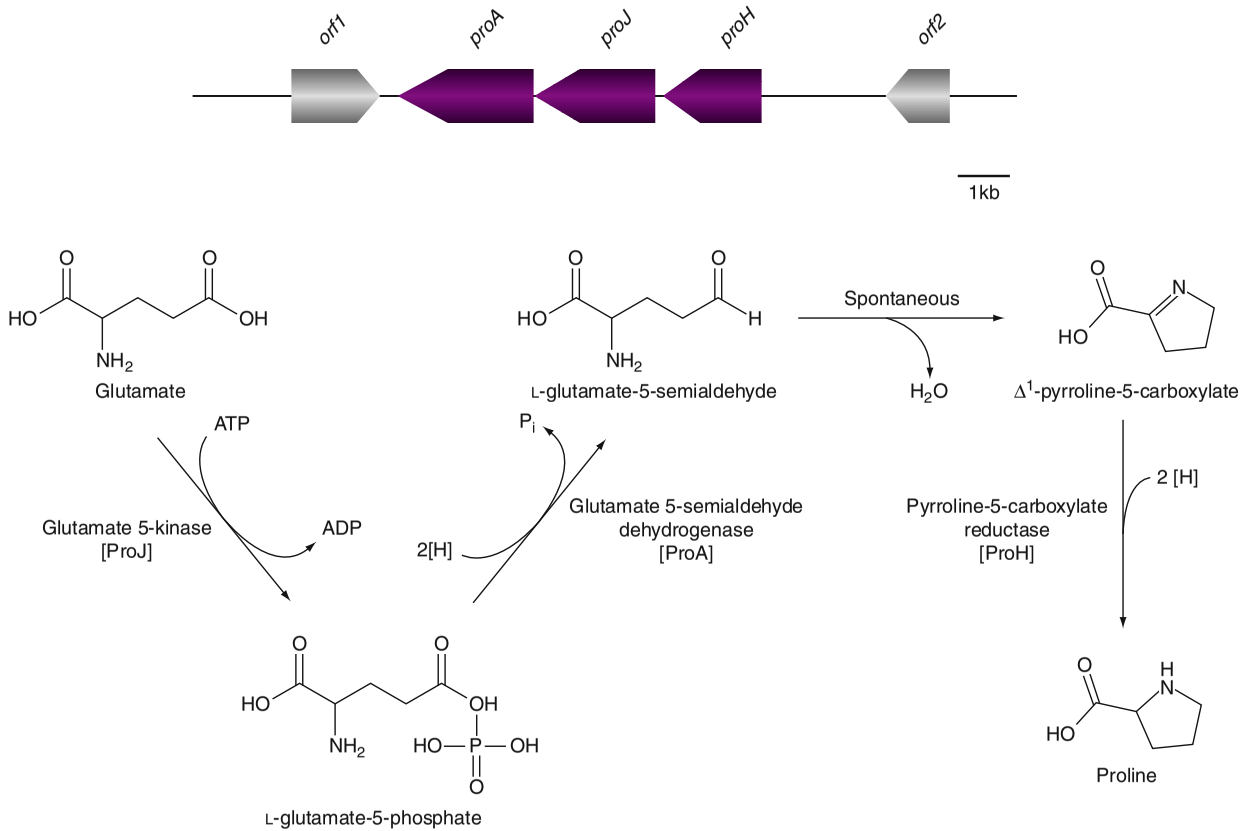

Fig. 3.6.2

Genetics and pathway of proline biosynthesis in *H. halophilus*. *proH:* pyrroline-5-carboxylate reductase, *proJ:* glutamate 5-kinase, *proA:* glutamate 5-semialdehyde dehydrogenase, *orf1*: hypothetical protein, *orf 2*: hypothetical protein

The function of these two genes is not yet known. But participation of at least three genes in the last step of proline biosynthesis may reflect specialized functions and/or regulation.

After an osmotic upshock with NaCl, expression of the *proHJA* operon was turned on immediately and transcript levels reached a maximum already 1.5 h after the upshock (Saum and Müller 2007). At the same time, the proline concentration increased and reached a maximum after 6 h. This result demonstrated that salinity (NaCl) triggers the production of proline. Interestingly, addition of (unphysiologically high) glutamate concentrations also led to an accumulation of proline and also turned on expression of the *proHJA* operon. The stimulating effect of glutamate on the transcription of the *pro* genes was addressed in more detail and it was found that a minimal concentration of 0.2 M glutamate is sufficient to stimulate *pro* gene transcription (Saum and Müller 2007).

The Biosynthesis of Ectoine

Ectoine is mainly produced at high salinities, but in contrast to other organisms ectoine is not the dominant solute at high salinities in *H. halophilus* (Saum and Müller 2008b). The biosynthetic genes (*ectABC*) for the production of ectoine from aspartate semialdehyde were identified and were shown to form an operon (*Fig. 3.6.3*). While expression of the *ectABC* operon and the ectoine concentrations were low at low salinities (0.4–1.5 M NaCl), they increased at elevated salinities (2–3 M NaCl). As observed for the *ect* promoter of *Chromohalobacter salexigens* fused to the *lacZ* gene (P_{ect}-*lacZ*) *ectABC* expression was maximal in the early stationary growth phase (Calderon et al. 2004). At the same time, the proline concentration decreased. This observation hints to a novel, additional layer of regulation and it can be assumed that ectoine production in the late stationary phase reflects an answer to the stress situation that the cells experience in stationary cultures. To resolve the time-dependent kinetics of ectoine production, cells were subjected to an osmotic upshock from 0.8 to 2 M NaCl. Transcripts were readily detectable already at time point 0, but increased dramatically with time and reached a maximum not before 3 h after upshock. Most important, expression of *ect* genes was preceded by expression of genes responsible for glutamine, glutamate, or proline biosynthesis. The signal leading to *ect* gene transcription is therefore assumed to be an indirect one mediated by one or more yet to be identified factors rather than by the presence of the osmolyte. The production of the ectoine synthase EctC nicely corresponded to the increase of *ectC* transcript. Both were found to increase twofold 4 h after upshock. Surprisingly, 4 h after upshock, the EctC content again decreased with time and the level reached a value only slightly above the value at the beginning, although the external stress was still present. This decrease, however, was not reflected in the ectoine concentration, which steadily increased and reached a maximum 18 h after upshock (Saum and Müller 2008b). Again, this demonstrates a great delay in accumulation compared to proline that reached its maximum already 6 h after upshock and hints to a role of ectoine not only in the immediate response to osmotic upshock, but also to a function as a more general protectant in the cell. This idea is corroborated by the results of different groups that showed that ectoine is able to function as a very powerful stabilizing agent of whole cells or enzymes against a number of stresses like thermal denaturation, cryo–damage, or UV radiation (Lippert and Galinski 1992; Louis et al. 1994; Canovas et al. 1999; Buenger and Driller 2004). An explanation for the only temporary increase of protein would be that the reaction catalyzed by this enzyme is not the rate-limiting step in ectoine biosynthesis. An increase in enzyme concentration is not necessary and translation

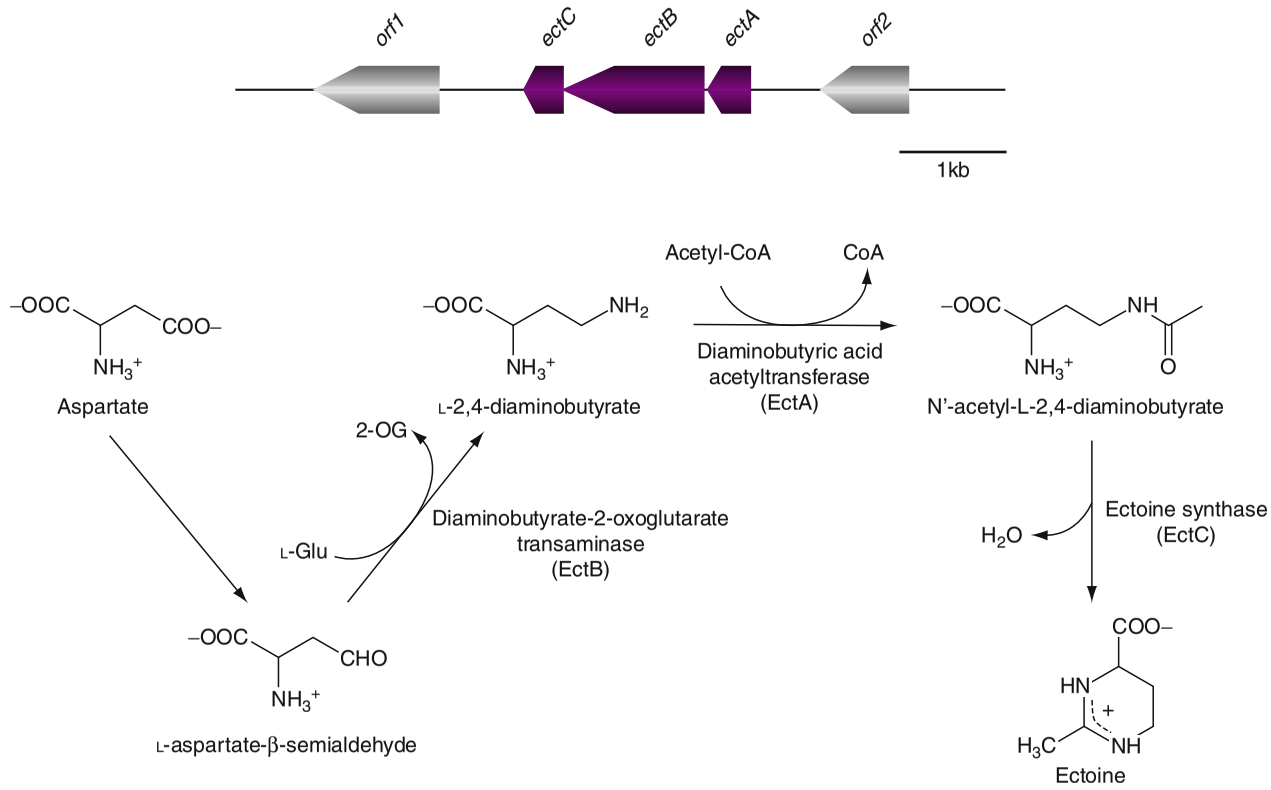

Fig. 3.6.3

Genetics and pathway of ectoine biosynthesis in *H. halophilus*. *ectA:* diaminobutyric acid acetyltransferase, *ectB:* diaminobutyrate-2-oxoglutarate transaminase, *ectC:* ectoine synthase, *orf1*: hypothetical protein, *orf2*: hypothetical protein

therefore reduced. It is also conceivable that the ectoine synthase is regulated on the activity level by the increase of chloride within the cell and, therefore, makes an increase of EctC almost unnecessary.

The Biosynthesis of Glycine Betaine from Choline

H. halophilus cannot synthesize glycine betaine de novo, but can take up the precursor choline and oxidize it in the cytoplasm to glycine betaine. Oxidation of choline is induced by the salinity of the environment and repressed by exogenous glycine betaine. The genes encoding the choline dehydrogenase (*gbsB*) and the glycine betaine aldehyde dehydrogenase (*gbsA*) were identified and shown to constitute an operon (*Fig. 3.6.4*) (Burkhardt et al. 2009). GbsB is similar to the choline dehydrogenases of the GMC (glucose methanol choline) oxidoreductase family that are known to use FAD as a cofactor (Cavener 1992). Moreover, a putative glycine box (GXGXXG) could be identified in the N-terminal sequence of GbsB, which characterizes flavin proteins. Localization experiments showed that similar to BetA from *E. coli* (Lamark et al. 1991), GbsB from *H. halophilus* is membrane bound (Burkhardt et al. 2009). GbsA is a soluble protein and predicted to be NAD^+ dependent. A similar cluster was found in *H. dabanensis,* and *gbsA* (1,472 bp) and *gbsB* (1,688 bp) of *H. dabanensis* have been shown experimentally to encode a glycine betaine aldehyde dehydrogenase and a choline

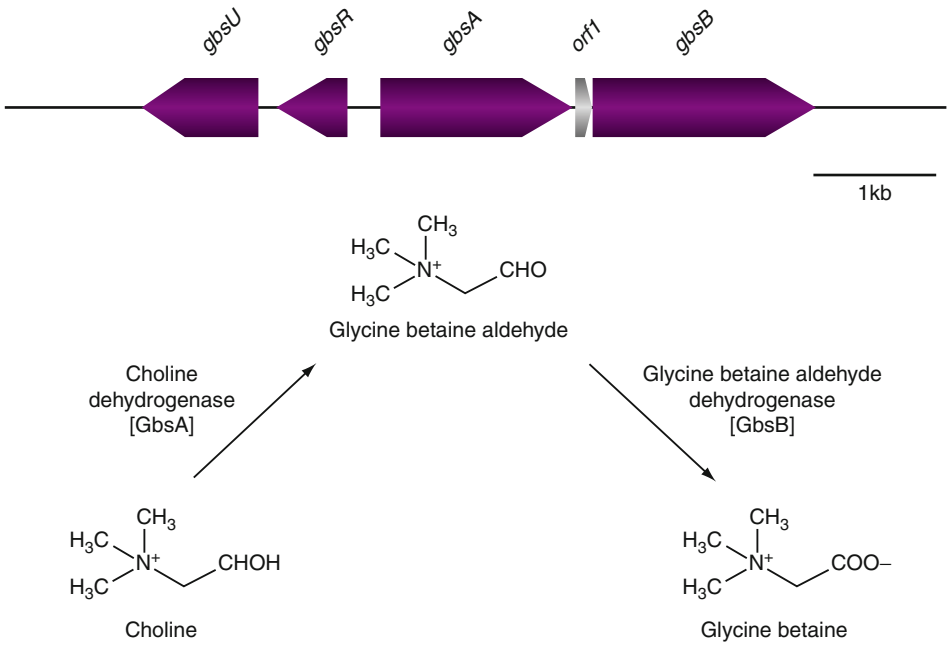

Fig. 3.6.4

Genetics and pathway of glycine betaine formation from choline in *H. halophilus*. *gbsA*: choline dehydrogenase, *gbsB*: glycine betaine aldehyde dehydrogenase, *gbsR*: transcriptional regulator, *gbsU*: glycine betaine binding protein, *orf1*: hypothetical protein

dehydrogenase, respectively (Gu et al. 2008). Divergent to this operon is another operon containing *gbsR* and *gbsU* that encode proteins with similarities to a transcriptional regulator and a glycine betaine-binding protein of ABC transporters, respectively (Burkhardt et al. 2009). Again, a similar cluster is present in *H. danabensis* (Gu et al. 2008). GbsR was found in the cytoplasm, but was also present in the cytoplasmic membrane although in a smaller fraction. The physiological relevance of the membrane localization of this protein remains unclear since hydrophobic domains that are common in these proteins may interact unspecifically with the membrane. GbsU has a hydrophobic N terminus that anchors the protein to the membrane and, therefore, was found experimentally in the membrane. It also carries a sequence L-A-A-C-G-S in the N-terminal part corresponding to the consensus (L-Y-Z-cleavage site-C-y-z; where Y is A, S, V, Q, T; Z is G or A; y is S, G, A, N, Q, D; z is S, A, N, Q) of the precursors of lipoproteins (Yamaguchi et al. 1988). Thus it appears very likely that GbsU is a lipoprotein, like other substrate-binding proteins in Gram-positives, for example, OpuAC of *B. subtilis* (Kempf et al. 1997) and, therefore, was found experimentally in the membrane.

The regulation of the *gbs* clusters is rather complex and involves several signals. Expression was strictly dependent on the choline concentration. There was no expression in the absence of choline and the cellular amount of Gbs proteins increased when the choline concentration increased from 0 to 1 mM (Burkhardt et al. 2009). A threshold of Gbs protein production was reached at about 20 μM choline. Compared to the articulate essentiality of choline, salinity has a less pronounced effect on cellular levels of GbsA, GbsR, and GbsU. At saturated amounts of choline (1 mM), the protein concentrations were already relatively high at the lowest salt concentration used, but increased by 47%, 40%, and 26% when the salinity was increased from 0.8 to 3 M NaCl. In contrast, the concentration of GbsB was strictly salt dependent. Therefore, Gbs protein synthesis and/or stability are regulated differentially by salinity. This additional layer of regulation may involve posttranscriptional mechanisms such as differentially regulated protein production or protein stability unknown at present time. The analysis of the regulatory patterns involved is a challenging task for future experiments.

Glycine betaine can also be taken up by *H. halophilus* and uptake is always preferred for energetic reasons over de novo synthesis. The presence of glycine betaine in the medium indeed caused a reduction of the cellular concentration of all four Gbs proteins. Half maximal inhibition was observed at 0.1 mM glycine betaine. Repression by glycine betaine may involve the glycine-binding protein GbsU.

The binding protein GbsU is a subunit of an ABC transporter. These types of transporter are composed of a substrate-binding protein, a transmembrane protein catalyzing transport, and a motor domain, the ATPase. However, there are no transporter or motor genes in the vicinity of the *gbs* genes. Often, the genes encoding the subunits of ABC transporter build an operon in prokaryotes. This might be different in *H. halophilus*. Inspection of the genomic sequence revealed genes for postulated ABC transporter-type permeases and ATP-hydrolyzing subunits that are not transcriptionally linked to a gene encoding a substrate-binding protein. The finding of an ABC transporter subunit in a gene cluster mediating the oxidation of choline is novel and the key question is the function of GbsU. Since *gbsU* and *gbsR* form an operon, it is clear that GbsU is involved in the pathway or its regulation. Since there are potential choline transporters encoded by the *H. halophilus* genome a function of GbsU in choline uptake is unlikely. Moreover, although the substrate specificity is hard to delineate from sequence alignments, GbsU is a glycine betaine rather than a choline-binding protein. The finding that GbsR is localized to some extent at the membrane together with the membrane localization of GbsU could also suggest a transmembrane communication of the signal “choline” to the

transcriptional regulator GbsR via GbsU. However, although attractive at a first glance, this seems to be unlikely since there is no unusual feature of GbsU that would allow it to transmit a signal to the cytoplasm. Apart from the N-terminal hydrophobic, α-helical stretch that serves as a membrane anchor in Gram-positives there is no other transmembrane region in GbsU.

Could GbsU be involved in measuring the intracellular glycine betaine concentration and in transferring a negative feedback regulation? In general, if a cell produces different solutes and the sum of the solutes determines the turgor, then measuring "concentrations" of single components of the solute cocktail makes no sense. Since glycine betaine contributes only a small fraction to the intracellular osmolyte pool in *H. halophilus,* we assume that GbsU is not involved in measuring intracellular glycine betaine concentrations.

Instead, a GbsU containing ABC transporter may be involved in repression of the system by glycine betaine. It may serve as a system to shut down expression of *gbsA* and *gbsB* immediately when the cells encounter exogenous glycine betaine. This would make sense only if the permease and motor component of the transporter are constitutively produced. This system would allow reacting on exogenous but not intracellular glycine betaine. To date, nothing is known about the regulation of osmolyte transporter in (moderate) halophiles. Once inside the cell, glycine betaine is bound by GbsR which then represses transcription of GbsA and GbsB. This regulatory network is now under investigation in our laboratory.

Uptake of Glycine Betaine from the Environment

Like other organisms, *H. halophilus* takes up glycine betaine from the environment. The intracellular glycine betaine concentration was strictly dependent on the extracellular NaCl concentration (Roeßler and Müller 2001a). The intracellular glycine betaine concentration was calculated using the experimentally determined intracellular volume to 60 mM at 250 mM NaCl and 365 mM at 1 M NaCl. Uptake rates for glycine betaine under isoosmotic conditions were stimulated by increasing salt concentrations. Uptake of glycine betaine required both, Na^+ and Cl^-. Cl^- could be substituted by nitrate and bromide, but not by sulfate. Glycine betaine transport was optimal at around 0.7 M Cl^-. Cells also accumulated glycine betaine after an osmotic upshock, but only in the presence of chloride. These studies revealed the first chloride-dependent glycine betaine transporter in a prokaryote (Roeßler and Müller 2001a).

Regulation of Compatible Solute Formation

A Chloride Modulon in *H. halophilus*

Chloride is absolutely necessary for growth of *H. halophilus* (Roeßler and Müller 1998). Measurement of internal Cl^- concentrations using $^{36}Cl^-$ revealed that chloride is taken up by *H. halophilus* in an energy-dependent process. The internal chloride concentration was roughly half of that of the environment (Roeßler and Müller 1998). Intense physiological and molecular studies revealed several genes and enzymes that are dependent on the chloride concentration (*Fig. 3.6.5*) (Saum and Müller 2008a). Historically, the subunit of the flagellum, flagellin, was the first protein for which a chloride dependence of production was shown (Roeßler et al. 2000). Later, this was also found for YvyD, a modulator of σ^S sigma factor, SodA, a superoxide dismutase (Roeßler and Müller 2002), and LuxS that plays a role in the

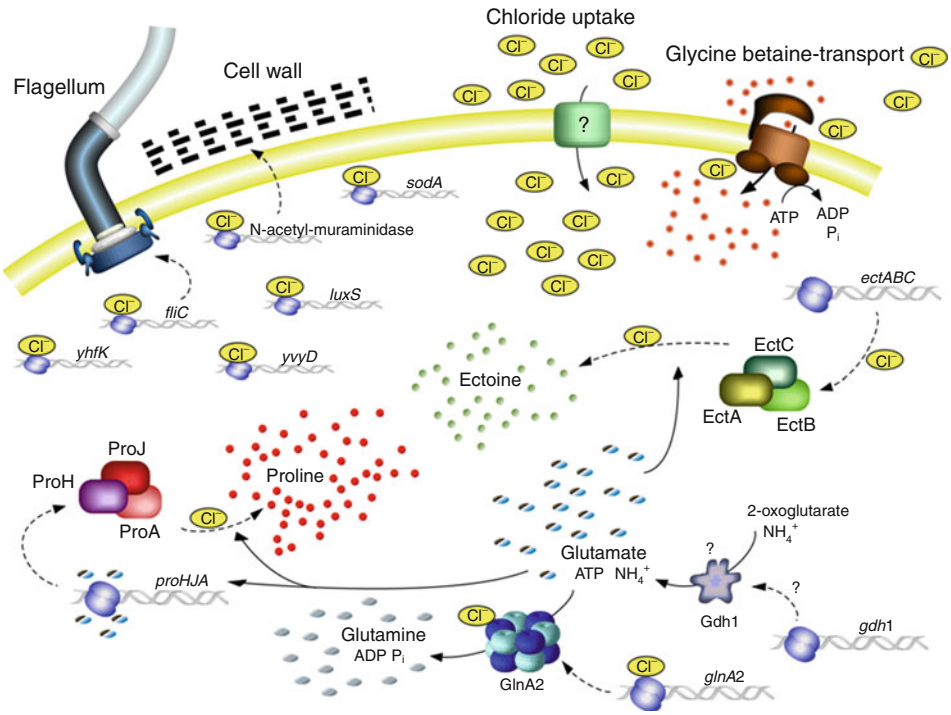

Fig. 3.6.5

The chloride modulon of *H. halophilus*. A summary of physiological processes, genes, and enzymes/proteins that depend on Cl^- for activity, expression, or synthesis. For explanations, see text. The chloride transporter has not been identified

biosynthesis of autoinducers of the furanone family (Sewald et al. 2007). This family of autoinducers is found in Gram-negative as well as in Gram-positive bacteria. The fourth protein was found to be a N-acetyl-muraminidase (previously annotated as ATP-binding subunit of an ABC transporter of unknown substrate specificity). Unfortunately, none of the proteins is known to be involved in a process essential for life. Generally, chloride has a much more pronounced effect on translation than on transcription and also on enzyme activities. Therefore, it is a real global player in the regulatory network(s) of *H. halophilus* and the expression "chloride modulon (regulon)" was termed (Saum and Müller 2008a).

Chloride, a "Salinity" Signal in *H. halophilus*

In search for an essential role of chloride we asked whether a truly essential property of a moderate halophile, sensing and responding to changing salinities may be chloride dependent. Luckily expression of the salinity-induced *glnA2* gene appeared to be stimulated only slightly, but significantly by chloride (Saum et al. 2006). A more striking influence of chloride was found on the level of glutamine synthetase activity, which appeared to be strictly chloride dependent (Saum et al. 2006). These were the first experiments that unraveled a function of one

component of salt, chloride, as a signal compound and laid the foundation for the hypothesis that *H. halophilus* senses the salinity concentration of its environment via the chloride concentration. Moreover, the finding of a chloride-dependent compatible solute production gave for the first time a rationale for the chloride dependence of growth of *H. halophilus*, since growth in saline environments is feasible only with a functionally active osmoregulation.

Chloride First, Glutamate Second: the Switch

At intermediate salinities (1–1.5 M NaCl), glutamate and glutamine are accumulated in correspondence to the external salinity and chloride is the signal that turns on *glnA2* expression and glutamine synthetase activity. Upon a further increase of the external salinity, the glutamate pool reaches a critical threshold value that turns on the transcription of the *pro* genes and, therefore, the production of proline (Saum and Müller 2007). Whether there is an additional stimulatory effect of glutamate on the production of proline biosynthetic enzymes or their activities remains to be identified in the future.

Based on these data, it is not surprising that glutamate is able to substitute chloride in growing cells (Saum et al. 2007). Glutamate was shown to be taken up in a chloride-independent manner. Within the cell, it (1) serves as an osmoprotectant and (2) turns on proline production. Therefore, growth is allowed in the absence of chloride. However, it should be kept in mind that 1 M Na-glutamate is artificial and not encountered in the ecosystem. Interestingly, glutamate cannot short-circuit the Cl^--regulon in general. The biosynthesis of flagellin and therefore motility requires chloride and is not turned on by glutamate.

Lastly, the Growth Phase Response

The ectoine biosynthesis and final concentration are dependent on the salinity of the medium and the availability of chloride, but an additional layer of regulation by the growth phase is encountered. Again, glutamate is crucial and serves as a NH_2-donor in this pathway (*Fig. 3.6.3*). The mode of sensing the growth phase is currently not known, but could be accomplished, for example, via quorum sensing. *H. halophilus* produces autoinducers of the furanone-type (autoinducer 2 [AI-2]) that accumulate in stationary phase cultures (Saum, Winzer, Müller unpublished data). Moreover, the transcription and translation of the key enzyme for AI-2 production was shown to be chloride dependent as already mentioned.

The Role of Ions in Gene and Enzyme Regulation

Generally, ions are involved and mediate a number of regulatory processes in prokaryotes in both gene activation/inactivation and enzymatic activities. The functionality and specificity is based mainly on the chemical properties of the specific ion. The regulating mechanisms require specific ion–target interactions and are functional already at very low, micromolar concentrations. The first example in which a specific interaction required millimolar intracellular concentration was the regulation of expression of the Na^+/H^+ antiporter NhaA from *E. coli* (Karpel et al. 1991). In whole cells maximal induction was obtained by 100 mM NaCl at pH 7.5, whereas at pH 8.6, 10 mM NaCl elicited a similar response. NhaR was identified as a positive

regulator responsible for the Na^+-specific induction of *nhaA* (Rahav-Manor et al. 1992; Carmel et al. 1994). Being both the sensor and the transducer of the Na^+ signal which regulates expression of *nhaA*, a maximal effect of Na^+ in vitro was observed at a concentration of 20 mM. The conformational change of NhaR upon Na^+ binding is also pH dependent (Carmel et al. 1997) within the same range affecting expression in vivo (Karpel et al. 1991).

With increasing ion concentrations, the physical effects of the salt solution become more pronounced and unspecific interactions (direct or indirect) become important. Among the effects that are caused by high salt concentrations are (1) the depletion of water molecules, (2) a change in water activity, (3) osmotic activity, or (4) hydrophobic interactions.

The effect of inorganic ions on biological matter was investigated already at the end of the 19th century. Franz Hofmeister detected that especially anions have the capacity to precipitate proteins (globuline) out of solutions. The capacity to do so is different for different anions. Based on this observation, he was able to rank the anions in a series that became known as the "Hofmeister series." In this series, sulfate had the strongest influence on precipitation followed by phosphate, acetate, citrate, tartrate, bicarbonate, chromate, chloride, nitrate, and chlorate (Hofmeister 1888). Centuries later, the exact molecular basis for the Hofmeister series is still enigmatic, but it becomes more and more obvious that it is due to direct ion–macromolecule interactions and interactions of ions with water molecules in the first hydration shell of the macromolecule. Thereby, kosmotropic ions are differentiated from chaotropic ions. Kosmotropic ions are strongly hydrated, act by stabilizing on macromolecules, and show a salting-out effect. In contrast, the chaotropic ions directly interact with macromolecular surfaces and destabilize folded proteins. They exhibit a salting-in behavior (for review and further reading, see (Westh et al. 2006; Zhang and Cremer 2006). The relevance of the Hofmeister series in biological systems was demonstrated by several studies that focused on enzyme activity (Pinna et al. 2005b; Pinna et al. 2005a; Bauduin et al. 2006), on protein stability (Ebel et al. 1999; Broering and Bommarius 2005), on protein–protein interactions (Perez-Jimenez et al. 2004; Curtis and Lue 2006), or on bacterial growth (Hallsworth et al. 2003; Lo Nostro et al. 2005).

A conceivable model for sensing osmolarity, therefore, could also imply the measurement of such physical parameters by cellular compounds. Based on the Hofmeister series and the effects of anions on proteins or whole cells, a concept can be proposed on how *H. halophilus* measures osmolarity. *H. halophilus* is a moderately halophilic organism that has adapted to saline environments. Since chloride is not excluded from the cytoplasm but rather accumulates, depending on the external salinity, to molar concentrations within the cell (Roeßler and Müller 1998), it is possible, in principle, that the physical effects caused by the presence of chloride is of regulatory importance. A prerequisite for the usage of such a strategy is the evolutionary development of proteins that are functional in the presence of such high ion concentrations. As a consequence, such enzymes might be dependent on the presence of minimal Cl^- concentrations for optimal functionality. Examples for such a behavior were already found in *H. halophilus* in the case of the glutamine synthetase that demands an optimal salinity of 1.5 M NaCl (Saum et al. 2006). Such an adaptation to high salinities is already observed in extremely halophilic organisms such as members of the *Halobacteriaceae* or *Salinibacter ruber* and it would be in good correlation with the identification of 7 positively charged residues in the deduced protein sequence of GlnA2 – the postulated chloride dependent glutamine synthetase – at which chloride could bind (Saum et al. 2006). The Hofmeister series not only offers an explanation of the regulatory influence of chloride on cell metabolism in *H. halophilus*, but also offers an explanation for the regulation of the expression of *pro* genes

by glutamate. At physiological pH, glutamic acid is deprotonated to the anion glutamate that accumulates within the cell to molar concentrations. Since too high concentrations of such anions are inhibitory for cell metabolism, the cell has to substitute glutamate by an electroneutral compatible solute like proline. The same mechanism was previously proposed for *E. coli* that substitutes glutamate by trehalose (Dinnbier et al. 1988). Again, the cell has to "sense" the concentration of an anion (in this case glutamate). For *H. halophilus,* it was shown that a minimal external glutamate concentration of 0.2 M is sufficient to stimulate *pro* gene transcription very effectively (Saum and Müller 2007). Following these data, it was speculated that not only NaCl is the initial signal that triggers the production of proline, but also the internal glutamate concentration, that increases with increasing salinity. Glutamate is therefore regarded as a "second messenger" in *H. halophilus* besides being a compatible solute. This idea was nicely corroborated by studies done in the group of Prof. Gralla that demonstrate the potential of glutamate as an activator or inhibitor of transcription (Lee and Gralla 2004; Gralla and Vargas 2006). In sum, based on the Hofmeister series, a very sophisticated model of regulatory sequences can be proposed for the osmoregulation in *H. halophilus* that is solely based on physico-chemical properties of anions.

Nature and Function of Carotenoids in *H. halophilus*

Carotenoids are naturally occurring pigments found in a wide variety of plants and microorganisms (Sandmann 2001). It is striking that carotenoids are widely distributed in extremophiles. Examples are the thermophilic bacterium *Thermus thermophilus* which synthesizes zeaxanthin and ß-cryptoxanthin glucoside fatty acid esters which help for membrane stabilization (Yokoyama et al. 1995; Yokoyama et al. 1996), high salt requiring *Salinibacter ruber* with the glycosyl fatty acid ester salinixanthin (Lutnaes et al. 2002), and the psychrotrophic bacterium *Arthrobacter agilis* which increases its content in C50 bacterioruberin glycosides in response to low temperature (Fong et al. 2001). Bacterioruberin is also the major carotenoid found in the radioresistant bacterium *Rubrobacter radiotolerans* (Saito et al. 1994) and typical for halophilic archaea like *Halobacterium salinarum* (Kelly et al. 1970). Quite often, extremophiles accumulate glycosides of carotenoids of C30, C40, and C50 carbon chain length as integral constituents of their membranes. By spanning through the membrane (Yokoyama et al. 1995), these carotenoids with the polar end groups influence membrane fluidity and stability (Britton 1995). Furthermore, they prevent oxidation of the membranes due to their antioxidative properties (Woodall et al. 1997).

Interestingly, especially many endospore forming bacteria isolated from saline environments like salt marshes contain carotenoids, whereas their nonhalophilic relatives do not (Turner and Jervis 1963), indicating that carotenoids play a crucial role in salt adaptation in these organisms. To get to know more about the role of carotenoids in the moderately halophilic *H. halophilus,* we identified and characterized the carotenoids of *H. halophilus.* The carotenoids were extracted 3 times with CH_2Cl_2:MeOH (1:1, [v/v]) and subjected to silica gel chromatography. The main compound was isolated and dissolved in CH_2Cl_2:MeOH (1:1, [v/v]). By positive ion HRAPCI-MS, the main carotenoid produced by *H. halophilus* could be identified as a C30 methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate.

The biosynthesis pathway is encoded by two gene clusters shown in *Fig. 3.6.6*. Three of the gene products, designated CrtNa, CrtNb, and CrtNc, show sequence homology to bacterial carotene desaturases. *crtM* compares best to a putative phytoene/diapophytoene synthase that

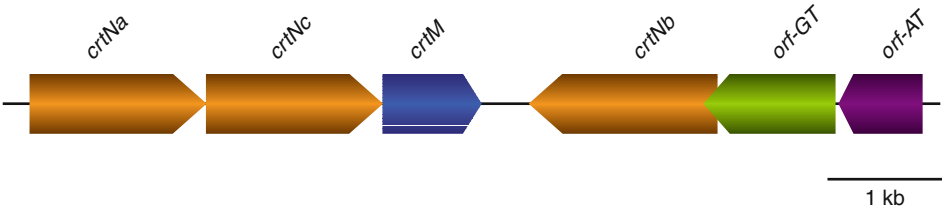

Fig. 3.6.6

Organization of carotenoid biosynthesis genes in *H. halophilus*. *crtNa:* diapophytoene desaturase; *crtNb:* diapophytoene desaturase; *crtNc:* diapophytoene desaturase; *crtM:*diapophytoene synthase; *orf-GT:*glycosyl transferase; *orf-AT:* acyl transferase

catalyses the condensation of two molecules of farnesyl pyrophosphate. Besides these typical proteins for carotenoid biosynthesis, we could also identify two additional genes that encode for proteins that appear to be involved in pigment biosynthesis in *H. halophilus*. The product of *orf-GT* is similar to a glycosyl transferase and *orf-AT* is a putative acyl-transferase (Köcher et al. 2009).

Although carotenoids of various structures are synthesized in bacteria, their common feature is their function as lipophilic antioxidants. For several bacteria, the presence of the antioxidative enzyme catalase was shown to be important for osmoprotection (Cho et al. 2000; Lee et al. 2005). In contrast, growth of *H. halophilus* at conditions where the synthesis of colored carotenoids was inhibited was comparable to the noninhibited culture. However, when oxidative conditions were applied that allowed about 50% growth of the noninhibited culture, the culture devoid of colored carotenoids did not grow. This result indicates that the carotenoids produced by *H. halophilus* cope with oxidative stress. This is in accordance with the fact that the main carotenoid produced by *H. halophilus* (methyl glucosyl-3,4-dehydro-apo-8′-lycopenoate) is a very potent antioxidant (Shindo et al. 2008). In future studies, the carotenoid protective function, analysis of salt-dependent carotenoid synthesis, and carotenoid pathway regulation in this halophilic bacterium will be investigated.

Concluding Remarks

Moderately halophilic bacteria are truly fascinating organisms. In their environment, they cope with changing salt concentrations that go from 0.4 to 3.5 M NaCl without problems. They adapt within short time frames and can resist a hypoosmotic shock during rain fall instantaneously. In recent decades, the nature of the solutes as well as their biosynthesis was in focus. We have learned a lot about the biochemistry of the biosynthetic enzymes and the industrial application of the products, but we don't know hardly anything about the regulation. Key questions like how "salinity" is measured and how the signal is transmitted through the cell as far as gene expression and protein activation are a black box. Furthermore, physiological adaptation such as Na^+ and K^+ homeostasis has not been addressed in detail and neither has the cell wall architecture and membrane composition been studied. Is there an effect of salinity on primary bioenergetics, compounds of the respiratory chain, coupling efficiencies, and ATP synthesis? We don't know! But the time is ripe to address these questions by the "omics" techniques; a couple of genomes from organisms whose physiology has been studied to some

extent are known (*H. halophilus, H. elongata*). Lastly, we have never considered multicellularity/biofilm formation as a means to protect from environmental stresses! This certainly has to be addressed in the future. The road is paved, let's move!

Acknowledgments

Work from the author's laboratory is supported by the Deutsche Forschungsgemeinschaft.

Cross-References

- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 3.1 Taxonomy of Halophiles
- 3.2 Diversity of Halophiles
- 3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in Methanosarcina mazei Gö1
- 3.4 Ecology of Halophiles

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Part 4

Extremophiles: Thermophiles

4.1 History of Discovery of Hyperthermophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.1,

Introduction: My Scientific Interests Before Woese's Discovery of the Archaea

In my Master Thesis work in 1969 in Otto Kandler's lab at the Botanical Institute in Munich, I studied kinetics of lactic acid isomer formation in a variety of Lactobacilli including some isolates obtained by myself. Otto Kandler told me personally how to hunt for new microbes and how to isolate and describe them.

Since Orla-Jensen (1919), the kind of lactic acid isomer formed had been taken as constant for a *Lactobacillus* species and, therefore served as an important taxonomic marker. However in my investigations several species (e.g., *Lactobacillus curvatus*) exhibited powerful changes of the isomer composition depending on the growth phase. In my following PhD work (finished in 1973) on the biochemistry of lactic acid formation in Lactobacilli, I discovered a novel lactic acid racemase in *L. curvatus*. In the presence of bivalent Manganese ions, it was induced by L (+) – lactate that was then converted into DL-lactate (Stetter and Kandler 1973). This finding was the basis for my research on transcription and DNA-dependent RNA-polymerases (RNAP) in Lactobacilli as a post doc in Wolfram Zillig's lab at the Max-Planck-Institute of Biochemistry, Munich-Martinsried. Wolfram Zillig was one of the internationally leading scientists in the field of bacterial transcription. As I found out, in contrast to other bacteria the RNAP in Lactobacilli uses bivalent Manganese ions (Stetter and Zillig 1974). This post doc research was the basis of my habilitation in Microbiology in November 1977 at the Ludwig-Maximilians-University in Munich.

How the Discovery of the Archaea Changed My Science

In January 1977, my teacher and mentor Otto Kandler visited Ralph Wolfe at the University of Illinois at Urbana. During this visit, he also met Carl Woese, who discussed with him his novel and revolutionary finding of the archaebacteria (now: archaea) as a so far unrecognized third superkingdom (now: domain) of life. Woese's findings were based on small subunit rRNA partial sequence comparisons among the bacteria, where the methanogens and extreme halophiles appeared to be extremely different from all other bacteria. As Otto Kandler had found out, methanogens had no murein in their cell walls, which appeared highly unusual for bacteria. Therefore, Woese's data had convinced him. After returning to Germany, Otto Kandler informed me immediately about Woese's findings, which made me very excited. In the following Friday afternoon seminar in Wolfram Zillig's department at the Max-Planck-Institute in Martinsried, I reported about Woese's discovery of a third urkingdom (in German: Ur-Reich) of life, what Otto Kandler had told me. However, nobody could believe it and Wolfram was kidding: "I don't believe a word, we are fed-up of a Third Reich" and there was a hell of laughter afterwards, which made pretty much of a fool of myself and ended the discussion. I urgently wanted to have a look onto the RNAP subunit pattern of a methanogen and Otto Kandler stimulated me to do it. All bacterial RNAP known so far exhibited the same structural subunit pattern ßß′α_2σ. If the methanogens were phylogenetically so different, this may become evident also by their RNAP structure. At that time, very few people in the world were able to grow methanogens, among those especially Ralph Wolfe and his students at the University of Illinois whom I asked for a cell mass of methanogens. Already 2 months after my request, Ralph Wolfe had been kind enough to donate to me a precious lump of freeze-dried cells of *Methanobacterium thermoautotrophicum ΔH*. Already in April 1977, I tried to purify the RNAP of this species. Highly unusual for a "bacterial" RNAP, I discovered that this enzyme was

resistant against the antibiotic Rifampicin! When I had mentioned this result to Wolfram Zillig, based on all his experience he concluded that this could then not be the RNAP. Unfortunately, this enzyme turned out to be highly oxygen-sensitive and, therefore became rapidly inactivated in the presence of air during the purification process. Although I worked day and night, I ended up with an inactive, but structurally almost pure (as it turned out later!) RNAP. In November 1977, Carl Woese published his discovery of the archaea, so far represented by the strictly anaerobic methanogens (see also ❯ Chap. 3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1) and the extreme halophiles (Woese and Fox 1977). Extreme halophiles grow best within saturated salt solutions (e.g., within salterns) and exhibit very high salt concentrations in their cytoplasm (see also ❯ Chap. 3.1 Taxonomy of Halophiles). Already in 1972, a Canadian group (Louis and Fitt 1972) had reported about a uniquely small RNAP in the extreme halophile *Halobacterium halobium*, which consisted only of two subunits of molecular weight as small as 18,000 each. Nobody had ever confirmed this finding. Was this the characteristic archaeal RNAP, possibly still rather primitive? I grew *H. halobium* and tried to find this small enzyme described by Louis and Fitt. However I was unable to reproduce their data. Then, Wolfram Zillig became interested too and we agreed to purify this difficult to handle, high ionic strength-requiring enzyme together. After about 1 month, we had the pure halobacterial RNAP in our hands and I reported about our findings in May 1978 on the International Congress on Halophiles at Rehovot, Israel (Stetter et al. 1978). Like in *Methanobacterium* (and in eukaryotes!), transcription in vitro turned out to be insensitive to Rifampicin. The purified enzyme consisted of at least 4–5 different subunits and had a molecular weight in the range of that of bacterial enzymes. However, no structural analogy to the composition and stoichiometry of known bacterial enzymes was evident, strongly supporting Woese's idea of a separate phylogenetic history. Wolfram and I became very enthusiastic about our finding and wanted to investigate the RNAP of further organisms proposed to be archaea by Carl Woese. In January 1978, we were informed via Otto Kandler that the thermoacidophiles *Sulfolobus* and *Thermoplasma* were archaea, too (see also ❯ Chap. 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles). In order to grow cell masses, I ordered *Sulfolobus acidocaldarius* from the German Culture collection (DSMZ). It had been the organism with the highest growth temperature (75°C) known at that time. *S. acidocaldarius* had been isolated and described by Tom Brock and his coworkers from Yellowstone National Park (Brock et al. 1972). However, my interest had been mainly its RNA polymerase structure and function and not so much its thermophily, which was spectacular, however. *S. acidocaldarius* grew best on yeast extract as carbon and energy source at pH 3 and 70°C under aerobic conditions in the medium described by Brock. In order to obtain enough cell mass, we grew 50 l of cultures under vigorous aeration in 60 l polyethylene bottles equipped with reflux condensers. The purification of the RNAP from *S. acidocaldarius* had been achieved by conventional procedures (Zillig et al. 1979). Most exciting, similar to the halobacterial enzyme its subunit pattern and stoichiometry was very different from that of the characteristic bacterial enzyme and consisted of even about ten different polypeptides. In addition, the *S. acidocaldarius* RNAP was again resistant to Rifampicin. In contrast to *S. acidocaldarius*, *Thermoplasma acidophilum* grew "only" at temperatures of about 60°C. Purification of its RNAP succeeded by employing standard methods (Sturm et al. 1980). This enzyme also exhibited a subunit pattern and stoichiometry, which was very different from the bacterial (e.g., mycoplasmal) ones and turned out to be resistant against Rifampicin. In the same year, by employing Wolfe's anaerobic technique, together with my colleague Josef Winter, I was able to purify the oxygen-labile RNAP of

M. thermoautotrophicum, which was again very different from bacterial RNAP and similar to the other archaeal RNAP, thus supporting Woese's theory about archaebacteria representing a different domain of life (Stetter et al. 1980).

"Falling in Love" with Hot Volcanic Environments and Their Microbes

In 1978, the group of extreme thermoacidophiles isolated from solfataric hot springs comprised *S. acidocaldarius* (Brock et al. 1972), an apparently similar organism (later *Acidianus brierleyi*) described by Corale and James Brierley from Yellowstone National Park (Brierley and Brierley 1973), and the strains MT3 and MT4 tentatively named "*Caldariella acidophila*" discovered by de Rosa and colleagues in an Italian hot spring (De Rosa et al. 1975). Together with *Sulfolobus*, "*Caldariella*" had been assumed to belong to a polyphyletic form habitat group, the apparent homogeneity of which resulted from convergence. Wolfram Zillig and I wanted to check this hypothesis by comparing the RNAP structures of these organisms. Unable to obtain the strains MT3 and MT4 of "*C. acidophila*," Wolfram Zillig and I, together with our technician Simon Wunderl drove to Naples in order to find Pisciarelli Solfatara. At this original site of isolation we wanted to take samples in order to re-isolate the *C. acidophila*," strains. After searching for 2 days, we finally had found this pretty much hidden site with its steam-heated acidic waterholes and its characteristic sulfur smell. This small solfataric field is situated at the backside of the world's famous Solfatara crater within the Campi Flegrei. The same Wolfram and I "fell in love" with this exciting environment, which at once reminded us of the primitive Earth, 3.5 billion years ago. However, in other people such places may cause pretty controversial feelings and imaginations: a great deal hate the sulfur smell as a touch of stink bombs, hell, and industrial smog. For us, from the first moment on it was "heaven." We took samples and were able to isolate strains with identical properties as had been originally described for the "*Caldariella*" MT strains. We deposited them at the German culture collection (DSM 1616 and DSM 1617). Based on its *Sulfolobus*-like RNAP, we named this new species *S. solfataricus* (Zillig et al. 1980). In June 1980, Wolfram Zillig and I decided to go onto a sampling trip to the boiling hot terrestrial and marine volcanic environments of Iceland (*Fig. 4.1.1*). The main target was to search for *Sulfolobus* viruses in order to use their DNA as homologous templates in in vitro transcription experiments. In addition, we wanted to search for further extreme thermophiles. Tom Brock had already reported on non-culturable rod-shaped microbes (now: *Thermocrinis*; Huber et al. 1998) growing in boiling (92°C, due to the high altitude) hot springs with neutral pH in Yellowstone National Park (Brock 1978). In addition to the samples taken aerobically for *Sulfolobus*, I also drew anaerobic samples (reduced by sodium sulfide). With the help of my colleague Josef Winter of Otto Kandler's group, I wanted to search for anaerobic extreme thermophiles. The possibility of the existence of anaerobic extreme thermophiles within boiling terrestrial and marine environments (now: hyperthermophiles) had never been taken into consideration and seemed highly unlikely: *Sulfolobus* had been commonly seen as a highly derived species adapted to extremely high temperatures, a kind of curiosity (due to misclassification!) among the Pseudomonads. Its aerobic lifestyle with its suggested much higher yield of energy appeared essential to resist thermal destruction (Castenholz 1979). The observed much lower growth temperatures within the anaerobic thermophilic methanogens seemed to confirm this prejudice (Zeikus et al. 1980). In sea water, the high salt concentration was seen as additional stress preventing an extremely hot

lifestyle (Castenholz 1979). However, I never pay attention to prejudices and want to find out the truth. Together with Wolfram Zillig, in our field microscope in Iceland we inspected samples of boiling mud pools and springs in several areas. To our big surprise, a great deal were teeming with microorganisms with very unusual appearances like antler-shaped cells with true branchings (› *Fig. 4.1.2*), which we described later as the *Thermoproteales* (Zillig et al. 1981). When I poured the redox indicator resazurin into such boiling environments, the blue clouds became immediately reduced, indicating that those environments in reality were highly anaerobic! I took several samples of boiling water and mud. In order to keep them anaerobic,

Fig. 4.1.1
K.O. Stetter (*left*) and W. Zillig (*right*) sampling within the Kerlingarfjöll solfataric fields, Iceland 1980

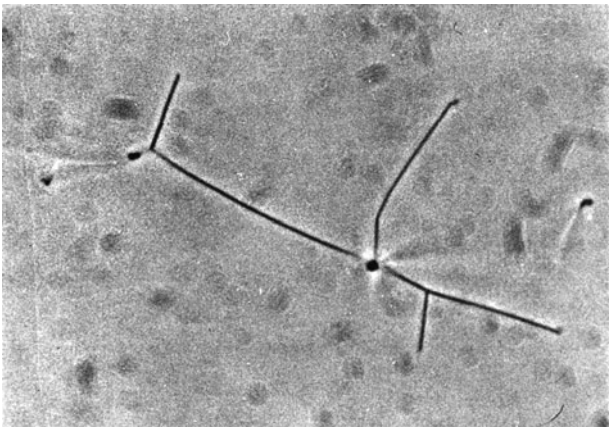

Fig. 4.1.2
Antler-shaped cell of *Thermoproteus* with true branchings, about 100 μm in length and 0.4 μm in diameter. Phase contrast micrograph

after adding resazurin, sodium sulfide, and sodium dithionite, I enclosed them in storage bottles with tightly fitting rubber stoppers. Back in the lab, together with my PhD student Michael Thomm (now my successor at the University of Regensburg), we designed hundreds of anaerobic enrichment culture attempts on a mixture of hydrogen and carbon dioxide as carbon and energy sources. These gasses are common within volcanic exhalations (see also Chap. 7.1 Microbiology of Volcanic Environments). We incubated them at "impossible" temperatures of 80°C and above. Due to our lack of experience in anaerobic culturing, almost all of these first cultivation attempts were negative. Therefore, my young students Harald Huber and Gertrud Wildgruber became already pretty bored in view of all the negative methane analyses they had performed from the culture bottles. Then came enrichment culture V24. It had been incubated for 3 days at 85°C and I never will forget this moment in my whole life. After injecting a sample from this enrichment, the pointer of the gas chromatograph rushed to the upper limit stop and remained there for quite a while, indicating lots of methane being formed from hydrogen and carbon dioxide at 85°C! The original sample had originated from a strongly gassed little hot waterhole in the Kerlingarfjöll mountains. Phase contrast microscopy revealed a dense enrichment culture of novel rod-shaped cells with the strong blue-green fluorescence at 420 nm characteristic of methanogens. Under the electron microscope above their cell wall, the cells showed an S-layer unique to methanogens (*Fig. 4.1.3*). For the first time, this methanogenic archaeon grew at temperatures of up to 97°C and exhibited fastest (optimal) growth at 82°C (*Fig. 4.1.4*)! Therefore, surprisingly this strictly anaerobic archaeon grew at much higher temperatures than the aerobic *S. acidocaldarius*. It became the key organism of my thinking "outside the box": only the laws of physics and chemistry limit the possibilities! In giving credit to its extremely high growth temperature, I named this novel methanogen *Methanothermus fervidus* (Stetter et al. 1981). In the following, from the same anaerobic samples taken at this trip, Wolfram Zillig and I were able to isolate the first members of the strictly anaerobic sulfur-reducing *Thermoproteales* (Zillig et al. 1981; Stetter and Zillig 1985). Similar to *Methanothermus*, the *Thermoproteales* exhibited growth temperatures of up to 97°C and were unable to grow at 65°C or below. Therefore, the novel isolates demonstrated a so far unknown kind of extremity in thermophily. Later, I designated such organisms that grow optimally (fastest) at 80°C and above as hyperthermophiles (Stetter 1992).

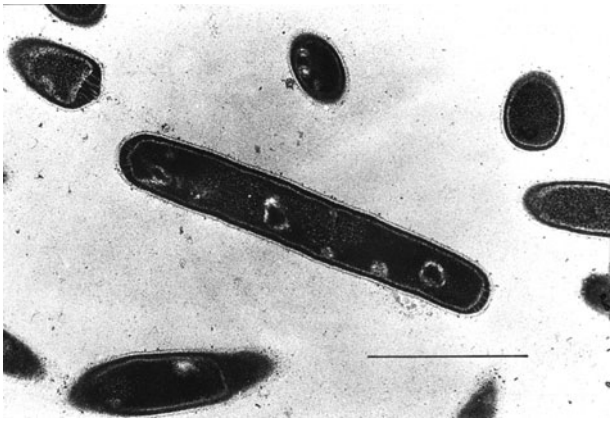

Fig. 4.1.3
***Methanothermus fervidus*, ultrathin section. Transmission electron micrograph. Scale bar, 1 µm**

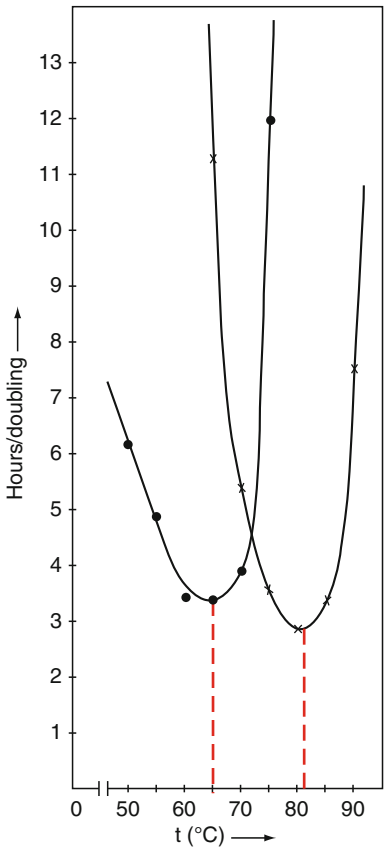

Fig. 4.1.4

Growth temperature curves of *Methanothermus fervidus* (*right, crosses*) and *Methanobacterium thermoautotrophicum* (*left, full circles*)

Discovery of Life Above 100 °C

Since Louis Pasteur it had been generally taken for granted that vegetative cells of bacteria are safely killed by boiling at 100°C, the regular boiling point of water. As I could confirm, this held also true for the novel hyperthermophilic archaea *M. fervidus* and *Thermoproteus tenax*. However, in contrast to Pasteur's experience, could there be life growing even at 100°C and above? Of course, life in steam would be impossible due to the lack of life-supporting components solubilized in liquid water. However, already an overpressure of 1bar above atmospheric pressure raises the boiling point of water from 100°C to 121°C and, therefore keeps water liquid up to 121°C. This pressure corresponds to a water depth of only 10 m, which is easily accessible by scuba diving. In order to hunt for life above 100°C, I intended to take samples at the submarine hot springs at Vulcano Island, Italy. When I discussed this idea with several colleagues, they found it just ridiculous: if such organisms would exist at all, they would have been found already long times ago. Contrariwise they were flouting, I should take care in order "not to boil in my own soup" during such a crazy undertaking. Later, after my success one

of those colleagues began himself to isolate microbes at 100°C! In view of all that prejudice, it appeared to me a waste of time attempting an application for a research grant to verify my assumption. I convinced my family to spend our holidays in 1981 at Vulcano Island, Italy which was great fun to all of us. At this occasion, I collected anaerobic samples from a submarine solfataric field close to the bay of Porto di Levante. The sea floor was situated at a depth between only 2 and 10 m and consisted mainly of sandy sediments with many small craters, and rock formations with gas-spewing, sulfur-encrusted holes and cracks (*Fig. 4.1.5*). I measured temperatures of up to 103°C in the sediments and in the cracks and holes. As I had tried out before in my home's bath tub, usual rubber gloves provided a pretty good protection from the heat of boiling water during sampling, but one has to be fast! In a small rubber boat at the surface, my wife Heidi and my young daughter Sabine were taking care of the still boiling hot samples, which I continuously brought up to the surface. They transferred them immediately into storage bottles, which they sealed with rubber stoppers. Then, they removed traces of oxygen by injecting sodium dithionite. From these samples, in the laboratory for about 3 months many different anaerobic culturing attempts had been executed. When I inoculated sterile sea water with the samples and incubated them for about 1 week at 85°C in the presence of sulfur and a hydrogen/carbon dioxide atmosphere, disk-shaped organisms grew up within a few bottles. One bottle (inoculated with sample PL-19) exhibited extremely slow growth at 85°C. In view of the other much faster growing cultures, my technician proposed to discard this experiment. First I suggested 85°C may have been already the upper limit for these organisms and, therefore asked her to repeat the PL-19 experiment at 75°C. As a result, 2 weeks later at 75°C, there had been not any growth at all, however! Then we did the incubation at 100°C and the consequence was incredibly exciting: already after 1 day, the sulfur in the PL-19 bottle had turned grayish and was covered with cobweb-like structures

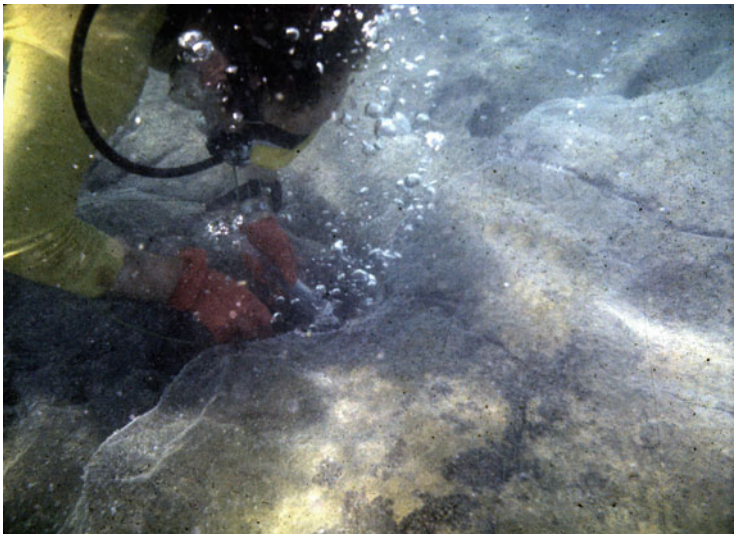

Fig. 4.1.5

Sampling at the submarine hydrothermal vents at Porto di Levante, Vulcano, Italy. Gasses and hot water escape from cracks within hot liparitic rocks

and large amounts of hydrogen sulfide had been formed (*Fig. 4.1.6*). Under the light microscope, for the first time highly unusual flat disk-shaped organisms were visible in high densities. Cells were usually arranged in single file or in other formations with constant distances between each other. Under the electron microscope, most of the disks appeared to be connected by novel ultrathin tubules up to 40 µm long and only about 25 nm in diameter (*Fig. 4.1.7*). Therefore, I named this novel organism *Pyrodictium occultum* (the "hidden fire network"). As it turned out, for the first time, it was able to grow above 100°C in superheated water with an optimal growth temperature of 105°C and an upper limit at 110°C (Stetter 1982; Stetter et al. 1983). This publication (my first one in Nature!) created a lot of attention in the public, too. When Sabine, in connection with her "holiday job" during sampling at Vulcano,

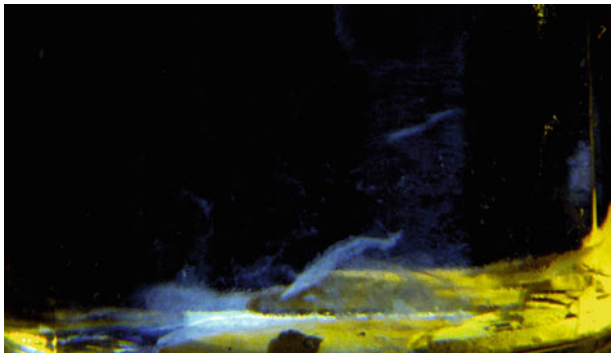

Fig. 4.1.6
Anaerobic culture of *Pyrodictium occultum* at 105°C within sea water in the presence of H_2/CO_2 and S^o with cobweb-like networks covering the sulfur

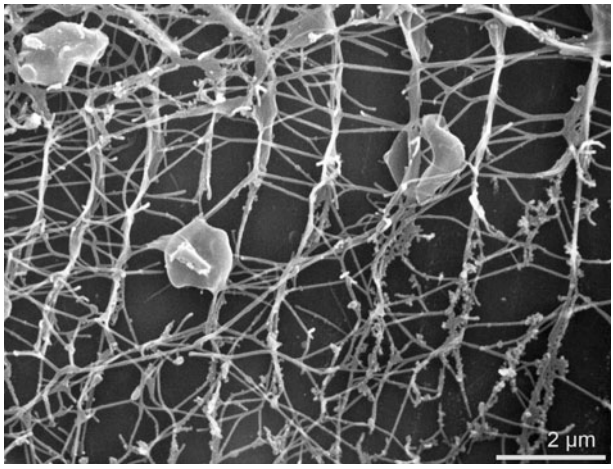

Fig. 4.1.7
Disk-shaped cells of *Pyrodictium* within a network of ultrathin tubules. Scanning electron micrograph

read her name in an article in the German news magazine "Der Spiegel," she was very proud, of course. Based on their Volcano-adapted primitive lifestyle, I raised the hypothesis that possibly similar hyperthermophilic organisms could have existed already at the Early Earth, 3.9 billion years ago. At those Hadean times, due to a still very brittle crust and very active volcanism, Earth had been much hotter than today.

Continued Hunting for Novel Hyperthermophiles

Based on my new cultivation experience, in order to find more exciting hyperthermophilic archaea, during the last 30 years I visited high temperature areas all over the world and isolated high temperature organisms from there. Hot environments like terrestrial and submarine heated soils, sediments, and hot springs are mainly found in areas of active volcanism along tectonic fracture zones and hot spots (see also Chap. 7.1). I had visited several of these sites including deep-sea hot vents with their spectacular "black smokers" (*Fig. 4.1.8*). In addition, I discovered communities of hyperthermophiles within deep, subterranean (nonvolcanic), geothermally heated, oil-bearing sandstone and limestone with in situ temperatures of about 100°C some 3,500 m below the bottom of the North Sea and the surface of the Alaskan North Slope permafrost soil (*Fig. 4.1.9*; Stetter et al. 1993). During that time, my lab isolated and described about 50 new species of hyperthermophiles, among those representatives of the novel bacterial genera *Thermotoga*, *Thermosipho*, *Aquifex*, *Thermocrinis*, and the novel archaeal genera *Acidianus*, *Metallosphaera*, *Stygiolobus*, *Thermoproteus*, *Pyrobaculum*, *Thermofilum*, *Desulfurococcus*, *Staphylothermus*, *Thermosphaera*, *Ignicoccus*, *Thermodiscus*, *Pyrodictium*, *Pyrolobus*, *Thermococcus*, *Pyrococcus*, *Archaeoglobus*, *Ferroglobus*, *Methanothermus*, *Methanopyrus*, *Nanoarchaeum*, and *Korarchaeum* (overview: Stetter 2005). In Woese's small subunit rRNA-based phylogenetic tree (Woese et al. 1990; *Fig. 4.1.10*), hyperthermophiles exclusively represent all extremely short and deeply branching-off lineages within the archaea and bacteria, indicating a slow rate of evolution.

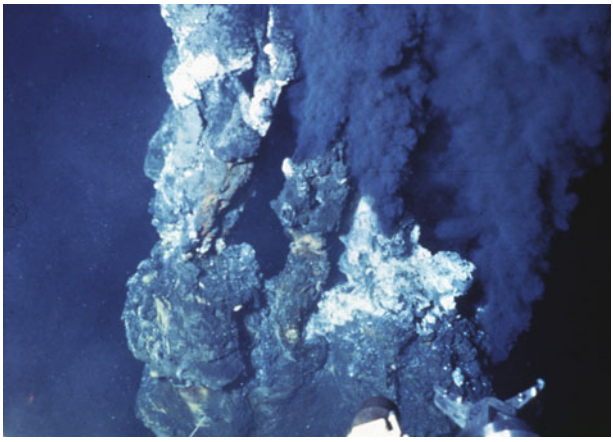

Fig. 4.1.8

Abyssal hot "black smoker" chimneys at the East Pacific Rise, 21°N. Depth: 2,500 m, maximal fluid temperature: 365°C

Fig. 4.1.9
Oil wells within the permafrost soil at Prudhoe Bay, North Alaska

Sampling and Cultivation

Samples of hot waters, soils, rock, and sediments may serve as primary material to set up enrichment cultures in the laboratory. Special care has to be taken to avoid contamination of the sample by oxygen. At the high temperatures of growth, it is toxic to anaerobic hyperthermophiles and may kill them within minutes. In contrast, at low temperatures (e.g., 4°C) in the presence of oxygen, anaerobic hyperthermophiles may survive for years. As a rule, the anaerobic samples are filled into 100 ml glass storage bottles. After reduction of oxygen that had penetrated during the sampling procedure, the bottles are tightly stoppered and transported to the lab at ambient temperature. Such samples can be stored at 4°C and used for successful cultivation experiments for 10 years and more.

Enrichment cultures can be obtained by simulating the varying geochemical and geophysical composition of the environments. Various plausible electron donors and acceptors may be used under anaerobic, microaerophilic, or (rarely) aerobic culture conditions. Depending on the (unknown) initial cell concentration and the doubling time of the organism, positive enrichment cultures of hyperthermophiles can be identified by microscopy within 1–7 days. For a deeper understanding of the organisms, the study of pure or defined mixed cultures is required. Due to the high incubation temperatures, the traditional way of cloning by plating does not perform well. Therefore, we developed a new procedure in order to clone single cells anaerobically under the laser microscope by employing optical tweezers (Ashkin and Dziedzic 1987; Huber et al. 1995). Large cell masses are required for biochemical and biophysical investigations. For mass culturing of hyperthermophiles, in collaboration with an engineering company, I developed a new type of high temperature fermentor (*Fig. 4.1.11*). Its steel casing is enamel-protected in order to resist the highly corrosive culture conditions. Sharp-edged parts like stirrers, gassing and sampling pipes, and condensers are made of titanium. The cell yield of a 300-l fermentation may vary from about 3 g to 2 kg (wet weight), depending on the hyperthermophilic isolate.

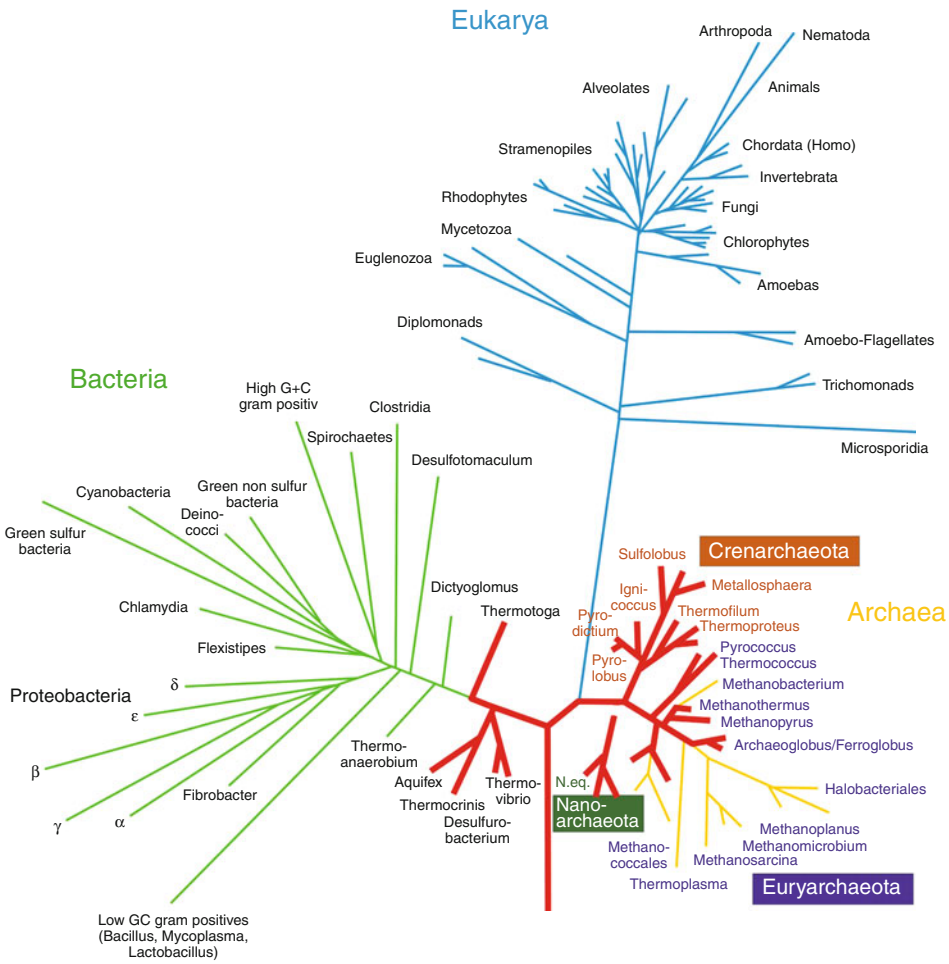

Fig. 4.1.10

Small subunit rRNA-based universal phylogenetic tree. The red bulky lineages represent hyperthermophiles

Energy Sources and Lifestyle

The energy sources of hyperthermophiles are very simple (see also Chap. 7.1). Most species exhibit a chemolithoautotrophic mode of nutrition (*Fig. 4.1.12*). Anaerobic and aerobic types of respiration follow inorganic redox reactions (chemolithotrophic), and CO_2 is the only carbon source required to build up organic cell material (autotrophic). Molecular hydrogen serves as an important electron donor. Other electron donors are sulfide, sulfur, and ferrous iron. In some hyperthermophiles oxygen may serve as an electron acceptor. However, these organisms are usually microaerophilic. Anaerobic respiration types are the nitrate, sulfate, sulfur, and carbon dioxide respirations. While chemolithoautotrophic hyperthermophiles produce organic matter, there are some obligate heterotrophic hyperthermophiles that depend

Fig. 4.1.11

Hyperthermophiles fermentation plant, University of Regensburg. Partial view, showing two 300 l and one 130 l fermenters

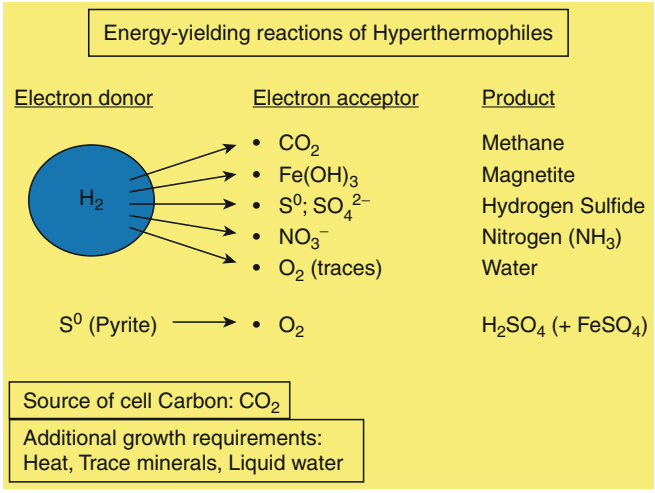

Fig. 4.1.12

Main energy sources of chemolithoautotrophic hyperthermophiles

on organic material as energy and carbon sources. In addition, several chemolithoautotrophic hyperthermophiles are opportunistic heterotrophs. Heterotrophic hyperthermophiles gain energy either by aerobic or different types of anaerobic respiration or by fermentation, using organic material like carbohydrates or peptides as electron donors.

Hyperthermophiles are adapted to distinct environmental factors including composition of minerals and gasses, pH, redox potential, salinity, and temperature (see also Chap. 7.1). In between their minimal and maximal growth temperature they grow – similar to mesophiles – within a range of about 25–30°C. Fastest growth is obtained at their optimal growth temperature, which may be up to 106°C. As a rule, hyperthermophiles do not propagate at 50°C or below, some not even below 80–90°C (e.g., *Pyrolobus*; Blöchl et al. 1997). Although unable to grow at ambient temperatures and temperatures of 140°C present in the interplanetary space, they are able to survive there for many years. Based on their simple growth requirements, hyperthermophiles could grow on any hot water-containing sites, even on other planets and moons like Mars and Europa.

Examples of Recent Hyperthermophilic Isolates

Within the bacterial domain, the deepest phylogenetic branch is represented by the hyperthermophilic *Aquifex* (Huber et al. 1992). Its type species *Aquifex pyrophilus* is a motile rod-shaped chemolithoautotroph (*Fig. 4.1.13*). This organism is a facultative microaerophile. Under anaerobic conditions, *A. pyrophilus* grows by nitrate reduction with H_2 and S^0 as electron donors. Alternatively, at very low oxygen concentrations (up to 0.5% after adaptation) it is able to gain energy by oxidation of H_2 and S^0 using oxygen as electron acceptor. We found the first members of *Aquifex* in shallow submarine vents. *A. pyrophilus* grows up to 95°C, the highest growth temperature observed within the bacteria. From the walls of a black smoker at the Mid-Atlantic Ridge, we had isolated the archaeon *Pyrolobus fumarii* (Blöchl et al. 1997). Cells are lobed cocci about 0.7–2.5 µm in diameter (*Fig. 4.1.14*). *P. fumarii* is optimally adapted to temperatures of superheated water, exhibiting an optimal growth temperature of 106°C and an upper temperature border of growth at 113°C. It is so dependent on high temperatures that it is unable to grow below 90°C. Cultures of *P. fumarii*, similar to *P. occultum* are able to survive autoclaving for 1 h at 121°C. From hydrothermally heated deep-sea sediments at the Guaymas Basin (Gulf of California) and from a shallow marine hydrothermal system at the Kolbeinsey Ridge (Iceland), we were able to isolate rod-shaped marine methanogens that grow up to 110°C and, therefore represent the methanogens with the highest growth temperature (*Fig. 4.1.15*). On the occasion of his 70th birthday, I dedicated this organism to Otto Kandler and named it *Methanopyrus kandleri* (Kurr et al. 1991). Otto Kandler had discovered pseudomurein, a cell wall component that is unique for the *Methanobacteriales* and *Methanopyrus*. As my PhD student Doris Hafenbradl found out, *M. kandleri* contains the non-reduced prae-archaeol as dominating core lipid, possibly a still primitive feature present in all isolates of *M. kandleri*. It may be one of the keys of the extreme thermotolerance of their membrane (Hafenbradl et al. 1993). Fifteen years later, Ken Takai in Koki Horikoshi's lab was able to isolate a further strain ("isolate 116") of *M. kandleri*. Under high pressure of 200 bar, it even grows up to 122°C and, therefore reports on the highest temperature of growth of a living being (Takai et al. 2008). Finally, I want to introduce the pheno- and genotypes of two members of novel groups of hyperthermophiles discovered recently in my lab. The first one is a virus-sized archaeon, which may have already existed at the dawn of life and that had been completely

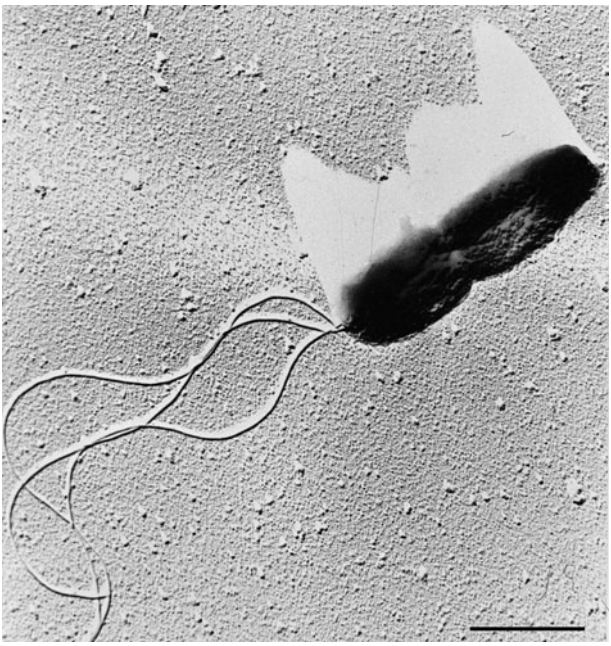

Fig. 4.1.13
***Aquifex pyrophilus*, dividing flagellated cell. Pt shadowing. Transmission electron micrograph. Scale bar, 1 μm**

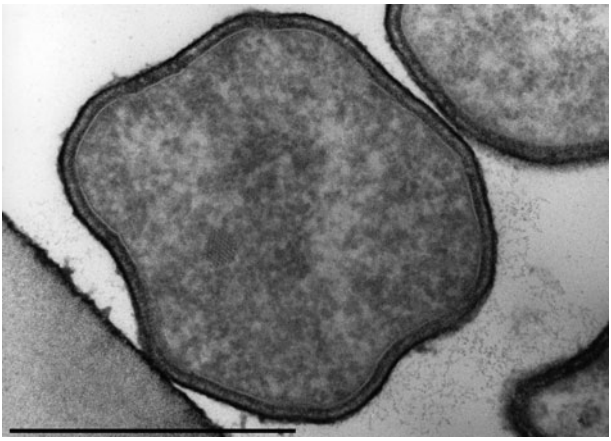

Fig. 4.1.14
***Pyrolobus fumarii*, lobed coccoid cell. Ultrathin section. Scale bar, 0.5 μm**

overlooked so far. From a submarine hydrothermal system at the Kolbeinsey Ridge, North of Iceland, we were able to obtain the coccoid-shaped *Nanoarchaeum equitans*, which represents a novel kingdom of archaea (Huber et al. 2002). With a cell diameter of only 400 nm, it is the smallest living organism known. Cells grow attached to the surface of a specific crenarchaeal

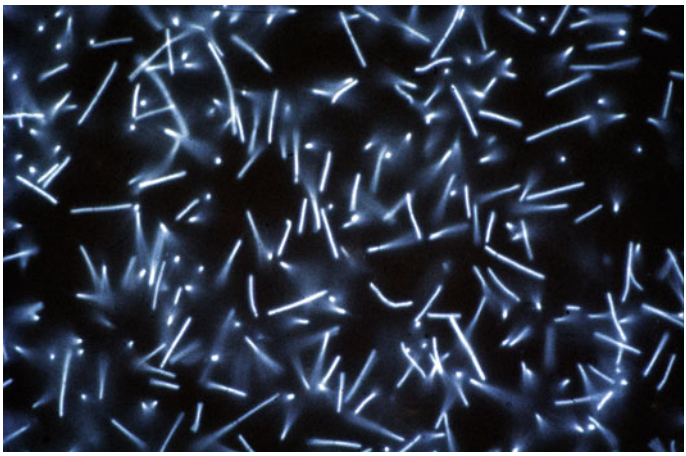

Fig. 4.1.15
Culture of *Methanopyrus kandleri* under the UV microscope. The cells show the characteristic blue-green fluorescence at 420 nm

host, which we named *Ignicoccus hospitalis* (Paper et al. 2007; *Fig. 4.1.16*). Owing to their unusual small subunit rRNA sequence, members of *N. equitans* remained undetectable by commonly used "universal" primers in ecological studies based on the polymerase chain reaction. Although its precise branching position is still unclear, *N. equitans* represents one of the most deeply branching positions, suggesting that the *Nanoarchaeota* diverged early within the archaea (*Fig. 4.1.10*). The genome of *N. equitans* harbors some big surprises (Waters et al. 2003). With only 490,885 base pairs, it is the smallest microbial genome known to date and also the most compact, with 95 % of the DNA predicted to encode proteins or stable RNAs (*Fig. 4.1.17*). This genome encodes the complete machinery for information processing and repair, but lacks genes for lipid, cofactor, amino acid, and nucleotide biosynthesis. *N. equitans* harbors further unexpected, possibly primitive features, such as separately encoded enzyme modules and tRNA gene fragments (Waters et al. 2003; Randau et al. 2005). The limited biosynthetic and catabolic capacity of *N. equitans* suggests that its symbiotic relationship to its *Ignicoccus* host could be parasitic. Unlike the small genomes of bacterial parasites, however, that are undergoing reductive evolution, the small genome of *N. equitans* has very few pseudogenes and a well-equipped DNA recombination system. In contrast to parasites, no free-living relatives of *Nanoarchaeum* could be detected so far. The genome of the host organism *I. hospitalis* has been analyzed too (Podar et al. 2008). It is the smallest among that of free-living bacteria and archaea, showing evidence of gene exchange with *N. equitans* and encoding streamlined biochemical functions necessary for a chemoautotrophic metabolism relying on carbon dioxide, hydrogen, and sulfur. Aside from selection pressure against genome expansion in a restrictive environmental niche, the two organisms have coevolved, leading to symbiotic specificity and gene exchange. In addition, *I. hospitalis* appears to have acquired a significant number of genes and predicted operons from bacteria and *Euryarchaeota*, some of them encoding membrane-associated complexes involved in transport and energy metabolism. This unicellular symbiotic system might resemble relationships that gave rise to eukaryotic organelles. At present, we are still far away from a deeper understanding

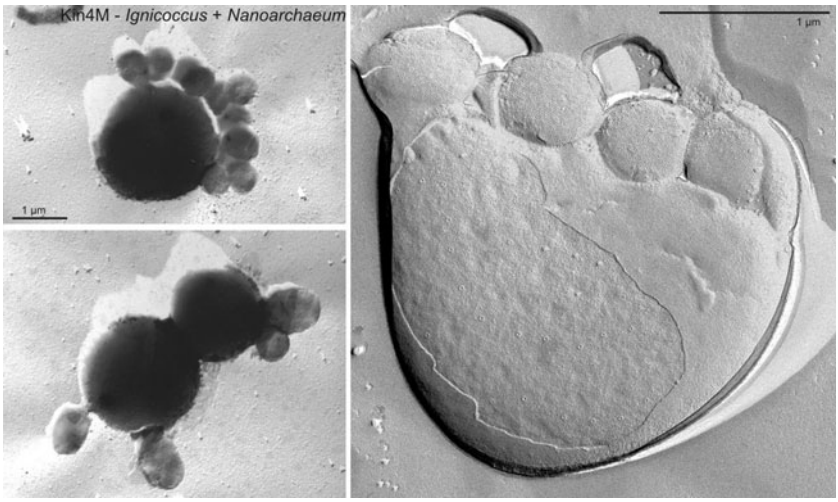

Fig. 4.1.16

Nanoarchaeum equitans **(tiny cocci) attached to** ***Ignicoccus hospitalis*** **(large cocci).** ***Left*****: Pt shadowing.** ***Right*****: Freeze etching. Transmission electron micrographs**

Nanoarchaeum equitans - Genomics

Length	490,885 bp
G+C content	31%
Open reading frames	539
Average ORF length	835 bp
Ribosomal RNAs	5S, 16S, 23S
Transfer RNAs	38
Total coding percentage	94%
ORF coding percentage	92%
ORFs similar to known proteins	360 (66%)
ORFs similar to orphan ORFs	32 (5%)
ORFs without a database match	147 (27%)

Fig. 4.1.17

Basic data of the genome of ***Nanoarchaeum equitans***

of the *Nanoarchaeum–Ignicoccus* relationship and further investigations are required. The *Nanoarchaeota* are inhabiting hot environments worldwide (Hohn et al. 2002). My most challenging task was the elucidation of the *Korarchaeota*, the deepest-branching phylogenetic lineage among the archaea, which went on in my lab since 1994. In their pioneering work on environmental ss rRNA gene sequences, Sue Barns and Norman Pace had discovered this extraordinarily deep-branching sequence within the hot Obsidian Pool in Yellowstone National Park (Barns et al. 1996). The unknown corresponding organisms had been tentatively named *Korarchaeota* ("the early archaea"). Norman Pace had informed me about his exciting finding very early and I promised to him to find the corresponding organisms that were most likely hyperthermophiles. Together with Norman Pace, we took samples at the site in the Mud

Volcano area, Yellowstone National Park. This project turned out to be one of the "toughest nuts to crack" in my life! In the lab, my coworkers Sigfried Burggraf and Nicole Eis were able to set up a stable continuous flow mixed culture at 85°C, which contained minor but significant amounts of the korarchaeal ss rRNA gene (Burggraf et al. 1997). Unfortunately, however, we were misled by the results of whole cell hybridization with fluorescently labeled gene probes ("FISH"-staining) targeted against sections of the ss rRNA taken as specific for the *Korarchaeota*. Very few rod-shaped cells – about 1 among 10^5 cells/ml culture – gave a (wrong!) positive hybridization signal. These cells appeared variable in length (between 5 and 10 µm), slightly curved, and had a diameter of about 0.5 µm. As my PhD student Gudrun Amann in DNA analyses found out much later, these cells in reality did not belong to the *Korarchaeota*, but were members of *Pyrobaculum*. Uniquely, they contained an intron sequence in its ss rRNA gene (which remained within the cells after excision!), which served as target for the hybridization probes applied. An incredible coincidence! As a consequence, the true *Korarchaeota* appeared to us as a kind of "phantom organism" with completely unknown morphology and properties. They could be detected only after PCR amplification of their ss rRNA gene. However, my team and I never gave up! Therefore, this setback even intensified our hunting after the real *Korarchaeota*. Together with my postdoc Brian Hedlund we developed an improved version of a strictly anaerobic continuous flow fermentor, which we inoculated with

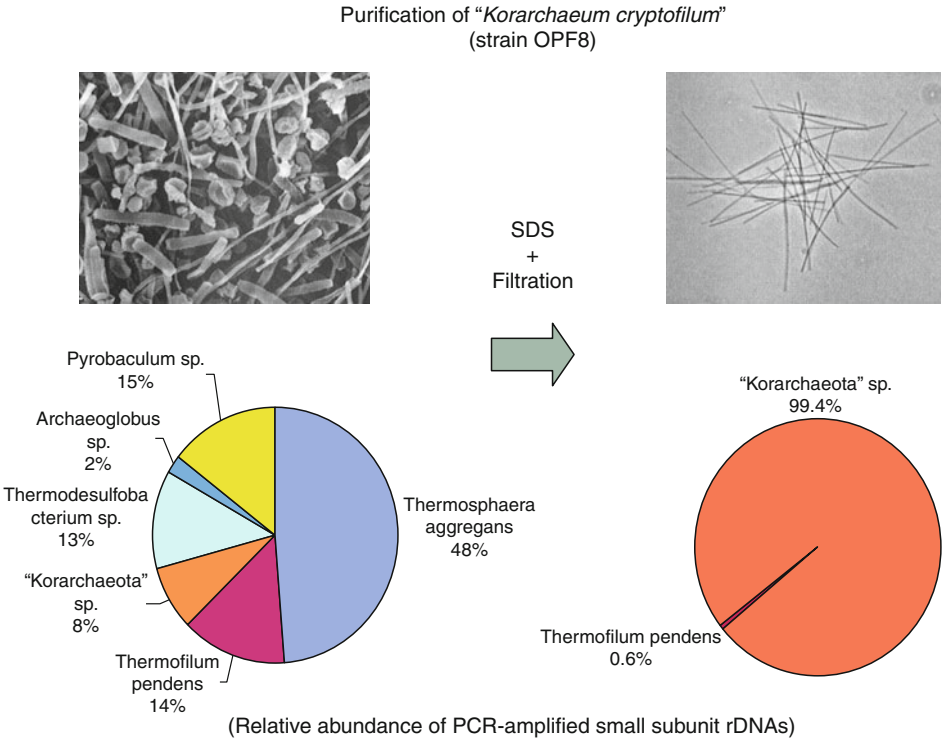

Fig. 4.1.18

Schematic: Physical enrichment of *Korarchaeum cryptofilum* cells from a continuous flow enrichment culture

fresh samples taken at Obsidian Pool. We operated the new fermentor at strictly anaerobic conditions at 85°C, pH 6.5, and continuously fed a dilute organic medium. A stable community of hyperthermophilic archaea and bacteria with a total cell density of about 1×10^8 cells/ml was supported for nearly 4 years. By PCR-amplified ss rDNAs, my PhD student James Elkins found a relative abundance of korarchaeal cells (strain OPF8) as high as 8% of the total mixed population, corresponding to about 6×10^6 korarchaeal cells/ml within the mixed culture. In addition, he observed that cells of the *Korarchaeota* (traced by its ss rDNA gene) were able to pass ultrafilters with 0.45 μm pore width and were resistant against 0.2% sodium dodecyl sulfate (SDS). By combining both procedures, he was able to physically enrich the *Korarchaeota* to a concentration of 99.4%. For the first time, their true morphology became evident: ultrathin rod-shaped cells, 10–100 μm long and only 0.16 μm in diameter, below the resolution of a regular light microscope (*Fig. 4.1.18*). Due to its difficult detection and unusual morphology, we named this organism "*Korarchaeum cryptofilum*" ("the hidden-thread-*Korarchaeum*"). Under the electron microscope, cells of *K. cryptofilum* can be easily recognized by their tiny cell diameter and their unique S-layer consisting of very tiny subunits (*Fig. 4.1.19*). After modification of the "FISH" staining method (by destaining of the background and increasing the SDS concentration), the true korarchaeal cells gave a clear signal after applying ss rRNA specific labeled oligonucleotide probes (Elkins et al. 2008). Although so far we cannot grow *K. cryptofilum* in pure culture (therefore its only the taxonomic "*Candidatus*" state) from the physically enriched cells, James Elkins was able to obtain sufficient DNA for

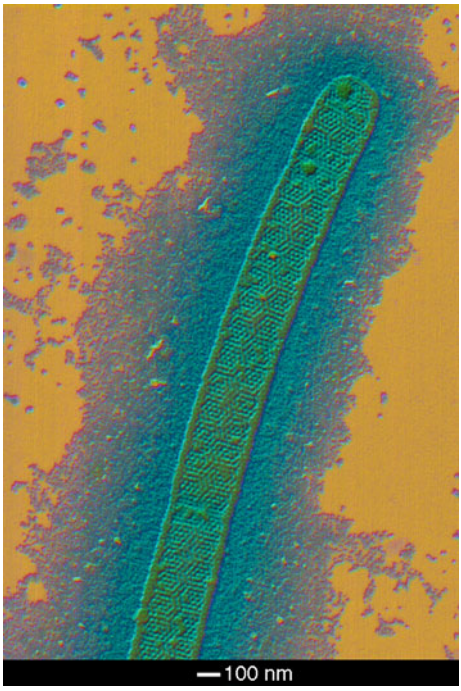

Fig. 4.1.19

Cell of *Korarchaeum cryptofilum* showing its S-layer composed of uniquely tiny subunits. Scanning electron micrograph by Gerhard Wanner, LMU Munich

Crenarchaeal and euryarchaeal arCOGs in "*Korarchaeum cryptofilum*"

arCOG	Cat.*	Function	Eur‡	Cr§
04447	L	DNA polymerase II, large subunit	27	0
04455	L	DNA polymerase II, small subunit	26	0
00872	L	ERCC4-like helicase	26	0
02610	L	Rec8/ScpA/Scc1-like protein	24	0
02258	L	subunit of RPA complex	20	0
00371	D	Chromosome segregation ATPase, SMC	24	0
02201	D	Cell division GTPase FtsZ	26	0
01013	R	Protein with L13E-like domain	0	11
04327	R	Ribosomal protein S25	0	13
04293	R	Ribosomal protein S30	0	13
04305	R	Ribosomal protein S26	0	13
04271	T	RNA polymerase, subunit RPB8	0	12
00393	T	Membrane-associated transcriptional regulator	0	9

*COG functional categories: L, Replication, recombination and repair; D, Cell cycle control, cell division, chromosome partitioning; R, Translation, ribosomal structure and biogenesis; T, Transcription, ‡Number of euryarchaeal (Eur) genomes containing that arCOG (out of 27 total). §Number of crenarchaeal (Cr) genomes containing that arCOG (13 total).

Fig. 4.1.20

Examples of genes thought to be characteristic either of the *Crenarchaeota* or the *Euryarchaeota* both occurring in *Korarchaeum cryptofilum*

a total sequencing of the genome in collaboration with JGI (Elkins et al. 2008). The korarchaeal genome turned out to be pretty small with a total number of 1,590,757 base pairs. By phylogenetic analyses of its concatenated small and large subunit rRNAs, *K. cryptofilum* represents the deepest branch-off within the *Crenarchaeota* (Elkins et al. 2008). However, by analyzing other genes, this korarchaeal genome revealed an unprecedented combination of genes thought to be characteristic either of the *Euryarchaeota* or the *Crenarchaeota* (*Fig. 4.1.20*). The heterogeneous gene compliment suggests that the *Korarchaeota* diverged from those two major archaeal lineages ("kingdoms") very early. Further comparisons may illuminate the early evolution of archaea and the nature with its shared ancestor with the *Eukarya*.

Time to Quit

For me, the age of retirement in Germany had come, and it was time to quit. I hope that younger people in the world will take over hyperthermophiles hunting and research. Further major exciting

groups may still be out so far unrecognized, and are waiting for their isolation in order to tell us more about the evolution and origins of life. In addition, without any doubt, hyperthermophiles and the basis of their hot lifestyle are still full of secrets (see also Chap. 4.9). My contribution revealed only the tip of a very hot (!) iceberg. Now, I became fascinated about another topic of challenging cultivation, however, at ambient temperatures: growth of rare "unculturable" species of mountain orchids like the South African *Disa*, *Dendrobium* from Papua New Guinea and *Telipogon* from South America, which are assumed to be unculturable so far. I will try to do a good job.

Cross-References

- 4.10 Physiology, Metabolism, and Enzymology of Thermoacidophiles
- 7.1 Microbiology of Volcanic Environments

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4.2 Carbohydrate-Active Enzymes from Hyperthermophiles: Biochemistry and Applications

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.2,

Background

Carbohydrate-active enzymes (cazymes) are enzymatic activities involved in the hydrolysis, synthesis, recognition, and binding of carbohydrates, thereby contributing to the metabolism and mobilization of sugars and glycoconjugates (glycoproteins and glycolipids). Cazymes play a central role in glycobiology, a relatively new discipline, which aims to recognize the confluence of the traditional disciplines of carbohydrate chemistry and biochemistry with modern understanding of the cellular and molecular biology of glycans (Varki et al. 2008). In the centrality of glycobiology in life science, the study of cazymes is fundamental for the understanding of the biochemical and enzymatic processes at the basis of glycan mobilization, which regulate important cellular events including energy metabolism, intracellular regulatory switch, protein trafficking, signal transduction, cell–cell interaction, host–parasite interaction, and many others.

The study of cazymes is also recognized as an indispensable prerequisite for their exploitation in biotechnology and biomedicine, for the development of new processes, for the conversion of saccharidic biomasses, for the synthesis of carbohydrate-based building blocks, for drugs and supplements, for nutrition (nutriceutical), and as possible targets for new diagnostic kits and drugs (Turner et al. 2007; Buchholz and Seibel 2008; Maiorano et al. 2008).

In this panorama, cazymes from *hyperthermophilic organisms* (Chap. 4.1 History of Discovery of Hyperthermophiles) (thriving at temperatures >80°C) are an interesting subject of study for several reasons. First, their genes, for the low complexity of the prokaryotic genomes, are easily accessible and can be expressed and manipulated in heterologous hosts. In addition, having intrinsic *remarkable resistance to heat* (Chap. 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles) and other protein denaturants, their enzymes and proteins can be easily subjected to biochemical, enzymatic, and crystallographic studies in mild conditions. Thus, they represent useful model systems of the more complex and less stable human counterparts. Finally, their stability is an added value for their exploitation in biotechnology at the harsh conditions encountered in industrial applications (Conners et al. 2006).

Each cazyme is classified on the basis of the amino acid sequence similarity in families compiled in the Carbohydrate-Active EnZYmes (CAZy) classification, which can be freely consulted on the web (http://www.cazy.org/) and that is continuously updated (Cantarel et al. 2009). Cazymes belonging to each family may have different substrate specificity, but follow the same reaction mechanism and have conserved fold and catalytic amino acids, thus, for every novel member of a family, these important functional features can be easily predicted. Moreover, CAZy families are grouped in superfamilies, or clans, which show common three-dimensional (3D) structures.

In this chapter we will focus on glycoside hydrolases (GH) and glycosyltransferases (GT). In addition, we will describe enzymes extracted from microorganisms requiring a temperature >80°C for their optimal growth, therefore, reviewing cazymes from Bacteria and *Archaea* only. We will make an overview of the main biochemical features of these classes of enzymes and we will focus on the most recent and promising fields of application for them.

Hyperthermophilic Glycoside Hydrolases

Biochemical Features

Glycoside hydrolases are widespread among bacterial and archaeal hyperthermophilic microorganisms as revealed by the inspection of the CAZy database. The function in vivo of these

enzymes has been rarely determined experimentally; presumably, they catalyze the hydrolysis of glycosidic bonds in *primary metabolism* (Chap. 4.10), and, being more common in heterotrophs, they allow the use of complex carbohydrates as energy sources. On the other hand, the increasing evidences of protein glycosylation in some bacterial phyla and in hyperthermophilic euryarchaeota and crenarcheota, strongly indicate that these enzymes might also be involved in the maturation and mobilization of the glycone component of glycoconjugates. This hypothesis is fostered by the observation that certain enzymatic activities, e.g., α-L-fucosidases and α-mannosidases belonging to families GH29 and GH38, respectively, which in Eukaryotes are in charge of the modification of the nascent oligosaccharides in protein N-glycosylation, are also found in extremophilic Archaea. The precise role in vivo of archaeal glycosidases will greatly profit from the combination of different approaches, including structural and enzymatic characterization and gene manipulation.

The two most common mechanisms used by glycosidases to promote oligosaccharide hydrolysis lead to the overall inversion or retention of anomeric stereochemistry of the products if compared to the substrate (*Fig. 4.2.1*). These mechanisms, proposed by Koshland more that 50 years ago, still hold and explain the behavior of most glycosidases (Ly and Withers 1999). Inverting enzymes effect bond cleavage through the action of two essential carboxylic acid residues typically located about 9 Å apart. The deprotonated residue in the active site acts as a general base, removing a proton from the incoming nucleophile (typically water under normal glycosidase mechanisms) during its attack to the anomeric carbon. The other carboxylic acid acts as a general acid residue, protonating the departing aglycone oxygen atom from the anomeric center. This single-displacement mechanism includes a single oxocarbenium ion-like transition state lacking any covalent intermediate and leads to a sugar product whose hemiacetal has the opposite configuration at the anomeric center to that of the starting material: for this reason the enzyme is named *inverting* (*Fig. 4.2.1a*).

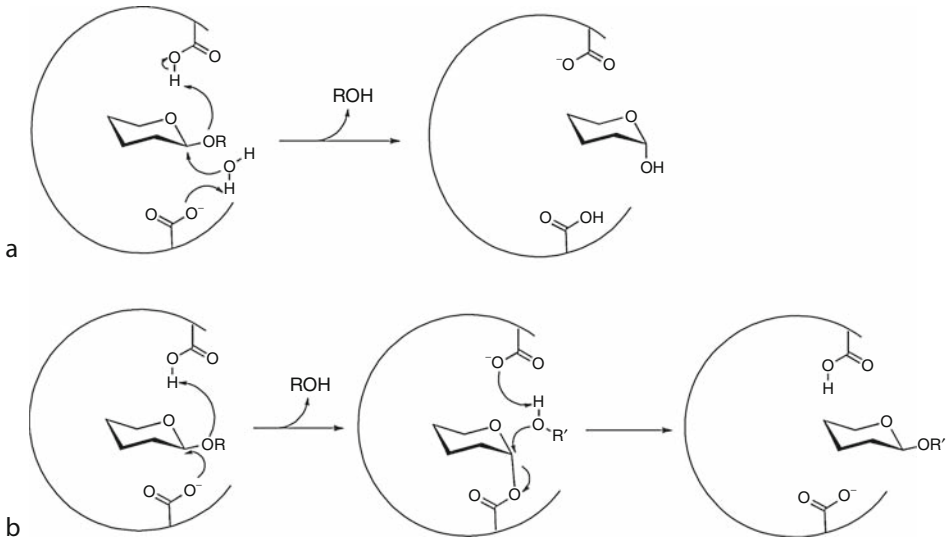

Fig. 4.2.1

Reaction mechanism of *inverting* (a) and *retaining* (b) β-glycosidases; in the latter, the R′ group is an alcohol or another sugar

Most *retaining* glycosidases also have a pair of essential Asp or Glu residues located in the active site, but, in this case, they are normally closer (about 5 Å) than those in inverting enzymes (Fig. 4.2.1b). These enzymes utilize a double-displacement mechanism showing a covalent a glycosyl-enzyme intermediate through two steps. In the first, termed the glycosylation step, one of the residues functions as a general acid by donating a proton during the departure of the aglycone from the substrate, which is also named *donor* of the sugar. In the same step, the second deprotonated carboxylate acts as a nucleophile, attacking the anomeric carbon and leading to the covalently linked glycosyl-enzyme intermediate that has an anomeric configuration opposite to that of the starting material. The second step of the reaction, termed de-glycosylation step, involved the hydrolytic breakdown of the glycosyl-enzyme intermediate. In this case, the carboxylate that first acted as acid now acts as a base by abstracting a proton from the incoming nucleophile that is termed *acceptor* of the sugar, and that, in hydrolytic reactions, is a water molecule. The acceptor molecule attacks the anomeric center of the sugar producing the departure of the carboxylate residue completing the hydrolysis of the glycosidic bond. The product thus obtained has the same anomeric configuration as the starting donor substrate, and for this reason this reaction mechanism is named *retaining* (Fig. 4.2.1b). When acceptors different from water, e.g., alcohols or other sugars, intercept the glycosyl-enzyme intermediate, transglycosylation reactions occur with the formation of a novel glycosidic bond. This approach gives yields usually not higher than 40%. However, *retaining* mesophilic and hyperthermophilic glycosidases, modified by site-directed mutagenesis, were converted in glycosynthases: enzymes able to synthesize oligosaccharides in quantitative yields. This is among the most promising applications of hyperthermophilic glycoside hydrolases in carbohydrate synthesis and it will be discussed in detail below together with the other possible applications of hyperthermophilic GHs.

Applications

The intrinsic remarkable resistance to heat and other protein denaturants of the enzymes from hyperthermophiles has always attracted industry. In fact, the exploitation of biocatalysts was precluded to conventional enzymes because of the harsh conditions used. Nevertheless, examples of industrial applications of hyperthermophilic enzymes are still limited for a number of reasons, including the difficulties in purchasing convenient amounts of enzyme at cheap prices and in adapting the existing industrial plants to the novel processes. So far, no other enzymatic activity from hyperthermophiles has overcome the blockbuster *DNA polymerases* (Chap. 4.4 Enzymes Involved in DNA Amplification and Modification (e.g. Polymerases) from Thermophiles: Evolution of PCR Enzymes). In this panorama, hyperthermophilic glycoside hydrolases are among the hyperthermozymes with the most promising applications. The exploitation of hyperthermophilic glycoside hydrolases in the conversion of biomass into sugars, e.g., for energy utilization, emerged as a renewed interest to cope the depletion of fossil oil resources and as an alternative to the first generation bioethanol. Here, thermostable glycosidases have an obvious advantage as catalysts in these processes, as the high temperatures often promote better enzyme penetration and cell wall disorganization of the raw materials (Blumer-Schuette et al. 2008).

This kind of applications aims to produce from a very cheap and renewable source, products (fuels, glucose, etc.) that have to be sold at low prices, but in large amounts. In this enormous market, the exploitation of hyperthermophilic enzymes is addressed to a so-called *low value, high volume* outcome. However, hyperthermophilic GHs can also be exploited for

the production of *high value, low volume* materials: substances that give to the formulation of different products a very high added value (i.e., precursors and chemical building blocks for pharmaceutics and diagnostics). In these cases, the final compounds, which result from long and expensive *Research and Development* studies, are produced in tiny amounts (if compared to the low value, high volume products), but have considerably higher prices. Here, we will describe examples of thermostable GHs involved in starch modification and in carbohydrate synthesis instead, Chap. 4.3 Lignocellulose Converting Enzymes from Thermophiles is specifically devoted to the description of the bioconversion of (hemi)cellulosic biomass by enzymes from thermophiles.

Starch Modifications

Starch, composed by amylose and amylopectin, is the polymer of plant origin that has the most common and long lasting application in biotechnology. Its modification is exploited for food, texture, pulp and paper, and several other industrial fields. The applications of hyperthermophilic enzymes involved in starch modification are briefly illustrated here.

Dextrin Syrups Production

Starch processing, which is widely used in the food industry for the production of maltodextrin and glucose syrups, is performed in a two-step process consisting in a liquefaction followed by saccharification. In the former, corn starch is first gelatinized at high temperature to allow the swelling of starch granules and to assure the removal of all lipid-amylose complexes and then enzymatically hydrolyzed. 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, which is performed for 1–2 h at 95°C. The thermostable enzymes withstand the pretreatment at high temperature and can be used for the whole process. Commercial preparations of α-amylases from hyperthermophilic Archaea, namely *Pyrococcus furiosus*, *Pyrococcus woesei*, and *Thermococcus litoralis*, exploit the ability of these enzymes to catalyze the hydrolytic reaction at 75–100°C and to resist for several hours at the operational conditions (Turner et al. 2007). These enzymes superseded the α-amylases from moderate thermophilic *Bacillus*, which requires Ca^{2+} ions. However, ideally, enzymes active and stable at low pH (about 4.5) and not demanding calcium for stability would be even more suitable for this process. In fact, liquefied starch is then subjected to saccharification into either maltose or glucose syrups by β-amylases (EC 3.2.1.2; family GH14) and glucoamylases (EC 3.2.1.3; GH15), respectively. To this aim, the system is cooled down to 60°C and the pH adjusted to 4.2–4.5 to allow to the saccharification enzymes to act properly. Therefore, thermostable β-amylases and glucoamylases, active in the same pH and temperature range as the α-amylases of the liquefaction step, would make the process more economically feasible. In these regards, a glucoamylase from *Sulfolobus solfataricus* and a β-amylase from *Thermotoga maritima* are enzymatic activities with promising applications in starch saccharification (Turner et al. 2007). In addition, two hyperthermostable amylopullulanases (EC 3.2.1.41; families GH13 and GH57) from *P. furiosus* and *T. litoralis* are also useful for the debranching of the amylose and the complete conversion of maltodextrins in maltose or glucose syrups (Brown and Kelly 1993).

Gelatin

Gelatinized starch can be modified by amylomaltases (EC 2.4.1.25; classified in families GH13, GH57, and GH77), which are glucanotransferases transferring α-1,4 linked glucan fragments

from starch to different acceptors bearing OH groups. This reaction results in the conversion of gelatinized starch into a thermoreversible gel, which contains amylopectin and is free of amylose. This polymer, which can substitute the product obtained from bone marrow that, being of bovine origin, raises concerns of contamination Bovine Spongiform Encephalopathy, has several applications in the food and cosmetic industry. Amylomaltases from hyperthermophilic Archaea, mainly *Thermococcus* species and *Pyrobaculum aerophilum*, with optimal temperatures between 75°C and 90°C, are industrially relevant enzymes (Turner et al. 2007). In fact, the latter, depending on the enzyme doses, produce gels melting at different temperatures (from 37°C to 70°C) thereby extending the variety of available gelling products with their own specific applications.

Texture Desizing

To prevent breaking of threads during weaving they are usually strengthened by the application of an adhesive size; the predominant size is starch as it is inexpensive and water-soluble. Before dyeing and bleaching, textures 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 texture at 75–80°C with a desizing liquor containing surfactants to wet starch and to make it available to hydrolysis. The enzyme is added together with calcium chloride and sodium hydroxide to raise the pH and bring the conditions to the optimum for the α-amylase hydrolysis. Conventional enzymes are limited by their relatively slow action and their sensitivity to the chemistry of the environment. Most of the problems have been overcome with the introduction of thermostable α-amylases that complete starch hydrolysis in as little as 30–60 s at 90–110°C (Godfrey 1996). The archaeal enzymes from *Pyrococcus* and *Thermococcus* species described above can further improve the process withstanding, for instance, to anionic surfactants that usually damage less stable enzymes.

Carbohydrates Synthesis

The importance of carbohydrates in industry as food ingredients and sizing component of fabric and paper is well known. Oligosaccharides, often in form of glycoconjugates, have instead considerable potential as therapeutic agents in biomedicine and pharmaceutical industry since they have important functions in biological systems such as fertilization, embryogenesis, neuronal development, cell proliferation, and metastasis. The exploitation of this potential is limited by the structural complexity of these molecules that hampers their study in vivo and their production in large scales. Classical organic chemistry requires many laborious manipulations to control the stereo- and the regiospecificity of the products with final yields often too low even for biological testing. (*Chemo*)-*enzymatic synthesis* can be a useful alternative, but the high concentrations of organics used are often detrimental for conventional enzymes; therefore, stable cazymes can be a promising option. Here is discussed their exploitation in cyclodextrin and trehalose synthesis and the advent of engineered hyperthermophilic glycosidases able to synthesize oligosaccharides in high yields.

Cyclodextrin and Trehalose Synthesis

Starch derived products showing a wide range of possible applications are cyclodextrins: covalently closed cycling polymers of different sizes that, showing an apolar interior, may act as carriers of different molecules (including peptides, proteins, oligonucleotides, pharmaceutical

small molecules, etc.). This property can be exploited in a variety of applications such as drug delivery, coating material, adhesives, food, cosmetics, and textiles. (van der Veen et al. 2000). The enzymes involved in the synthesis of these compounds are cyclodextrin glucosyltransferases (CGTase) (EC 2.4.1.19; also classified in family GH13), which are described in more detail in Chap. 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic Bacillus for the Production of Beta-Cyclodextrin. Regarding CGTases from hyperthermophilic Archaea the enzyme from *Thermococcus kodakaraensis* showed for the first time a C-terminal domain whose deletion abolished the cyclization activity, but left unaltered the hydrolytic activity (Rashid et al. 2002), confirming that the biodiversity of archaeal enzymes could be used for different applications.

Enzymes involved in trehalose biosynthesis are also attracting for their potential exploitation. This *compatible solute* (Chap. 4.5 Organic Compatible Solutes of Prokaryotes that Thrive in Hot Environments: The Importance of Ionic Compounds for Thermostabilization), which allows the organisms to adapt to different stresses, such as cold, heat, desiccation, dehydration, osmotic, and/or oxidative stress, can be usefully exploited to preserve biological materials in nutriceuticals, drugs, cell lines, etc. Several pathways of trehalose synthesis and degradation have been described (Kouril et al. 2008). The TPS/TPP (OtsA/OtsB) pathway involves a glycosyltransferase (see below) while the trehalose synthase pathway, which was recently found in *Picrophilus torridus*, catalyzes the reversible hydrolysis of trehalose, and the TreY/TreZ pathway, well known in *Sulfolobales*, involves a maltooligosyl-trehalose synthase and a maltooligosyl-trehalose trehalohydrolase forming and releasing, respectively, a trehalose molecule from the reducing end of a linear maltodextrin. Another pathway, where a trehalose phosphorylase synthesizes this disaccharide from glucose-1-phosphate and glucose (Glc), has never been observed in hyperthermophiles. Instead, more recently, enzymatic studies revealed in the euryarchaea *T. litoralis* and *Pyrococcus horikoshii* a novel pathway in which the glycosyltransferase TreT catalyzes the formation of trehalose from NDP-Glc and Glc (see below).

The trehalose synthase and the enzymes of the TreY/TreZ pathways have more promising applicative perspectives being stable enzymes and utilizing cheap substrates as maltodextrins and starch. Instead, GT-based pathways have reduced prospects for the limitations of glycosyltransferases discussed below.

Oligosaccharide Synthesis

Enzymatic synthesis of oligosaccharides has emerged as an interesting alternative for the preparation of oligosaccharides in large scale because the regio- and stereospecificity of the reaction can be controlled by exploiting the properties of biocatalysts. The approaches available so far are based on two major class of enzymes: glycosyl transferases and glycosidases. The formers are still poorly exploited because of their scarcity and for the high costs of their substrates (see below). A valid alternative is that of *retaining* glycoside hydrolases (*Fig. 4.2.1b*), which can be exploited in synthetic reactions by transglycosylation. This method exploits the ability of the enzymes of transferring the donor sugar to an acceptor different from water yielding a glycoside (*Fig. 4.2.1b*). Normally, to avoid the competition of the water solvent, the acceptor substrate is used in large excess. Nevertheless, transglycosylation usually provides yields not higher than 10–40% because of the competition of the hydrolytic reaction and as a consequence of the high concentration of organics that can be denaturing for conventional enzymes. In these regards, hyperthermostable glycosidases, such as the β-glycosidases, α-xylosidase and α-L-fucosidases from various hyperthermophilic sources as *S. solfataricus*, *P. furiosus*, and *T. maritima*, stable and active for several hours at the operational

conditions, were used in the synthesis of a wide range of oligosaccharides (Moracci et al. 2001; Osanjo et al. 2007). Despite the advantages of using cheap substrates and convenient enzymes, generally, carbohydrate synthesis by transglycosylation is not economical for large-scale synthesis since the product of reaction is a new substrate of the glycosidase and it can be hydrolyzed reducing the final yields of the reaction.

An interesting alternative was the approach utilizing engineered glycosidases. The hydrolytic reaction of *retaining* glycosidases, eliminated by site-directed mutagenesis, led to the production of a novel class of enzymatic activities named *glycosynthases* (for reviews see Perugino et al. 2004; Hancock et al. 2006). In these enzymes the carboxylate working as nucleophile (Asp or Glu) of the reaction was mutated into a non-nucleophile residue (typically Ala or Gly, but also Ser) producing an inactive enzyme. Under these conditions, the novel glycosynthase could use glycoside fluorides with an anomeric configuration opposite to that of the normal substrate producing oligosaccharides by transglycosylation (› *Fig. 4.2.2a*). The mutant enzyme was not able to hydrolyze the products that accumulate in the reaction and the novel enzymatic activities were named *glycosynthases*. By following a complementary approach, the inactive mutant was reactivated in the presence of an external nucleophile

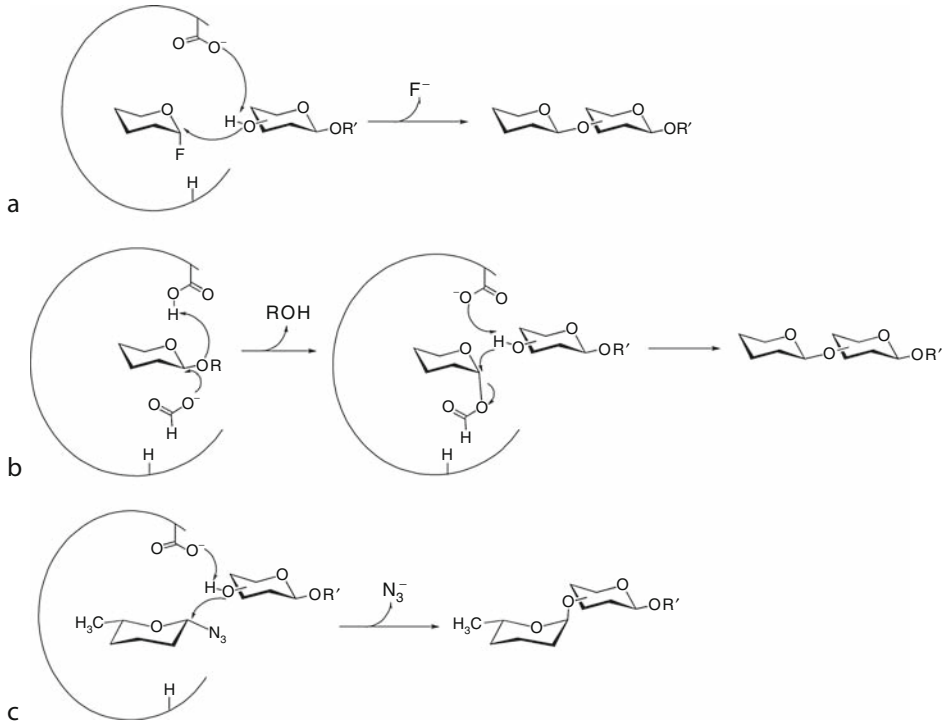

Fig. 4.2.2

Reaction mechanism of *mesophilic* (a) and *thermophilic* (b) β-glycosynthases, and (c) of α-fucosynthases. R and R′ groups are an alcohol or another sugar in the donor and the acceptor substrates, respectively

(such as sodium formate ion) and of an activated substrate donor (typically, a nitrophenyl-glycoside) with the same anomeric configuration of the normal substrate (Fig. 4.2.2b). The mutant enzyme promoted a transglycosylation reaction similar to that of the wild type enzyme, but with the assistance of the external nucleophile, mimicking the natural nucleophilic carboxylate. In this case the product accumulation was due to the inability of the mutant, even in the presence of the external ion, in hydrolyzing a disaccharide, which was more stable than the activated donor. Hyperthermophilic glycosidases could be converted in efficient glycosynthases following the reaction mechanism described in Fig. 4.2.2b. In particular, mutants of the β-glycosidases from the Archaea *S. solfataricus*, *Thermosphaera aggregans*, and *P. furiosus* were efficient β-glycosynthases catalyzing the synthesis of oligosaccharides at yields higher than 90%. The three enzymes showed different and peculiar regiospecificity and higher conversion rates, than mesophilic β-glycosynthases, in terms of total donor substrate converted *per* time *per* amount of enzyme used (Perugino et al. 2005).

More recently, the hyperthermostable α-L-fucosidases from *S. solfataricus* and *T. maritima* were also converted in efficient glycosynthases. The enzymes followed the reaction mechanism described in Fig. 4.2.2c. Again, the donor had an anomeric configuration opposite to that of the natural substrate as in Fig. 4.2.2a, but in this case a more stable and convenient azide derivative rather than a fluoride glycoside, was used, widening the repertoire of substrates available for this important class of engineered enzymes (Cobucci-Ponzano et al. 2009). Yields were again >80% and these were the first α-glycosynthases produced so far demonstrating that this approach is of general applicability to both α- and β-glycosidases (Wang 2009).

The overall superior efficiency of hyperthermophilic glycosynthases if compared to conventional enzymes can be ascribed to their intrinsic operational stability that allows their endurance in harsh conditions (high concentrations of external nucleophiles) and mutated in residues often destabilizing the protein fold (the modification of the catalytic Asp/Glu into a non-nucleophilic Gly/Ala can be detrimental for the stability of the enzyme). For these reasons, hyperstable glycosynthetic enzymes have high chances of being usefully exploited in carbohydrate synthesis in recent future.

Hyperthermophilic Glycosyltransferases

Biochemical Features

Glycosyltransferases catalyze the synthesis of carbohydrates by transferring the glycosyl moiety from donors activated with phosphates to an acceptor. GTs utilizing sugar mono- or diphosphonucleotides are sometimes termed Leloir enzymes (in honor of the Nobel prize winner Luis F. Leloir) while GTs that utilize non-nucleotide donors, such as polyprenol pyrophosphates, polyprenol phosphates, sugar-1-phosphates, or sugar-1-pyrophosphates, are termed non-Leloir enzymes. The donor glycoside may be a mono-, oligo-, or polysaccharide. The acceptors are, most commonly, other sugars, but can also be lipids, proteins, nucleic acids, antibiotics, or other small molecules. The nucleophile of the acceptor molecule is most commonly oxygen, but it can also be nitrogen (in the N-glycosylation), sulfur (thioglycosides), and carbon (antibiotics).

Based on their amino acid sequence similarities, also glycosyltransferases, as glycosidases described above, are classified in families listed in the CAZy database (http://www.cazy.org).

Further classification is based on the three-dimensional folding, with three groups recognized so far, GT-A, GT-B, and GT-C. In contrast to GHs, the GT 3D-structures are much less diverse, with the vast majority belonging to GT-A and GT-B folds, as the GT-C folds was experimentally determined only recently and predicted for only a dozen of GT families. Both GT-A and GT-B folds contain at least one nucleotide-binding domain of the Rossman fold type; in the former two, β/α/β Rossman domains lie adjacent, while in GT-B, they face each other and are linked flexibly. Interestingly, the nucleotide-binding domain is observed not only in GTs belonging to the Leloir pathway but also for the enzymes utilizing donor substrates activated with a phosphate group, suggesting an evolutionary link between enzymes utilizing these two substrate forms (Lairson et al. 2008).

Instead, the GT-C fold, which has been identified for the first time in an oligosaccharyltransferase (OST) from the hyperthermophilic Archaeon *P. furiosus*, has a different architecture, with, at the N-terminal, a predicted 13 transmembrane helices domain and a soluble C-terminal domain consisting of a central core (made of α-helices) and two peripheral domains (β-strands) (Igura et al. 2008).

As described above for GHs, also glycosyltransferases are classified on the basis of the catalytic mechanism used, with enzymes *retaining* or *inverting* the stereochemistry of the substrate (*Fig. 4.2.3*). However, in comparison with GHs, the information available, especially for the mechanisms of retaining GTs, is by far less complete.

In *inverting* GTs, an active site carboxylate serves as a base catalyst that deprotonates the incoming nucleophile of the acceptor, facilitating the displacement of the activated phosphate leaving group (*Fig. 4.2.3a*). The reaction, most probably occurring through a single oxocarbenium ion-like transition state, results in an inversion of the anomeric configuration of the donor sugar. The key questions in elucidating this mechanism are to identify the residue acting as a base and to characterize the catalytic machinery assisting the departure of the phosphate leaving group. In many *inverting* GT-A enzymes it has been reported that a divalent cation (usually Mn^{2+} or Mg^{2+}), coordinated by a conserved motif called DXD (D = Asp and X = any amino acid), facilitates the departure of the nucleoside diphosphate, but alternative roles for the cation binding and for the leaving group assistance have also been described specially in GT-B enzymes (Lairson et al. 2008).

By direct comparison to that of GHs, the mechanism of *retaining* GTs has been proposed to be a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate (*Fig. 4.2.3b*). Here, an active site carboxylate serves as nucleophile attacking the anomeric center of the substrate while a divalent cation or a suitably positioned positively charged residue would play the role of an acid catalyst promoting the departure of the substitute phosphate group. This latter group itself probably plays the role of a base catalyst activating the acceptor hydroxyl group (*Fig. 4.2.3b*). Unfortunately, despite considerable efforts, there is little work supporting the double-displacement mechanism as the canonical GT mechanism and the nature of the intermediate is also not completely clear. In particular, several lines of evidence suggest that an oxocarbenium ion intermediate rather than the covalent intermediate could be the case for several GTs. The detailed reasons why the mechanistic characterization of this class of enzymes is a challenging task go beyond the scope of this review and are treated elsewhere (Lairson et al. 2008). The difficulties in purifying stable and soluble GTs, which are often membrane enzymes, and the strict requirements of these enzymes for expensive (substituted) phosphodonor sugar substrates are the major limits for the biochemical characterization of the reaction mechanisms.

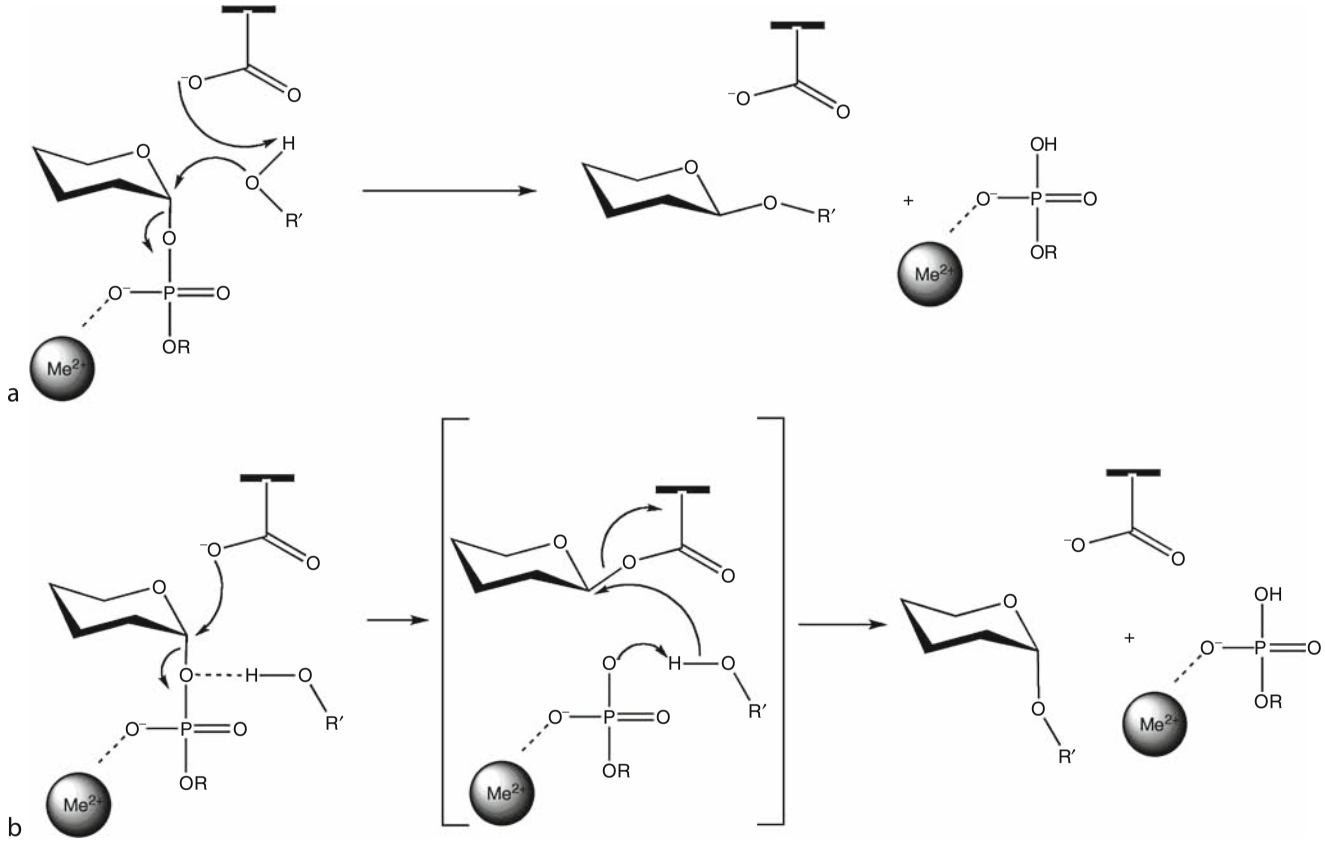

Fig. 4.2.3

Reaction mechanism of *inverting* (a) and *retaining* (b) glycosyltransferases; R is the molecule substituting the phosphate, R′ group is an alcohol or another molecule, Me^{2+} is a metal cation. In (b) a proposed intermediate is shown in brackets

Characteristics of Hyperthermophilic GTs

Glycosyltransferases in hyperthermophiles are involved in the synthesis of carbohydrates, such as intracellular polysaccharides and exopolysaccharides, and of glycoconjugates, as glycoproteins and glycolipids. The formers are used as storage polymers and as mediators of the cellular adhesion to each other or to solid surfaces (biofilm) (VanFossen et al. 2008). Instead, protein N-glycosylation, which for long time was considered a prerogative of Eukaryotes and of few pathogenic Bacteria, has been identified in Archaea (Abu-Qarn et al. 2008). The study of carbohydrate synthesis in this domain of life is in its infancy: which of the many GTs identified in the genomic sequence are involved in polysaccharide synthesis is mostly unknown and more detailed insights into GTs involved in N-glycosylation are available only for halophile and methanogen model organisms. Very few archaeal glycoproteins have been identified, and the detailed structural characterization of the glycosidic antennae is missing in most of them. The studies made on *Haloferax volcanii*, *Methanococcus voltae*, and *Methanococcus morismortui* showed that N-glycosylation in Archaea, though showing aspects unique to this form of life, shares much in common with its eukaryotic counterpart. In particular, Archaea exploit dolichol as the lipid upon which the oligosaccharide is assembled, lipid-linked antenna are modified after its translocation across the membrane, N-acetylglucosamine is the sugar linked to the Asn of the nascent protein, and the N-glycosylation motif is Asn-X-Ser/Thr. The single-subunit oligosaccharyltransferase (OST), the enzyme responsible for the *en-bloc* transfer of the oligosaccharide from its lipid carrier to the Asn residue, is, instead, a treat common to Bacteria; therefore, N-glycosylation in Archaea can be considered a biological mosaic (Abu-Qarn et al. 2008).

In this panorama, the information available on GTs from hyperthermophilic Bacteria and Archaea involved in polysaccharide synthesis and protein glycosylation can be obtained mainly from the inspection of the sequenced genomes and the consultation of the CAZy database. However, it is worth noting that experimental studies on GTs from hyperthermophilic Archaea are of arising interest as the information derived thereof is contributing significantly to our knowledge in general on N-glycosylation in Eukaryotes (see below).

Hyperthermophilic Bacteria, namely *T. maritima* and *Aquifex aeolicus*, show 19 and 22 GTs, respectively; a limited number if compared to archaeal counterparts. In addition, no enzymes belonging to GT66, which group OSTs, are found in these organisms, suggesting that N-glycosylation is missing in hyperthermophilic Bacteria. On the other hand, it cannot be excluded that a more detailed inspection of the genomic data might reveal homologs of GT66 members. This was indeed the case for three hyperthermophilic Archaea, namely *Nanoarchaeum equitans*, *P. torridus*, and *Thermoplasma volcanium*, in which an OST homolog that was apparently missing was found by searching the available database with the WWDXG motif implicated in the catalytic mechanism of the enzyme (Magidovich and Eichler 2009).

Despite the apparent lacking of N-glycosylation in hyperthermophilic Bacteria, the study of the peptidoglycan glycosyltransferase belonging to family GT51 from *A. aeolicus* was particularly interesting. This GT showed a unique λ lysozime-like 3D-structure different from all folds reported to date in GTs and its inspection supported by biochemical data demonstrated the processivity of the enzymes belonging to this family giving the basis for a new model in peptidoglycan biosynthesis (Yuan et al. 2007).

Hyperthermophilic Archaea, though showing a reduced number of GTs if compared to halophiles and methanogens, apparently display the catalytic apparatus for N-glycosylation. By following the general trend, families GT2 and GT4 are the families with the highest number of

enzymes in hyperthermophilic Archaea. These families include activities involved in cellulose, chitin, and sucrose biosynthesis, but also in N-glycosylation. In addition, a recent study on 56 complete archaeal genomes revealed that all but two hyperthermophilic species (*Aeropyrum pernix* and *Methanopyrus kandleri*) have at least one homolog of OST (GT66) (Magidovich and Eichler 2009). In addition to GT2 and GT4, Archaea also encode other GTs belonging to clan GT-A (GT-2 (*inverting*), GT55 (*retaining*), GT81 (*r*), and GT84 (*i*)) and to clan GT-B (GT1 (*i*), GT3 (*r*), GT4 (*r*), GT5 (*r*), GT9 (*i*), GT20 (*r*), GT28 (*i*), and GT35 (*r*)). Finally, the only family found in hyperthermophilic Archaea belonging to GT-C is GT66 (*i*) while the remaining families GT11 (*i*) and GT75 (*i*) have unknown 3D-structure.

The inspection of the GT in Archaea classification is the first step to identify their role in vivo and it is extremely useful to restrict the number of possible substrates to test for a certain enzyme. However, unfortunately, GTs have very strict substrate specificity and some families group very diverse enzymatic activities (i.e., GT2 and GT4) making the prediction of the substrate specificity very hard and of the role in vivo totally unpredictable from a simple amino acid sequence analysis. This explains why most of the information available on archaeal glycosylation came from deletion and complementation of GT genes and mass spectrometry analysis of glycoproteins rather than from biochemical and enzymatic approaches (Abu-Qarn et al. 2008). On the other hand, genetic tools for hyperthermophilic Archaea are by far less sophisticated than those available for halophiles and methanogens; thus, most of the studies on hyperstable GTs are structural and biochemical approaches.

The recent characterization of hyperthermophilic GTs gave useful hints on their function in vivo. A GT2 encoded by the PH0051 ORF in *P. horikoshii* was expressed by using an *E. coli* in vitro translation system and resulted to be a dolichol phosphoryl mannose synthase, a key enzyme in both N- and O-glycosylation of proteins (Urushibata et al. 2008). Instead, the only GT from hyperthermophiles characterized in GT4, the other major family of these enzymes, is a trehalose synthase encoded by ORF PH1035 in *P. horikoshii*, which catalyzed the transfer of UDP-Glc to free glucose; intriguingly, the enzyme was able also to hydrolyze trehalose back to glucose (Ryu et al. 2005). So far, GTs belonging to GT4 involved in protein glycosylation have never been identified in Archaea.

Another GT involved in the synthesis of a compatible solute, in this case α-mannosylglycerate, was the product of the ORF PH0927 in *P. horikoshii*, belonging to GT55. This enzyme catalyzed the transfer of α-mannose from GDP-Man to D-3-phosphoglycerate yielding α-mannosyl-3-phosphoglycerate while the downstream ORF (PH0926) promoted the subsequent dephosphorylation to α-mannosylglycerate (Empadinhas et al. 2001).

GT5 groups enzymes involved in starch and glycogen biosynthesis. Three enzymes from hyperthermophilic Archaea, *Methanocaldococcus jannashii, P. furiosus,* and *Pyrococcus abyssi*, have been isolated and characterized. The analysis of the 3D-structure of the GT from *P. abyssi*, which was the smallest member of the family, allowed to demonstrate that this enzyme had an unusual promiscuity in using both ADP-Glc and UDP-Glc as donor substrates in the transferring to the non-reducing end of a growing α-1,4 glucan chain (Horcajada et al. 2006). Several GTs from hyperthermophilic Archaea have been identified also in GT35, grouping glycogen phosphorylases. One of these, encoded by the ORF SSO2538 in *S. solfataricus*, could not be expressed in *E. coli*, but only in *T. kodakaraensis* in tiny, but sufficient amounts to demonstrate its pyridoxal 5′-phosphate-dependent activity (Mueller et al. 2009).

This small survey gives a general view of the difficulties commonly encountered in the study of glycosyltransferases: scarce expression yields, difficult strategies to identify the substrate of the enzyme, and laborious and expensive enzymatic assays. On the other hand, the advantages

of adopting hyperthermophilic GTs as model systems is also clear: (1) the remarkable stability of enzymes from this source allow easy purification and manipulation for 3D-structure determination (Horcajada et al. 2006; Igura et al. 2008); (2) their stability is a promising feature for the applicative exploitation of hyperstable GTs in large-scale carbohydrate synthesis; (3) the similarity between archaeal and eukaryotic N-glycosylation allow the use of the formers as a model system to study this process, which is important in several aspects of the biology of the cell and, therefore, in biomedicine and pharmaceutical industry.

Cross-References

- 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic Bacillus for the Production of Beta-Cyclodextrin
- 3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1
- 4.1 History of Discovery of Hyperthermophiles
- 4.3 Lignocellulose Converting Enzymes from Thermophiles
- 4.4 Enzymes Involved in DNA Amplification and Modification (e.g., Polymerases) from Thermophiles: Evolution of PCR Enzymes
- 4.9 Thermophilic Protein Folding Systems
- 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles

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4.3 Lignocellulose Converting Enzymes from Thermophiles

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Introduction

Due to the limitation of fossil resources, increasing CO_2 emission, and high fuel demands, the efficient utilization of renewable resources will play a crucial role in the future. Biomass derived from plants is considered to be a suitable resource for the production of energy carriers, e.g., ethanol, methane, hydrogen as well as high value products such as fine chemicals, building blocks, pharmaceuticals, and biopolymers (Biorefinery). Accordingly, the industrial (white) biotechnology with its current world market of more than 90 billion dollars will have a great impact on our life. The bottle neck in this technology is the development of robust enzymes that are able to convert the complex plant material to fermentable products such as glucose and xylose. Microorganisms that live at elevated temperatures are suitable candidates for the production of thermoactive biopolymer degrading enzymes. In most cases enzymes of thermophiles are superior to the traditional catalysts, because they allow performing industrial processes even under harsh conditions, under which conventional proteins are completely denatured. Furthermore, the solubility and accessibility of the complex substrates to the enzymes are improved at high temperatures and risk of contamination is reduced. However, the success of this technology demands the production of recombinant enzymes at high level in suitable industrial host microorganisms such as *Bacillus* sp. or yeast. The modern methods of genomics, genetic engineering, and synthetic biology combined with an increasing knowledge of structure/function and process engineering will allow further adaptation of enzymes to the needs of the industry.

There are a number of fungal, bacterial, and archaeal species that are able to break down lignocellulose that is composed of approximately 30–60% cellulose, 20–40% hemicellulose, and 10–30% lignin (Peters 2007). Aerobic and anaerobic microorganisms produce a large variety of glycosyl hydrolases, often acting in synergy to degrade cellulose and hemicellulose. Glycosyl hydrolases hydrolyze the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Henrissat and Coutinho 2001). They are classified into glycosyl hydrolase families (GH families) based on amino acid sequence similarities and up to date 118 GH families exist (Henrissat 1991; Cantarel et al. 2009; CAZy database www.cazy.org). Enzymes within a particular family exhibit a similar three-dimensional structure and a similar catalytic mechanism. Therefore, this classification system reflects both structural and mechanistic features (Henrissat and Davies 2000).

In this review we will focus on thermophilic microorganisms including bacteria and archaea that are able to degrade cellulose and hemicellulose as carbon and energy source. These enzymes are listed in the CAZy database and are classified into different glycosyl hydrolase families.

Cellulose-Degrading Enzymes

Cellulose is the most abundant organic biopolymer in nature and the main component of plant cell walls with 35–50% of the dry weight (Lynd et al. 2002). The unbranched cellulose polymer consists of up to 15,000 glucose units which are joined together by β-1,4-glycosidic bonds (Brett 2000). Natural cellulose fibers are structurally heterogenous and contain amorphous and highly ordered crystalline regions (Hazlewood and Gilbert 1998). The degree of crystallinity depends on the origin of the cellulose and constitutes 40–90% (Hilden and Johansson 2004; O'Sullivan 1997). The amorphous regions enhance the enzymatic hydrolysis, whereas the

crystalline regions are more resistant to enzymatic hydrolysis (Zhang and Lynd 2004). Efficient cellulose degradation requires the synergistic action of three different classes of glycosyl hydrolases: endoglucanases (EC 3.2.1.4), cellobiohydrolases or exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Endoglucanases hydrolyze β-1,4-glycosidic bonds in cellulose chains randomly at internal sites, preferring amorphous regions of cellulose, and producing oligosaccharides of various lengths and new chain ends. Cellobiohydrolases (Exoglucanases) cleave the β-1,4-glycosidic bonds of cellulose chains by removing cellobiose either from the reducing or the non-reducing ends. Cellobiohydrolases can also act on microcrystalline cellulose. β-Glucosidases catalyze the hydrolysis of β-1,4-linkages of cellodextrines and cellobiose to glucose.

Endoglucanases

Many Endoglucanases from various thermophilic bacteria and archaea have been investigated and these enzymes belong the glycosyl hydrolase families 5, 6, 8, 9, 12, 48, 51, and 74 (*Table 4.3.1*).

Among the archaea thermostable endoglucanases are produced by the hyperthermophilic species *Pyrococcus furiosus*, *Pyrococcus horikoshii*, and *Sulfolobus solfataricus*. A very heatstable recombinant endoglucanase from *P. furiosus* with a half-life of 40 h at 95°C has a temperature optimum of 100°C and the highest specific activity on cellopentaose and cellohexaose. This enzyme hydrolyzes only β-1,4- but not β-1,3-glycosidic linkages (Bauer et al. 1999). The recombinant hyperthermostable endoglucanase from *P. horikoshii* with maximal activity at 97°C also hydrolyzes β-1,4 glycosidic bonds with highest activity towards carboxymethyl cellulose (CMC), lichenan, and avicel but no xylanase activity was detected (Ando et al. 2002). The genome of the thermoacidophilic archaeon *Sulfolobus solfataricus* encodes three thermostable endoglucanases. The purified recombinant endoglucanase SSO1949 hydrolyzes CMC and cellooligosaccharides, with cellobiose and cellotriose as the main hydrolysis products. This enzyme is acid-stable with maximal activity at pH 1.8 and 80°C (Huang et al. 2005). The endoglucanase CelS from *S. solfataricus* is active towards CMC and the production of the enzyme is induced by the presence of CMC in the medium (Limauro et al. 2001). *Sulfolobus solfataricus* also produces the endoglucanase SSO1354, an enzyme with endoglucanase and xylanase activity. Degradation of cellulosic and hemicellulosic fractions using a mixture of SSO1354 and additional glycosyl hydrolases of *S. solfataricus* yielded in the production of glucose and xylose. Therefore SSO1354 represents an interesting candidate for utilization of agro-industrial waste for fuel production (Maurelli et al. 2008).

Several endoglucanases from thermophilic bacteria have been characterized (*Table 4.3.1*). Many endoglucanases are produced by the members of the genus *Clostridium*, especially by *Clostridium thermocellum*. The genes of *C. thermocellum* encoding endoglucanases have been cloned and expressed in *Escherichia coli*. These enzymes show optimal activity between 60°C and 75°C and pH 5.4–7.0. *C. thermocellum* produces a large number of cellulosome-bound or non-cellulosomal extracellular endoglucanases with activity towards many cellulose substrates like barley β-glucan, CMC, lichenan, avicel, acid swollen cellulose, p-nitrophenyl-β-cellobioside (pNP-cellobioside), and xylan (Béguin et al. 1983; Schwarz et al. 1986; Pétré et al. 1986; Hall et al. 1988; Chauvaux et al. 1990; Navarro et al. 1991; Lemaire and Béguin 1993; Hazlewood et al. 1993; Ahsan et al. 1997; Arai et al. 2001; Kurokawa et al. 2002; Zverlov et al. 2003). The activity of CelD from *C. thermocellum* is

Table 4.3.1

Endoglucanases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Acidothermus cellulolyticus	E1	5	81°C	5.25			Dai et al. (2000)
Alicyclobacillus acidocaldarius	CelA	9	70°C	5.5	30 min at 75°C	59	Eckert et al. (2002)
	CelB	51	80°C	4.0	>60 min at 80°C	100	Eckert and Schneider (2003)
Anaerocellum thermophilum	CelA	9, 48	85–95°C	5.0–6.0	40 min at 100°C	230	Zverlov et al. (1998a)
Aquifex aeolicus	CelY	8	80°C	7.0	4 h at 90°C 2 h at 100°C	36.8	Kim et al. (2000)
Caldicellulosiruptor saccharolyticus	CelB	5	85°C		29 h at 70°C	118–120	Saul et al. (1990)
	CelA	9, 48	70°C			194.6	Te'o et al. (1995)
Clostridium stercorarium	CelZ	9	80–90°C	6.0–6.5	>12 h at 80°C	100	Bronnenmeier and Staudenbauer (1990)
Clostridium thermocellum	CelB	5	60°C[a]	6.3		53–55	Beguin et al. (1983)
	CelC	5	60°C	6.0		39	Pétré et al. (1986)
	EGE	5	60°C[a]	6.5[a]		90.2	Hall et al. (1988)
	CelG	5	60°C[a]	5.5	8 min at 75°C	63.1	Lemaire and Beguin (1993)
	CelA	8	75°C	5.5–6.5	>2 h at 60°C	49–52	Schwarz et al. (1986)
	CelD	9	60°C	6.3[a]	13 min at 75°C		Chauvaux et al. (1990)
	CelF	9				82	Navarro et al. (1991)
	CelI	9	60°C	6.5[a]		98.5	Hazlewood et al. (1993)
	CelJ	9	70°C	6.5		60.3	Ahsan et al. (1997)
	CelN	9	70°C	5.4			Zverlov et al. (2003)
	CelQ	9	60°C	5.5		89.8	Arai et al. (2001)
	Cel9R	9	87.5°C	6.0	30 min at 90°C	74.4	Zverlov et al. (2005a)
	CelT	9	70°C	7.0		68.5	Kurokawa et al. (2002)

Fervidobacterium nodosum	CelI5A	5	80°C		48 h at 80°C		Zheng et al. (2009)
Geobacillus sp. 70PC53	CelA	5	65°C	5.0	>6 h at 65°C >4 h at 75°C	43	Ng et al. (2009)
Pyrococcus furiosus	EglA	12	100°C	6.0	40 h at 95°C	35.9	Bauer et al. (1999)
Pyrococcus horikoshii	EGPh (PH1171)	5	97°C	5.4–6.0	>3 h at 97°C	43	Ando et al. (2002)
Rhodothermus marinus	CelA	12	100°C	6.0–7.0	>4 h at 90°C	28.8	Halldórsdóttir et al. (1998)
Sulfolobus solfataricus	CelS	12	65°C[a]	5.8[a]		36.7	Limauro et al. (2001)
	SSO1354	12	90–95°C	3.5–4.0	53 min at 95°C	57	Maurelli et al. (2008)
	SSO1949	12	80°C	1.8	8 h at 80°C	37.5	Huang et al. (2005)
Thermobifida fusca	E5	5	50°C[a]	5.5[a]		46.3	Irwin et al. (1993)
	Cel5B	5	77°C	8.2	3 h at 77°C	67.7	Posta et al. (2004)
	E2	6	55°C[a]	6.5[a]		43	Ghangas and Wilson (1988)
	E1	9	50°C[a]	5.5[a]			Jung et al. (1993)
	E4	9	50°C[a]	5.5[a]			Jung et al. (1993)
Thermotoga maritima	CelI5A	5	80°C	6.0	18 h at 80°C	37.4	Chhabra et al. (2002)
	CelA	12	90°C	5.0–6.0	<6 h at 95°C 50 h at 85°C	29.7	Liebl et al. (1996)
	CelB	12	85–90°C		12 h at 90°C	31.7	Liebl et al. (1996)
	Cel74	74	90°C	6.0	5 h at 90°C	77	Chhabra and Kelly (2002)
Thermotoga neapolitana	CelA	12	95°C	6.0		29	Bok et al. (1998)
	CelB	12	106°C	6.0–6.6	130 min at 106°C 26 min at 110°C	30	Bok et al. (1998)
Thermus caldophilus	CelI5A	5	70°C	7.0[a]		44	Kim et al. (2006)

[a]Assay conditions.

stimulated by the presence of Ca^{2+}. Ca^{2+} enhances the activity of this enzyme towards avicel twofold and towards CMC and pNP-β-cellobioside fourfold (Chauvaux et al. 1990). The endoglucanase CelA of *C. thermocellum* is remarkably resistant to thiol reagents, SDS, and divalent cations and is not significantly affected by these various chemicals (Schwarz et al. 1986). The recombinant and purified endoglucanase CelC of *C. thermocellum* shows endo- and exoglucanase activity, since it has high activity on CMC and pNP-β-cellobioside and releases cellobiose units from cellotriose, cellotetraose, and cellopentaose (Pétré et al. 1986). The thermophilic bacterium *Clostridium stercorarium* produces a highly thermostable endoglucanase with maximal activity at 80–90°C and no loss of activity after incubation for 12 h at 60°C was observed. The incubation for 12 h at 80°C leads to the loss of one-third of its activity. This enzyme hydrolyzes avicel and produces cellotriose and cellotetraose as intermediates (Bronnenmeier and Staudenbauer 1990).

Further endoglucanases have been detected in the bacteria *Thermotoga maritima* and *Thermotoga neapolitana*. The expression of the gene encoding endoglucanase Cel5A is induced only when *T. maritima* is grown on glucomannan (Chhabra et al. 2002). Other endoglucanases produced by *T. maritima* are optimally active at 80–90°C and are highly thermostable with half-lives of 50 h at 85°C for CelA and 12 h at 90°C for CelB (Liebl et al. 1996). The endoglucanase Cel74 is active towards barley glucan, CMC, glucomannan, and xyloglucan but no activity towards avicel was observed. Avicelase activity was detected after creation of a fusion protein of Cel74 associated with a carbohydrate binding domain of a *Pyrococcus furiosus* chitinase (Chhabra and Kelly 2002). *T. neapolitana* produces two thermoactive endoglucanases with maximal activity at 95°C (CelA) and 106°C (CelB). CelB shows very high thermostability with a half-life of 130 min at 106°C and 26 min at 110°C. This enzyme also exhibits transglycosylation activity (Bok et al. 1998).

Recently, a thermostable endoglucanase with a half-life of 48 h at 80°C has been identified in *Fervidobacterium nodosum*, which is thermally activated by heating (Zheng et al. 2009).

Further endoglucanases from the thermoacidophilic bacteria *Acidothermus cellulolyticus* and *Alicyclobacillus acidocaldarius* were investigated. The endoglucanase gene of *A. celluloyticus* was expressed in transgenic tobacco and the resulting recombinant protein shows temperature and pH optima at 81°C and pH 5.25 (Dai et al. 2000). *A. acidocaldarius* produces the endoglucanase CelA which displayed a temperature optimum of 70°C and a pH optimum of 5.5. No signal peptide for transport across the cytoplasmic membrane was detected and, together with the pH optimum near neutral, the endoglucanase may be a cytoplasmic enzyme (Eckert et al. 2002). By contrast the endoglucanase CelB of *A. acidocaldarius* is an extracellular enzyme which is remarkably stable at acidic pH with an optimum pH at 4.0 (Eckert and Schneider 2003).

The endoglucanases produced by *Anaerocellum thermophilum* and *Caldicellulosiruptor saccharolyticus* are multidomain enzymes composed of two catalytic domains. The enzymes contain carbohydrate binding domains which are linked by proline-threonine-rich regions to the catalytic domains (Saul et al. 1990; Te´o et al. 1995; Zverlov et al. 1998a).

Bacteria belonging to the genera *Aquifex*, *Rhodothermus*, and *Thermobifida* are also good cellulose degraders. A recombinant thermostable endoglucanase of *Aquifex aeolicus* was produced in *E. coli* showing maximal activity at 80°C and pH 7.0 with half-lives of 2 h at 100°C and 4 h at 90°C (Kim et al. 2000). *Thermobifida fusca* produces a number of endoglucanases consisting of a catalytic and a carbohydrate binding domain and the enzymes show activity between 50°C and 77°C and pH 5.5–8.2 (Ghangas and Wilson 1988; Irwin et al. 1993; Jung et al. 1993; Posta et al. 2004). The recombinant endoglucanase of *Rhodothermus marinus*

has a pH optimum of 6.0–7.0 and a temperature optimum at 100°C (Halldórsdóttir et al. 1998). The aerobic thermophilic bacterium *Thermus caldophilus* also produces an endoglucanase which exhibits high activity on CMC with cellobiose and cellotriose as products (Kim et al. 2006).

Cellobiohydrolases

To date only few thermophilic bacteria are able to produce cellobiohydrolases and no archaeal gene encoding cellobiohydrolase has been identified so far (Mardanov et al. 2009). The investigated exoglucanases belong to glycosyl hydrolase families 5, 6, 9, and 48 and are produced by the genera *Clostridium*, *Thermobifida*, and *Thermotoga* (Table 4.3.2).

The cellobiohydrolases of *Clostridium thermocellum* are components of the cellulosome and are most active between 60°C and 65°C and pH 6.0–6.6. The cellobiohydrolase CelO consists of a catalytic domain and a carbohydrate binding module. The recombinant enzyme is active on cellodextrins, barley β-glucan, CMC, and insoluble cellulose, whereas cellobiose is the only product released from these substrates (Zverlov et al. 2002). CbhA also reveals a multidomain structure, it consists of two carbohydrate binding domains and a catalytical domain (Zverlov et al. 1998b). Another cellobiohydrolase produced by *C. thermocellum* is thermostable, with 97% of the original activity remaining after 200 h of incubation at 60°C. This enzyme is strongly inhibited by cellobiose (Kataeva et al. 1999).

The gene enconding cellobiohydrolase E3 of *Thermobifida fusca* was cloned and expressed in *Streptomyces lividans*. The recombinant enzyme is thermostable, retaining full activity after

Table 4.3.2

Cellobiohydrolases from thermophilic bacteria

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Clostridium stercorarium	CelY	48	75°C	5.0	1–2 h at 75°C	103	Bronnenmeier et al. (1997)
Clostridium thermocellum	CelO	5	65°C[a]	6.6[a]		71.7	Zverlov et al. (2002)
	CbhA	9	60°C[a]	6.0		136.1	Zverlov et al. (1998b)
	CelK	9	65°C	6.0	>200 h at 60°C	98	Kataeva et al. (1999)
Thermobifida fusca	E3	6	50°C[a]	7.0–8.0	>16 h at 55°C	59.7	Zhang et al. (1995)
	Cel48A	48				104	Irwin et al. (2000)
Thermotoga maritima			95°C	6.0–7.5	30 min at 95°C	29	Bronnenmeier et al. (1995)
Thermotoga sp. FjSS3-B.1			105°C	6.8–7.8	70 min at 108°C	36	Ruttersmith and Daniel (1991)

[a]Assay conditions.

incubation for 16 h at 55°C (Zhang et al. 1995). The multidomain enzyme Cel48A of *T. fusca* is active towards cellotetraose and cellopentaose and the major product of swollen cellulose hydrolysis is cellobiose (Irwin et al. 2000).

The most thermostable cellobiohydrolase reported to date is produced by *Thermotoga* sp. FjSS3-B.1. with a half-life of 70 min at 108°C. This enzyme is active against amorphous cellulose and CMC but only effects limited hydrolysis of filter paper (Ruttersmith and Daniel 1991).

β-Glucosidases

Thermoactive β-glucosidases are produced by a variety of thermophilic bacteria and archaea and belong to glycosyl hydrolase families 1 and 3 (› *Table 4.3.3*).

The β-glucosidase from *Caldicellulosiruptor saccharolyticus* was overproduced in *E. coli* and *Bacillus subtilis*. The recombinant enzyme is remarkably stable with a half-life of 38 h at 70°C (Love et al. 1988). The β-glucosidase produced by *Clostridium stercorarium* is existent as an extracellular and membrane-bound enzyme. Thiol groups are essential for its activity and the enzyme has a half-life of 5 h at 60°C in the presence of reducing agents and divalent cations (Bronnenmeier and Staudenbauer 1988). Two β-glucosidases are produced by *Clostridium thermocellum* which display optimal activity between 45°C and 65°C and pH 5.6–7.0 (Ait et al. 1979; Romaniec et al. 1993). The enzyme BglA of *C. thermocellum* is localized in the periplasmic space (Ait et al. 1979). The β-glucosidase BglB of *C. thermocellum* exhibits cellobiase and aryl-β-glucosidase activity, whereas the temperature optimum depends on the substrate. The cellobiase activity shows an optimum at 45°C, whereas the aryl-β-glucosidase has an optimum at 60°C (Romaniec et al. 1993).

Thermoanaerobacter brockii produces a β-glucosidase which releases glucose from the non-reducing end of β-1,4-cellooligomers and from various disaccharides. The enzyme exhibits activity towards glucosides, galactosides, and fucosides (Breves et al. 1997). The β-glucosidase of *Thermobifida fusca* is part of an operon, which includes also two sugar permeases. This enzyme is most active against cellobiose, cellotriose, cellotetraose, and sophorose; it also shows aryl-β-glucosidase activity and is insensitive to end-product inhibition by glucose (Spiridonov and Wilson 2001).

The gene encoding a β-glucosidase of *Thermobispora bispora* was cloned and expressed in *E. coli*. The enzyme shows maximal activity at 60°C and pH 6.2. This β-glucosidase is activated two- to threefold in the presence of glucose (Wright et al. 1992).

Several β-glucosidases are produced by the hyperthermophilic bacteria *Thermotoga maritima* and *Thermotoga neapolitana*. The enzymes display optimal activity at 90–95°C and pH 5.5–7.0 (Gabelsberger et al. 1993; Zverlov et al. 1997; Park et al. 2005). The activity of BglA from *T. neapolitana* markedly increases in the presence of reducing agents and the enzyme also exhibits transglycosylation activity (Park et al. 2005). The β-glucosidase BglB of *T. neapolitana* also shows laminaribiase activity. Together with a laminarinase of *T. neapolitana* the enzyme is capable of complete degradation of laminarin to glucose (Zverlov et al. 1997).

The β-glucosidases produced by the thermophilic bacterium *Thermus* are most active between 80°C and 90°C and pH 4.5–6.5 and the enzyme of *Thermus nonproteolyticus* also exhibits transglycosidic activity (Takase and Horikoshi 1988; Xiangyuan et al. 2001; Choi et al. 2003). The β-glucosidase of *Thermus* sp. Z-1 is extremely thermostable with a half-life of 5 days at 75°C (Takase and Horikoshi 1988).

Table 4.3.3

β-Glucosidases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Caldicellulosiruptor saccharolyticus	BglA	1	85°C	6.25[a]	38 h at 70°C	54.4	Love et al. (1988)
Clostridium stercorarium	BglZ	3	65°C	5.5	>5 h at 60°C	85	Bronnenmeier and Staudenbauer (1988)
Clostridium thermocellum	BglA	1	65°C	6.0	>7 h at 60°C	50	Ait et al. (1979)
	BglB	3	45°C and 60°C	5.6 and 7.0	10 h at 45°C 40 min at 60°C	84	Romaniec et al. (1993)
Fervidobacterium sp. YNP	BglA	1	85°C	7.3[a]		50	Lima et al. (2009)
Pyrococcus furiosus	CelB	1	102–105°C	5.0	85 h at 100°C 13 h at 110°C	58	Kengen et al. (1993)
Pyrococcus horikoshii	BGPh	1	>100°C	6.0	15 h at 90°C	35	Matsui et al. (2000)
Sulfolobus acidocaldarius	BgaS	1	90°C	5.5	494 h at 70°C 60 h at 80°C 0.2 h at 90°C	57	Park et al. (2010)
Sulfolobus shibatae	Bgly	1	95°C	5.0	15 h at 75°C 3.9 min at 95°C	57	Park et al. (2007)
Sulfolobus solfataricus	LacS	1	80°C[a]			56.9	Moracci et al. (1995)
Thermoanaerobacter brockii	CglT	1	75°C	5.5	>24 h at 60°C	50	Breves et al. (1997)
Thermobifida fusca	BglC	1	50°C	7.0		53.4	Spiridonov and Wilson (2001)
Thermobispora bispora	BglB	1	60°C	6.2	>48 h at 60°C	51.2	Wright et al. (1992)
Thermosphaera aggregans		1			>130 h at 80°C		Chi et al. (1999)
Thermotoga maritima	BglA	1	90°C	6.0–6.2	>6h at 95°C	47	Gabelsberger et al. (1993)

Table 4.3.3 (Continued)

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Thermotoga neapolitana	BglA	1	95°C	5.0–7.0	3.6 h at 100°C 12 min at 105°C	56.2	Park et al. (2005)
	BglB	3	90–95°C	5.5	1 h at 95°C 3 h at 90°C >18 h at 85°C	81	Zverlov et al. (1997)
Thermus caldophilus	BglT	1	80°C	6.0			Choi et al. (2003)
Thermus nonproteolyticus	Gly	1	90°C	5.6	2.5 h at 90°C	48.9	Xiangyuan et al. (2001)
Thermus sp. Z-1		1	85°C	4.5–6.5	5 d at 75°C	48	Takase and Horikoshi (1988)

[a]Assay conditions.

Several β-glucosidases have been detected in the archaeal genera *Sulfolobus* and *Pyrococcus*. The β-glucosidase of the hyperthermophilic *Pyrococcus furiosus* is remarkably thermostable with a half-life of 85 h at 100°C and 13 h at 110°C. This enzyme also exhibits high β-galactosidase and β-xylosidase activity, but no activity towards α-linked disaccharides or β-linked polymers (Kengen et al. 1993). The β-glucosidase BGPh of *Pyrococcus horikoshii* exhibits a temperature optimum over 100°C and maximal activity at pH 6.0. The enzyme´s stability is dependent on the presence of Triton X-100 with a half-life of 15 h at 90°C (Matsui et al. 2000).

The β-glucosidase of *Sulfolobus solfataricus* is very resistant to various denaturants with activity up to 85°C (Antranikian et al. 2005). The gene for this β-glucosidase has been cloned and overexpressed in *E. coli* (Moracci et al. 1995). *Sulfolobus acidocaldarius* produces a highly thermostable β-glucosidase with a half-life of 494 h at 70°C and 60 h at 80°C (Park et al. 2010).

Hemicellulose

Hemicellulose is the second most abundant polysaccharide in nature and represents about 20–40% of the lignocellulosic biomass (Peters 2007). Hemicelluloses are structural polysaccharides of the plant cell wall which consists of heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Classes of hemicellulose are named according to the main sugar unit of the backbone chain (Polizeli et al. 2005). The composition of hemicelluloses varies and depends on the plant source. Hardwood hemicelluloses contain mostly xylans, whereas in softwood hemicelluloses glucomannans are most common (Kumar et al. 2008). Due to the complexity of hemicellulose, various enzymes are necessary for its degradation. These enzymes can be distinguished into xylan- and mannan-degrading enzymes.

Xylan-Degrading Enzymes

Xylan, the major component of hemicelluloses, is a heterogenous polysaccharide consisting of a backbone of xylopyranosyl units linked by β-1,4-glycosidic bonds. Depending on its origin, the degree of polymerization varies from 70–130 in softwood xylans and from 150–200 in hardwood xylans (Salles et al. 2000). The xylan main chain contains mostly various substituent side groups such as acetyl, arabinosyl, and glucuronosyl residues. However, unsubstituted linear xylans also exist. Thus, xylans can be categorized as homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan (Saha 2003).

For the efficient hydrolysis of xylan several enzymes are necessary. The main enzymes involved in hydrolysis are endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Endoxylanase hydrolyzes the β-1,4-glycosidic linkages in the xylan backbone resulting in a decreased degree of polymerization of the substrate and formation of xylooligosaccharides. β-Xylosidase hydrolyzes small oligosaccharides and xylobiose by removing successive xylose residues from the non-reducing terminus. The side-chain substituents are removed by several accessory enzymes such as α-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72). Arabinose residues are released by the action

of α-arabinofuranosidase, which hydrolyzes the terminal non-reducing α-arabinofuranose from arabinoxylans. α-Glucuronidase hydrolyzes the α-1,2-linkages between the β-xylopyranosyl backbone and the glucuronic acid found in glucuronoxylans. Acetylated xylan is hydrolyzed by acetyl xylan esterase, which removes the acetyl groups from the xylopyranosyl residues of acetyl xylan.

Endoxylanases

Endoxylanases are widespread in thermophilic bacteria and are produced by the thermophilic archaea *Pyrodictium*, *Sulfolobus*, and *Thermococcus*. Thermoactive endoxylanases are members of glycosyl hydrolase families 10, 11, and 43 and they are mainly produced by species of the genera *Clostridium* and *Thermotoga* (*Table 4.3.4*).

The most thermostable bacterial endoxylanases are produced by *Thermotoga* species. XynA and XynB of *Thermotoga maritima* MSB8 are optimally active at 90°C and 105°C with half-lives of 40 min at 90°C for XynA and more than 3 h at 90°C for XynB. XynA is a modular enzyme composed of five domains, and the enzyme is able to adsorb to microcrystalline cellulose (Winterhalter et al. 1995). The activity of XynB of *T. maritima* MSB8 is stimulated twofold by the addition of 500 mM NaCl and both enzymes are tolerant to relatively high salt concentration up to 2 M NaCl (Winterhalter and Liebl, 1995). Endoxylanases produced by *T. maritima* FjSS3-B.1 are also remarkably thermostable with half-lives of 12 h at 95°C and 22 h at 90°C for XynA and 3 h at 85°C for XynB (Saul et al. 1995; Reeves et al. 2000). Like the endoxylanases of *T. maritima* MSB8 they also belong to the glycosyl hydrolase family 10. *Thermotoga neapolitana* also produces endoxylanases, which are highly stable with half-lives of 5 h at 100°C for XynA and 162 min at 90°C for XynB. XynA exhibits a multidomain structure and consists of three different domains. The core domain displays homology to glycosyl hydrolase familiy 10 and the N- and C-terminal are dispensable for thermostability and substrate binding (Zverlov et al. 1996). Like XynA, the second endoxylanase produced by *T. neapolitana*, namely XynB, exhibits activity towards xylan, lichenan, pNP-cellobioside, and pNP-xyloside (Velikodvorskaya et al. 1997).

Further endoxylanases from the thermophilic bacterium *Caldicellulosiruptor* have been investigated. The gene encoding endoxylanase XynA from *Caldicellulosiruptor saccharolyticus* was expressed in *E. coli* and the resulting recombinant protein is most active at 70°C and pH 5.5–6.0. This enzyme is stable for at least 72 h if incubated at 60°C, with half-lives of 8–9 h at 70°C (Lüthi et al. 1990). The multimodular xylanase produced by *Caldicellulosiruptor* sp. Rt8B.4 acts as endoxylanase, β-xylosidase, and α-arabinofuranosidase (Dwivedi et al. 1996). The two endoxylanases XynB and XynC from *Caldicellulosiruptor* sp. Rt69B.1 are highly modular enzymes, and they are composed of 10 and 8 discrete domains (Morris et al. 1999).

Several endoxylanases from the thermophilic anaerobic bacterium *Clostridium* are known. The enzyme XynC of *Clostridium stercorarium* consists of six domains and hydrolyzes xylan and xylooligosaccharides larger than xylotriose with the liberation of xylose and xylobiose (Ali et al. 1999). Another endoxylanase of *C. stercorarium* exhibits maximal activity at 75°C and pH 7.0 (Sakka et al. 1994). Xylanase activity of *C. stercorarium* was enhanced up to 9.5-fold if the organism is grown on arabinoxylan (Adelsberger et al. 2004). The endoxylanases from *Clostridium thermocellum* display optimal activity at 60–80°C and pH 5.5–6.8. The endoxylanase XynA produced by *C. thermocellum* is a component of the cellulosome and consists of four different domains. The enzyme was the first xylanase from *C. thermocellum*

Table 4.3.4

Endoxylanases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Caldicellulosiruptor saccharolyticus	XynA	10	70°C	5.5–6.0	8–9 h at 70°C 2–3 min at 80°C	40.5	Lüthi et al. (1990)
Caldicellulosiruptor sp. Rt8B.4	XynA	10	70°C	6.5	>24 h at 70°C	36	Dwivedi et al. (1996)
Caldicellulosiruptor sp. Rt69B.1	XynB	10	70°C	6.0–6.5			Morris et al. (1999)
	XynC	10 & 43	65°C	6.0–6.5			Morris et al. (1999)
Clostridium stercorarium	XynB	10	80°C	7.0	10 min at 100°C	44.4	Fukumura et al. (1995)
	XynC	10	85°C	5.0	>10 min at 75°C	115.3	Ali et al. (1999)
	XynA	11	75°C	7.0	>10 min at 70°C	54	Sakka et al. (1994)
Clostridium thermocellum	XynC	10	80°C	5.5	>10 min at 70°C	69.5	Hayashi et al. (1997)
	XynD	10	80°C	6.4	>30 min at 90°C	71.7	Zverlov et al. (2005b)
	XynX	10	60°C[a]			105	Jung et al. (1998)
	XynY	10	75°C	6.8		116	Fontes et al. (1995)
	XynZ	10	60°C[a]	6.3[a]		90	Grepinet et al. (1988)
	XynA	11	65°C	6.5		74.5	Hayashi et al. (1999)
Dictyoglomus thermophilus	XynA	10	85°C	6.5	>24 h at 85°C		Gibbs et al. (1995)
	XynB	11	85°C	6.5		39.8	Morris et al. (1998)
Geobacillus stearothermophilus	XynA	10	60°C	7.0	>1 h at 60°C	39.5	Nanmori et al. (1990)
	XT6	10	65°C	6.5	>10 h at 65°C	43	Khasin et al. (1993)
Pyrodictium abyssii			110°C	6.0[a]	>100 min at 105°C		Andrade et al. (2001)
Rhodothermus marinus	Xyn1	10	80°C	7.5	1 h 40 min at 80°C	48	Karlsson et al. (1998)
Sulfolobus solfataricus			90°C	7.0	47 min at 100°C	57	Cannio et al. (2004)

Table 4.3.4 (Continued)

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Thermoanaerobacterium saccharolyticum	XynA	10	70°C	5.5	>1 h at 75°C 35 min at 80°C	130	Lee et al. (1993)
Thermoanaerobacterium sp. JW/SL-YS 485	XynA	10	80°C	6.3	1 h at 77°C	150	Liu et al. (1996)
Thermobacillus xylanilyticus	XynA	10	75°C	6.5	30 min at 75°C	42	Connerton et al. (1999)
	Xyl11	11	75°C		12 h at 60°C 18 min at 70°C	20.7	Paës et al. (2006)
Thermobifida fusca	Xyl10B	10			16 h at 62°C	43	Kim et al. (2004)
	TfxA	11	50°C[a]	7.0	>18 h at 75°C	32	Irwin et al. (1994)
Thermococcus zilligii			80°C[a]	6.0	4 h at 95°C 8 min at 100°C	95	Uhl and Daniel (1999)
Thermotoga maritima FjSS3-B.1	XynA	10	85°C[a]	6.3	12 h at 95°C 22 h at 90°C	40.5	Saul et al. (1995)
	XynB	10	87°C	6.5	1.5 min at 95°C 4 min at 90°C 3 h at 85°C		Reeves et al. (2000)
Thermotoga maritima MSB8	XynA	10	90°C	6.2	40 min at 90°C	120	Winterhalter et al. (1995)
	XynB	10	105°C	5.4	>3 h at 90°C 125 min at 95°C	40	Winterhalter and Liebl (1995)
Thermotoga neapolitana	XynA	10	102°C	5.5	2 h at 100°C	116	Zverlov et al. (1996)
	XynB	10	90°C	5.5	162 min at 90°C 30 min at 100°C	39	Velikodvorskaya et al. (1997)

[a]Assay conditions.

classified in gylcosyl hydrolase familiy 11 (Hayashi et al. 1999). The multidomain enzyme XynC of *C. thermocellum* is active towards xylan and low activity against cellulosic substrates was observed. Immunological and sequence analyses demonstrated that XynC is one of the major components of the cellulosome (Hayashi et al. 1997). *C. thermocellum* also produces the endoxylanase XynD which hydrolyzes xylan and mixed-linkage 1,3-1,4-β-glucan with a temperature optimum at 80°C (Zverlov et al. 2005b). The endoxylanase XynX of *C. thermocellum* has a molecular weight of 105 kDa and exhibits the endoxylanase activity besides the activity against CMC and other soluble and insoluble cellulosic substrates (Jung et al. 1998). Another xylanase produced by *C. thermocellum* is part of the cellulosome and the enzyme shows a multidomain structure. This enzyme contains a central catalytical domain and an adjacent domain, which may be involved in thermostability of the endoxylanase (Fontes et al. 1995).

The thermophilic bacterium *Dictyoglomus thermophilum* is also an endoxylanase producer; two endoxylanases from this organism have been investigated. One of them, the xylanase XynA, is a single domain enzyme which displays maximal activity at 85°C and pH 6.5 and hydrolyzes xylan to xylotriose and xylobiose (Gibbs et al. 1995). In contrast, the other endoxylanase is a multidomain enzyme comprising a catalytical and a substrate-binding domain (Morris et al. 1998).

Several thermophilic bacteria belonging to the genera *Geobacillus*, *Rhodothermus*, and *Thermoanaerobacterium* are able to produce xylanases. The xylanase XynA of *Geobacillus stearothermophilus* liberates xylobiose and xylotriose from xylan at optimal temperature of 60°C (Nanmori et al. 1990). Another endoxylanase of *G. stearothermophilus* is most active at 65°C and pH 7.0 with a half-life of 6 h at 65°C (Khasin et al. 1993). The catalytical domain of *Rhodothermus marinus* endoxylanase was produced in *E. coli*. The activity of the recombinant protein is stabilized by addition of 1 mM calcium and the enzyme shows highest specific activity on oat spelt xylan with xylobiose and xylotriose as hydrolysis products (Karlsson et al. 1998).

The endoxylanases produced by the thermophilic anaerobic bacterium belonging to the genus *Thermoanaerobacterium* are large proteins with a molecular mass of 130 kDa for XynA of *Thermoanaerobacterium saccharolyticus* and 150 kDa for XynA of *Thermoanaerobacterium* sp. JW/SL-YS 485. They are most active at temperatures of 70–80°C and pH 5.5–6.3 (Lee et al. 1993; Liu et al. 1996). The single domain xylanase XynA from *Thermobacillus xylanilyticus* is able to bind and hydrolyze insoluble xylan efficiently, which is an unusual feature for a single catalytical domain protein (Connerton et al. 1999).

Thermobifida fusca is able to degrade xylan and produces a number of xylanases when grown on xylan or Solka Floc. The major hydrolysis product liberated by Xyl10B from birchwood and oat spelt xylan is xylobiose (Kim et al. 2004). Another endoxylanase produced by *T. fusca* is extremely stable, the enzyme retains 96% of its activity after incubation for 18 h at 75°C. The native enzyme consists of a catalytical and a substrate-binding domain and binds strongly to cellulose and xylan, whereas no cellulase activity was detected (Irwin et al. 1994).

Among the thermophilic archaea, a xylanase from *Pyrodictium abyssi* has been characterized with an optimal temperature at 110°C, one of the highest values reported for a xylanase (Andrade et al. 2001). The xylanase of *Thermococcus zilligii* is active up to 100°C and can attack different xylans but shows no activity towards cellulose (Uhl and Daniel 1999). The thermoactive endoxylanase of *Sulfolobus solfataricus* was purified and characterized. The products of xylan hydrolysis were xylooligosaccharides and xylobiose (Cannio et al. 2004).

β-Xylosidases

A large number of β-xylosidases are produced by thermophilic bacteria including species of the genera *Clostridium*, *Geobacillus*, *Caldicellulosiruptor*, *Thermoanaerobacter*, and *Thermoanaerobacterium*. The bacterial β-xylosidases belong to glycosyl hydrolase families 3, 39, 43, and 52 (*Table 4.3.5*), and to date only a bifunctional β-xylosidase/α-arabinofuranosidase of the archaeon *Sulfolobus solfataricus* was identified (Morana et al. 2007).

β-Xylosidases produced by *Clostridium stercorarium* are most active between 50°C and 80°C and pH 5.5–6.5. The enzyme Bxl3B of *C. stercorarium* hydrolyzes xylobiose and β-xyloside end groups of oligosaccharides from the non-reducing end and releases limited amounts of xylose from xylan (Adelsberger et al. 2004).

The β-xylosidase XylA of *Geobacillus stearothermophilus* produces xylose from xylobiose and xylotriose and has an optimal activity at a temperature of 70°C and pH 6.0 (Nanmori et al. 1990). A further characterized β-xylosidase of *G. stearothermophilus* is a member of glycosyl hydrolase family 52 and the enzyme appears to operate with an overall retention of the anomeric configuration. It is most active at 65°C and at pH 5.6–6.3 (Bravman et al. 2001). The gene encoding XynB1 of *G. stearothermophilus* is part of a gene cluster consisting of xylan utilization genes. The cluster includes the gene for both an extracellular and intracellular xylanase, two β-xylosidases, and genes involved in the utilization of α-glucuronic acid (Shulami et al. 1999). *Geobacillus thermoleovorans* produces a β-xylosidase belonging to family 43. The recombinant protein exhibits a half-life of 35 min at 57.5°C and a broad substrate specificity. It hydrolyzes xylooligosaccharides up to xylopentaose, and also releases xylose from birch and beechwood xylan (Wagschal et al. 2009).

The β-xylosidase of *Thermoanaerobacterium saccharolyticum* shows hydrolytic activity towards xylopentaose, xylotriose, xylobiose, and pNP-xylopyranoside, but activity towards xylan was not detected (Lee and Zeikus 1993).

α-Arabinofuranosidase

Among the thermophilic archaea, only *Sulfolobus solfataricus* shows α-arabinofuranosidase activity. Bacterial α-arabinofuranosidases are produced by members of the genera *Clostridium*, *Geobacillus*, *Thermoanaerobacter*, *Thermobacillus*, and *Thermotoga*. The α-arabinofuranosidases from thermophilic bacteria and archaea are members of gylcosyl hydrolase families 3, 43, and 51 (*Table 4.3.6*).

The archaeal bifunctional β-xylosidase/α-arabinosidase from *Sulfolobus solfataricus* exhibits the highest activity towards pNP-α-arabinoside followed by pNP-β-xylopyranoside. β-Xylosidase activity of this enzyme has a half-life of 10.5 h at 90°C whereas the α-arabinosidase activity has a shorter half-life of 2 h at 90°C (Morana et al. 2007).

Among the thermophilic bacteria members of the genus *Clostridium* are known to be good α-arabinofuranosidase producers. The α-arabinofuranosidase Arf43A of *Clostridium stercorarium* is optimally active at 55°C and pH 6.5. This enzyme hydrolyzes pNP-β-xylopyranoside and pNP-α-arabinofuranoside but has no activity on arabinoxylan or xylobiose (Adelsberger et al. 2004). A second α-arabinofuranosidase of *C. stercorarium* is a thermoactive bifunctional enzyme with β-xylosidase- and α-arabinofuranosidase activity. The purified enzyme has hydrolytic activity towards xylobiose, pNP-β-xylopyranoside, and pNP-α-arabinofuranoside with a maximal activity at 65°C and pH 7.0 (Sakka et al. 1993). Another

Table 4.3.5

β-Xylosidases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Caldicellulosiruptor saccharolyticus	XynB	39	70°C[a]	6.5[a]		56	Lüthi et al. (1990)
Clostridium stercorarium	Bxl3B	3	50–55°C	6.0	75 min at 50°C	79	Adelsberger et al. (2004)
	BxlA	39	60°C	5.5–6.5		58	Adelsberger et al. (2004)
	Xyl43B	43	80°C	3.5	>15 min at 70°C	56.4	Suryani et al. (2004)
Geobacillus stearothermophilus	XynB1	39					Czjzek et al. (2005)
	XynB3	43	65°C	6.5	>30 min at 60°C		Shallom et al. (2005)
	XylA	52	70°C	6.0	>1 h at 60°C	75	Nanmori et al. (1990)
	XynB2	52	65°C	5.6–6.3	2 h at 65°C	79.9	Bravman et al. (2001)
Geobacillus thermoleovorans	Xyl	43	40°C[a]	5.0	35 min at 57.5°C	58.1	Wagschal et al. (2009)
Sulfolobus solfataricus	XarS	3	80–85°C	6.5	10.5 h at 90°C	82	Morana et al. (2007)
Thermoanaerobacter brockii	XglS	3	60°C[a]	6[a]		81	Breves et al. (1997)
Thermoanaerobacterium saccharolyticum	XynB	39	70°C	5.5	55 min at 75°C	55	Lee and Zeikus (1993)
Thermoanaerobacterium sp. JW/SL-YS 485	XylB	39	65°C	5.5–6.0		58.5	Lorenz and Wiegel (1997)

[a]Assay conditions.

Table 4.3.6

α-Arabinofuranosidases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Clostridium stercorarium	ArfA	43	55°C	6.5			Adelsberger et al. (2004)
	XylA	43	65°C	7.0	>10 min at 65°C	53.3	Sakka et al. (1993)
	ArfB	51	70°C	5.0	0.7 h at 90°C	52	Schwarz et al. (1995)
Clostridium thermocellum	Araf51	51	82°C	7.0[a]			Taylor et al. (2006a)
Geobacillus caldoxylolyticus	AbfA	51	75–80°C	6.0	>48 h at 70°C	58	Canakci et al. (2007)
Geobacillus stearothermophilus	AbfA	51	70°C	5.5–6.0	53 h at 60°C 15 h at 65°C 1 h 70°C	64	Gilead and Shoham (1995)
Sulfolobus solfataricus	XarS	3	80–85°C	6.5	10.5 h at 90°C	82	Morana et al. (2007)
Thermoanaerobacter ethanolicus	XarB	3	75–93°C	5.7–5.9	15 min at 86°C >3 h at 82°C	85	Shao and Wiegel (1992)
Thermobacillus xylanilyticus	AbfD3	51	75°C	5.6–6.2	2 h at 90°C	56.1	Debeche et al. (2000)
Thermotoga maritima	AraA	51	90°C	7.0[a]	>24 h at 90°C 20 min at 100°C	55.3	Miyazaki (2005)

[a]Assay conditions.

α-arabinofuranosidase produced by *C. stercorarium* is the enzyme ArfB with optima for temperature and pH of 70°C and 5.0. ArfB hydrolyzes aryl and alkyl α-L-arabinofuranosides and cleaves arabinosyl side-chains from arabinoxylan (Schwarz et al. 1995). The thermophilic bacterium *Clostridium thermocellum* also produces an α-arabinofuranosidase and the crystal structure of the enzyme is solved. It catalyzes the hydrolysis of α-1,5-linked arabinooligosaccharides and the α-1,3-arabinosyl side-chains of xylan (Taylor et al. 2006a).

Further α-arabinofuranosidases are produced by members of the genus *Geobacillus*. The gene encoding an α-arabinofuranosidase of *Geobacillus caldoxylolyticus* was cloned and expressed in *E. coli*. The recombinant purified enzyme has an optimum pH of 6.0 and an optimum temperature of 75–80°C. This enzyme is highly thermostable and does not lose any activity after 48 h incubation at 70°C (Canakci et al. 2007). A thermostable α-arabinofuranosidase of *Geobacillus stearothermophilus* has been characterized. The enzyme releases arabinose from arabinan and has low activity on oat spelt xylan (Gilead and Shoham 1995). The enzyme XarB from *Thermoanaerobacter ethanolicus* also exhibits activity towards pNP-α-arabinofuranoside and pNP-β-xylopyranoside. The temperature optimum of this enzyme depends on the substrate, the maximal activity of the β-xylosidase is at 90–93°C whereas the α-arabinofuranosidase exhibits maximal activity at 75–80°C (Shao and

Wiegel 1992). The thermophilic bacterium *Thermobacillus xylanilyticus* produces an α-arabinofuranosidase which does not hydrolyze pNP-α-arabinopyranoside but shows activity on wheat arabinoxylan, larchwood xylan, and oat spelt xylan and liberates arabinose (Debeche et al. 2000). The α-arabinofuranosidase produced by *Thermotoga maritima* is extremely thermostable. No loss of activity was observed after 24 h incubation at 90°C. This is the most thermostable α-arabinofuranosidase described to date (Miyazaki 2005).

α-Glucuronidase

Among the thermophilic bacteria known so far, only *Geobacillus stearothermophilus* and *Thermotoga maritima* are able to produce α-glucoronidases; no archaeal α-glucoronidases have been identified (Table 4.3.7). The characterized α-glucuronidases of *G. stearothermophilus* and *T. maritima* belong to glycosyl hydrolase families 4 and 67.

Two different strains of *G. stearothermophilus* produce α-glucuronidases with optimal activity between 40°C and 65°C and pH 5.5–6.5 (Choi et al. 2000; Zaide et al. 2001). The α-glucuronidase of *G. stearothermophilus* 236 acts mainly on small substituted xylo-oligomers, and synergism in the release of xylose from xylan was observed when the enzyme was added to a mixture of endoxylanase and β-xylosidase (Choi et al. 2000). The α-glucuronidase Agu4B of *T. maritima* exhibits activity towards pNP-α-glucuronopyranoside but no activity towards 4-*O*-methyl-glucuronoxylan was detected. The enyzme is most active at 80°C and pH 7.8 and requires Mn^{2+} and thiol compounds for its activity (Suresh et al. 2003). Another gene encoding a thermoactive α-glucuronidase of *T. maritima* was cloned and expressed in *E. coli*. The enzyme shows highest activity at 85°C and pH 6.3 and hydrolyzes 2-*O*-(4-*O*-methyl-α-glucopyranosyluronic acid)-xylobiose to xylobiose and 4-*O*-methylglucuronic acid (Ruile et al. 1997).

Acetyl Xylan Esterase

The acetyl xylan esterases of thermophilic bacteria are produced by *Clostridium thermocellum* and *Thermoanaerobacterium* sp. and are members of carbohydrate esterase family 2, 3, 4, and 7 (Table 4.3.8). No archaeal acetyl xylan esterase has been described so far.

Table 4.3.7

α-Glucuronidases from thermophilic bacteria

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Geobacillus stearothermophilus T-6	AguA	67	65°C	5.5–6.0	>20 min at 70°C	78.5	Zaide et al. (2001)
Geobacillus stearothermophilus 236	AguA	67	40°C	6.5	50 min at 50°C	78.2	Choi et al. (2000)
Thermotoga maritima	Agu4B	4	80°C	7.8	>30 min at 80°C		Suresh et al. (2003)
	AguA	67	85°C	6.3		78.6	Ruile et al. (1997)

Table 4.3.8

Acetyl xylan esterases from thermophilic bacteria

Organism	Enzyme	CE family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Clostridium thermocellum	CtCel5C-CE2	2		7.0[a]		90.2	Montanier et al. (2009)
	CtCes3	3		7.0	>15 min at 70°C		Correia et al. (2008)
	CtCE4	4					Taylor et al. (2006b)
Thermoanaerobacterium sp. JW/SL YS485	Axe1	7	75°C	7.0		31	Lorenz and Wiegel (1997)

[a]Assay conditions.

Domains of acetyl xylan esterases are often found in multimodular plant cell wall-degrading enzymes (Taylor et al. 2006b). The multifunctional protein CtCel5C-CE2 of *Clostridium thermocellum* contains an N-terminal domain that displays cellulase activity and a C-terminal domain which exhibits a noncatalytical cellulose-binding function and catalytic esterase activity. The catalytic esterase module catalyzes deacetylation of noncellulosic polysaccharides (Montanier et al. 2009). Another acetyl xylan esterase produced by *C. thermocellum* is a thermostable acetyl-specific esterase with high activity on acetylated xylan. This protein is part of the cellulosome and it consists of two catalytical modules and a type I dockerin (Correia et al. 2008). The acetyl xylan esterase CtCE4, which is also produced by *C. thermocellum*, derives from the xylanase Xyn11A and is a metal ion-dependent enzyme. This multimodular protein consists of an N-terminal xylanase domain, a carbohydrate binding module, a dockerin domain, and a C-terminal carbohydrate esterase domain (Taylor et al. 2006b). The structure of the acetyl xylan esterases of *C. thermocellum* has been solved and the proteins display an α/β hydrolase fold. The thermophilic bacterium *Thermoanaerobacterium* sp. JW/SL YS485 produces also an acetyl xylan esterase. The gene was cloned and expressed in *E. coli* and the purified enzyme exhibits the highest specific activity towards xylose tetraacetate and triacetin substrates (Lorenz and Wiegel 1997).

Mannan-Degrading Enzymes

Besides xylan, mannan is the other major constituent of hemicelluloses. Mannan consists of a β-1,4-linked backbone containing mannose residues or a combination of mannose and glucose. Additionally, α-1,6-linked galactose residues can be substituted to the mannan backbone. Different forms of mannan like linear mannan, glucomannan, galactomannan, and galactoglucomannan exist. Mannan is widespread in softwoods, which mainly consists of galactoglucomannan and glucomannan. In contrast, hardwoods contain only a small amount of glucomannan (Moreira and Filho 2008).

The degradation of mannan involves β-mannanases (EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25). The internal β-1,4-linkages of the mannan main chain are cleaved by the

β-mannanase, liberating new chain ends. β-Mannosidase hydrolyzes mannan and mannooligosaccharides from the non-reducing end and liberates mannose residues. α-Galactosidase (EC 3.2.1.22) is required for the removal of side-chain substituents. The enzyme hydrolyzes α-1,6-linked galactopyranosyl side-chains of galactomannan and galactoglucomannan.

β-Mannanases

A number of thermophilic bacteria produce β-mannanases but until today no mannanases from archaea were identified. The bacterial mannanases are distributed in glycosyl hydrolase families 5, 26, and 113 (Table 4.3.9).

The crystal structure of the intracellular mannanase ManA from *Alicyclobacillus acidocaldarius* was solved and the catalytical residues were identified. The protein shows the

Table 4.3.9

β-Mannanases from thermophilic bacteria

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Alicyclobacillus acidocaldarius	ManA	113	65°C	5.5			Zhang et al. (2008)
Caldanaerobius polysaccharolyticus	ManA	5	65°C 75°C	5.8		119.6	Cann et al. (1999)
Caldibacillus cellulovorans	ManA	5	85°C	6.0	15 min at 80°C >24 h at 70°C	31	Sunna et al. (2000)
Caldicellulosiruptor saccharolyticus	ManA	5	80°C	6.0	1 h at 80°C	38.9	Lüthi et al. (1991)
Caldicellulosiruptor sp. RT8B.4	ManA	26	60–65°C	6–6.5			Gibbs et al. (1996)
Clostridium thermocellum	ManA	26	65°C	6.5	30 min at 74°C	66.8	Halstead et al. (1999)
	Man26B	26	75°C	7.0		67	Kurokawa et al. (2001)
Dictyoglomus thermophilum	ManA	26	80°C	5.0	5.4 min at 90°C	40	Gibbs et al. (1999)
Geobacillus stearothermophilus	ManF	5	70°C[a]	5.5–7.5	>24 h at 70°C	73	Talbot and Sygusch (1990)
Rhodothermus marinus	ManA	26	85°C	5.4	>1 h at 70°C	113	Politz et al. (2000)
Thermobifida fusca	Man	5	80°C			38	Hilge et al. (1998)
Thermotoga maritima	Man5	5	90°C	7.0		76	Parker et al. (2001)

[a]Assay conditions.

architecture of a $(\beta/\alpha)_8$-barrel motif and two glutamate residues are involved in catalysis (Zhang et al. 2008). The multidomain β-mannanase of *Caldanaerobius polysaccharolyticus*, consisting of a mannanase-cellulase catalytical domain and two carbohydrate binding domains, exhibits optimal activity between 65°C and 75°C, whereas the temperature optimum depends on the substrate (Cann et al. 1999). ManA from *Caldibacillus cellulovorans* exhibits activity towards substituted substrates but no activity towards unsubstituted mannan was observed. The enzyme is most active at 85°C with a half-life of 15 min at 80°C and it reveals a multidomain structure. It consists of one domain with unknown function, two carbohydrate binding domains, and a catalytic β-mannanase domain (Sunna et al. 2000). The β-mannanase produced by *Caldicellulosiruptor saccharolyticus* has an optimal activity at 80°C and pH 6.0 and hydrolyzes locust bean gum, guar gum, and glucomannan (Lüthi et al. 1991).

The anaerobic bacterium *Clostridium thermocellum* produces two β-mannanases which belong to glycosyl hydrolase family 26. Both enzymes are components of the cellulosome (Halstead et al. 1999; Kurokawa et al. 2001). *Dictyoglomus thermophilum* produces a single domain β-mannanase, which liberates mannose, mannobiose, and mannotriose from locust bean gum (Gibbs et al. 1999). The β-mannanase of *Geobacillus stearothermophilus* is highly thermostable, it retains nearly full activity after incubation for 24 h at 70°C (Ethier et al. 1998). The thermophilic bacterium *Rhodothermus marinus* is able to hydrolyze carob-galactomannan. The corresponding recombinant enzyme reveals a temperature and pH optimum of 85°C and pH 5.4 (Politz et al. 2000).

β-Mannosidases

Only few β-mannosidases from thermophilic archaea and bacteria have been characterized so far. These thermostable enzymes belong to glycosyl hydrolase families 1 and 2 and are produced by the archaeon *Pyrococcus* and the bacteria *Thermobifida* and *Thermotoga* (*Table 4.3.10*).

The β-mannosidase from *Pyrococcus furiosus* has an optimum temperature of 105°C and is highly stable with a half-life time of more than 60 h at 90°C. The enzyme shows wide substrate specificity and is active against pNP-β-mannopyranoside, pNP-β-galactopyranoside, pNP-β-glucopyranoside, and pNP-β-xylopyranoside (Bauer et al. 1996). *Pyrococcus horikoshii* also

Table 4.3.10

β-Mannosidases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Pyrococcus furiosus	BmnA	1	105°C	7.4	>60 h at 90°C 77 min at 110°C	59	Bauer et al. (1996)
Pyrococcus horikoshii	BglB	1	90°C	4.75	27 min at 102°C	56.5	Kaper et al. (2002)
Thermobifida fusca	ManB	2	53°C	7.17	30 h at 40°C 10 min at 60°C	94	Béki et al. (2003)
Thermotoga neapolitana	Man2	2	90°C	7.0	38 min at 90°C	92	Parker et al. (2001)

produces a β-mannosidase, which is optimally active at 102°C (Kaper et al. 2002). Both enzymes belong to glycosyl hydrolase family 1. The β-mannosidase from the thermophilic actinomycete *Thermobifida fusca* shows optimal activity at 53°C and possesses also glycosyl transferase activity. The enzyme, however, is active only against pNP-mannopyranoside (Béki et al. 2003). The recombinant enzyme Man2 from *Thermotoga neapolitana* is active towards pNP-β-mannopyranoside and β-1,4-mannobiose with an optimal activity at 90°C (Parker et al. 2001).

α-Galactosidases

The majority of α-galactosidases were investigated from thermophilic bacteria and all belong to glycosyl hydrolase family 36 (*Table 4.3.11*).

The α-galactosidase produced by the crenarchaeon *Sulfolobus solfataricus* represents the first thermostable α-galactosidase from archaea. The enzyme optimally hydrolyzes α-linked galactosides at 90°C and pH 5.0 and has a half-life of 30 min at 90°C (Brouns et al. 2006).

Table 4.3.11

α-Galactosidases from thermophilic bacteria and archaea

Organism	Enzyme	GH family	T_{opt}	pH_{opt}	Thermostability (half-life)	MW (kDa)	Reference
Clostridium stercorarium	Aga36A	36	70°C	6.0		84.8	Suryani et al. (2003)
Geobacillus stearothermophilus	AgaA	36	65–67°C				Fridjonsson and Mattes (2001)
	AgaB	36		7.0[a]			Dion et al. (2001)
	AgaN	36	75°C	6.5[a]	3.5 h at 75°C 19 h at 70°C	80.3	Fridjonsson et al. (1999a)
Sulfolobus solfataricus	GalS	36	90°C	5.0	30 min at 90°C >2.5 h at 80°C	74.7	Brouns et al. (2006)
Thermotoga maritima	GalA	36	90–95°C	5.0–5.5	6.5 h at 85°C	63.7	Liebl et al. (1998)
Thermotoga neapolitana	AglA	36	93°C	7.0	>4 h at 80°C	61	King et al. (1998)
Thermus brockianus	AgaT	36	93°C	5.5–6.5	100 min at 92°C 17 h at 80°C	53.8	Fridjonsson et al. (1999b)
Thermus sp. T2	AglA	36	75°C	6.0[a]	1 h at 70°C	53.5	Ishiguro et al. (2001)
Thermus thermophilus	AgaT	36					Fridjonsson et al. (2000)

[a]Assay conditions.

Several thermophilic bacteria, belonging to the genera *Clostridium*, *Geobacillus*, *Thermotoga*, and *Thermus*, are able to produce α-galactosidases. *Clostridium stercorarium* produces an α-galactosidase which hydrolyzes raffinose and guar gum and is optimally active at 70°C and pH 6.0 (Suryani et al. 2003). The gene for a recombinant α-galactosidase of *Geobacillus stearothermophilus* was cloned and expressed in *Thermus thermophilus*. The enyzme displays maximal hydrolyzing activity at 65–67°C and a high affinity for melibiose and raffinose (Fridjonsson and Mattes 2001). Another α-galactosidase produced by *G. stearothermophilus* is optimally active at 75°C and has a half-life of 19 h at 70°C (Fridjonsson et al. 1999a). The α-galactosidases derived from the genus *Thermotoga* are thermostable exhibiting half-lives of 6.5 h at 85°C for GalA of *Thermotoga maritima* and more than 4 h at 80°C for AglA of *Thermotoga neapolitana*. The potential application of AglA of *T. neapolitana* to high temperature processing of soy molasses has been demonstrated (King et al. 1998). The gene of *T. maritima* α-galactosidase is part of a galactoside utilization operon (Liebl et al. 1998).

The gene encoding AgaT of *Thermus brockianus* is also located in a gene cluster of closely linked galactoside utilization genes. Inactivation of the gene by insertional mutagenesis results in the inability of *T. brockianus* to use melibiose or galactose as single carbohydrate source (Fridjonsson et al. 2000). The recombinant protein AgaT is thermostable with half-lives of 100 min at 92°C and 17 h at 80°C (Fridjonsson et al. 1999b). The gene encoding an α-galactosidase of *T. thermophilus* on the other hand is only located adjacent to a galactose-1-phosphate uridyltransferase gene and no galactose utilization genes were identified upstream of the *agaT* gene (Fridjonsson et al. 2000). Another α-galactosidase is produced by the *Thermus* strain T2 which acts only on terminal α-galactosyl residues of galactomannan-oligosaccharides and not on the side chain residues. The gene encoding this enzyme was also cloned and expressed in *E. coli* and the purified α-galactosidase is most active at 75°C (Ishiguro et al. 2001).

Conclusions

Owing to their properties such as activity over a wide temperature range, diverse substrate range, stability at elevated temperatures and in organic solvents, the polymer degrading enzymes from thermophiles will represent the choice for future countless applications in industry. Especially novel lignocellulose degrading enzymes will be needed to develop sustainable bioindustry. To date, the main feedstock for biorefineries is based on starch, however, lignocellulose is the more attractive renewable resource since it is available in large quantities and does not compete with food industry. The main challenge in the future will be the development of a fully integrated and sustainable bioindustry based on the renewable resource lignocellulose (second generation biorefinery).

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4.4 Enzymes Involved in DNA Amplification (e.g. Polymerases) from Thermophiles: Evolution of PCR Enzymes

Towards a Better PCR System Based on a KOD DNA Polymerase

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.4,

Abstract: *Taq* DNA polymerase from *Thermus aquaticus* and *Tth* DNA polymerase from *Thermus thermophilus* are thermostable DNA polymerases conventionally used in PCR (polymerase chain reaction) and they are classified in pol I like bacterial DNA polymerase family (family A). However, recently archaeal DNA polymerases classified in α-like DNA polymerase family (family B DNA polymerase) from *Pyrococcus furiosus, Pyrococcus* GB-D, *Thermococcus litoralis* are often used in PCR because of their high fidelity in DNA synthesis based on 3′–5′ exonuclease activity for proofreading of misincorporated nucleotides. Indeed high fidelity is ideal for PCR but these family B polymerases often require longer reaction time (at least 2 min) because of their low elongation speed. We have recently reported a new thermostable family B polymerase, KOD DNA polymerase from hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, which is an efficient PCR enzyme with high fidelity and extension rate.

In this article, development of PCR enzymes including (1) Characterization of a new PCR enzyme, KOD DNA polymerase, (2) Engineering of a new long and accurate (LA) PCR enzyme, and (3) Improvement of PCR by neutralizing monoclonal antibodies, (4) High success-rate DNA polymerase KOD-FX, will be reviewed.

Introduction

The melding of a technique for repeated rounds of DNA synthesis with the discovery of a thermostable DNA polymerase has given scientists the very powerful technique known as PCR (Saiki et al. 1988). PCR is based on three simple steps required for any DNA synthesis reaction; (1) denaturation of the template into single strands; (2) annealing of primers to each original strand for new strand synthesis; (3) extension of the new DNA strands from the primers (Delidow et al. 1993). To perform more than one round of synthesis, the templates must again be denatured at high temperature. Enzymes from hyperthermophiles are extremely thermostable and of industrial importance. PCR, which uses the thermostable DNA polymerase, is one of the most important contributions to protein and genetic research (Saiki et al. 1988).

Recently, it was shown that Archaea constitute a third domain of living organisms that is distinguishable from the domains Bacteria and Eucarya (Woese et al. 1990). On the phylogenetic tree, the hyperthermophiles are the deepest and shortest branches, and the last common ancestor on this tree, most likely thrived in extreme thermal environments (Rivera and Lake 1992). We are currently screening hyperthermophilic archaea to find new thermostable enzymes useful for research, diagnosis, and industrial applications. One strain, *Thermococcus kodakaraens* is KOD1, isolated from a solfataric hot spring at Kodakara Island in Japan, was identified (Morikawa et al. 1994; Atomi et al. 2004), and characterization of many genes and their products was performed. Complete sequence of its whole genome was also determined (Fukui et al. 2005), indicating that at most 2,306 open reading frames are contained and the gene structure of this archaeon is more closely related to that of eucarya than that of bacteria.

Cloning and Analyses of KOD DNA Polymerase Gene

KOD DNA polymerase gene was cloned and the most interesting features of the gene are the existence of two intervening sequences (*Fig. 4.4.1*). Sequences similar to those of KOD1 were found in the *Tli* DNA polymerase (Perler et al. 1992), and they are designated inteins. However, the *Tli* inteins are in conserved regions III(*Tli* pol intein-1, 538 amino acid residues) and I (*Tli* pol intein-2, 390 amino acid residues). The amino acid sequence of intein-2 from KOD

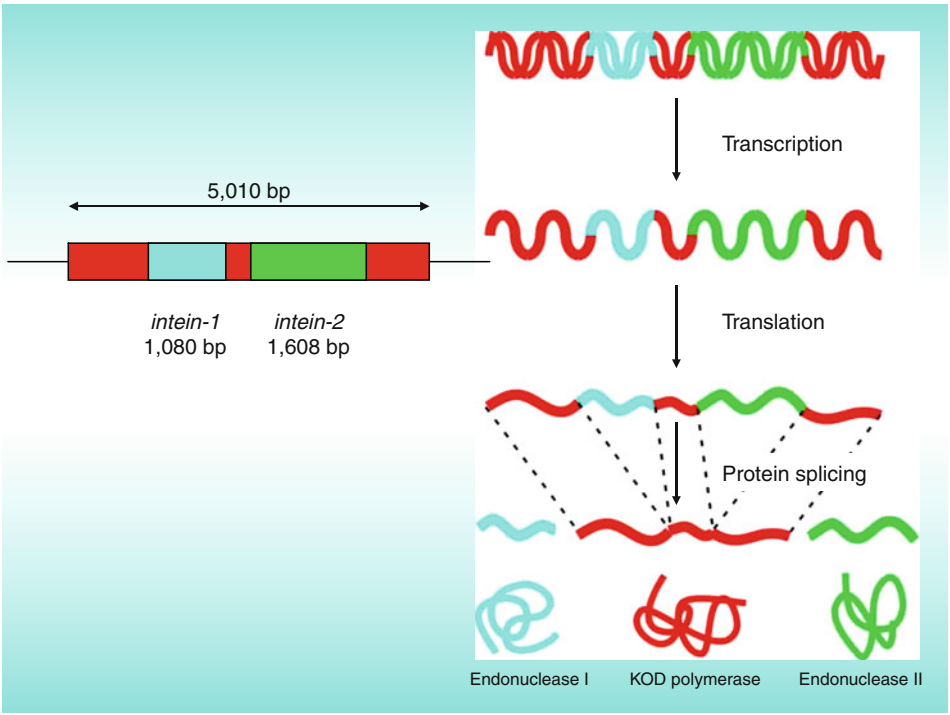


Fig. 4.4.1

The structure of the DNA polymerase (KOD polymerase) gene from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1

DNA polymerase exhibited lower similarity, 62% identity, to intein-1 from *Tli* DNA polymerase than to that of the mature DNA polymerase portion (78% identity). KOD pol intein-1 did not show significant similarity to either *Tli* pol intein-1 or *Tli* intein-2 (32% identity). The splice sites of these inteins could be predicted by amino acid sequence, since they are found in conserved regions. Cys or Ser at the N-terminal and His-Asn-Cys/Ser/Thr at the C-terminal splice junctions are very well conserved (Hodges et al. 1992). These residues might be important for the inteins to be spliced out from the KOD DNA polymerase precursor at the protein level (Takagi et al. 1997). Recently we have performed precise characterization of two inteins (Nishioka et al. 1998), moreover, intein-2 has been crystallized to perform tertiary structural study (Hashimoto et al. 2000).

The amino acid sequence of KOD DNA polymerase was compared from different sources. The amino acid sequence of the mature form of KOD DNA polymerase was similar to those of *T. litoralis* (Tli DNA polymerase; 78% identity), *Pyrococcus furiosus* (*Pfu* DNA polymerase; 79% identity), *Pyrococcus* sp. strain GB-D (Deep Vent DNA polymerase; 81% identity), and *Thermococcus* sp. Strain 9°N-7 (90% identity) (Takagi et al. 1997).

Characteristics of KOD DNA Polymerase

Recombinant KOD DNA polymerase was purified, and its characteristics were studied and compared with those of DNA polymerases from different sources. Frequency of

misincorporation of deoxyribonucleotide was 0.35%, indicating that KOD DNA polymerase has high fidelity, equivalent to that of *Pfu* DNA polymerase (Table 4.4.1). Processivity (persistence of sequential nucleotide polymerization) of the KOD DNA polymerase appears to be 10- to 15-fold greater than those for *Pfu* DNA polymerase and Deep Vent DNA polymerase. Moreover, even when the amount of enzyme was decreased, large products of the same size could be obtained. The extension rate of KOD DNA polymerase was 106–138 bases/s (Fig. 4.4.2), which was much higher than those of *Pfu* (25 bases/s), Deep Vent (23 bases/s), and *Taq* (61 bases/s) DNA polymerases. The characteristics of KOD DNA polymerase are summarized and compared with those of representative DNA polymerases (*Pfu*, Deep Vent, and *Taq* DNA polymerases) in Table 4.4.2 and Fig. 4.4.3.

Accurate and Time-Saving PCR by KOD DNA Polymerase

The major uses of thermostable DNA polymerases are for in vitro amplification of DNA fragments and for determination of DNA sequence. *Taq* DNA polymerase was used in many cases, but its fidelity is not high. To improve low fidelity in PCR, new thermostable DNA polymerases isolated from several hyperthermophiles have been characterized and used for PCR. These DNA polymerases exhibit proofreading ability based on exonuclease activity that edits out mismatched nucleotides. In general, these DNA polymerases can amplify DNA more accurately than *Taq* DNA polymerase. However, *Taq* DNA polymerase was not replaced with these DNA polymerases because of their low extension rates among other factors. Our experimental results clearly show that KOD DNA polymerase has excellent characteristics in fidelity (low mutation frequency), processivity, and elongation speed, suggesting that the KOD DNA polymerase is suitable for long, accurate, and time-saving PCR. Since the high processivity and high extension rate of KOD DNA polymerase may contribute to a decrease in the total reaction time, we performed PCR using various reaction times (1–120 s) with λDNA as a template (Fig. 4.4.4). When the KOD DNA polymerase was used, an amplified DNA product could be observed even with a very short reaction time (1 s). In the case of *Taq* and *Pfu* DNA polymerases, the DNA band could be observed at the minimum tested reaction

Table 4.4.1

Comparison of fidelities of thermostable DNA polymerases by the method of Kunkel et al.

Enzyme	No. of plaques scored[a]		Mutation frequency[b] (%)
	Mutant	Total	
KOD	23	6,619	0.35
Pfu	30	7,691	0.39
Taq	54	4,141	1.3
Tth	115	7,375	1.6
Taq-Pfu (20:1)	30	4,238	0.71
Taq-Pfu (50:1)	23	4,489	0.51

[a]Mutant, colorless plaques plus lighter bluc plaques; total, blue plaques plus mutant plaques.
[b]Mutant plaques/total plaques.

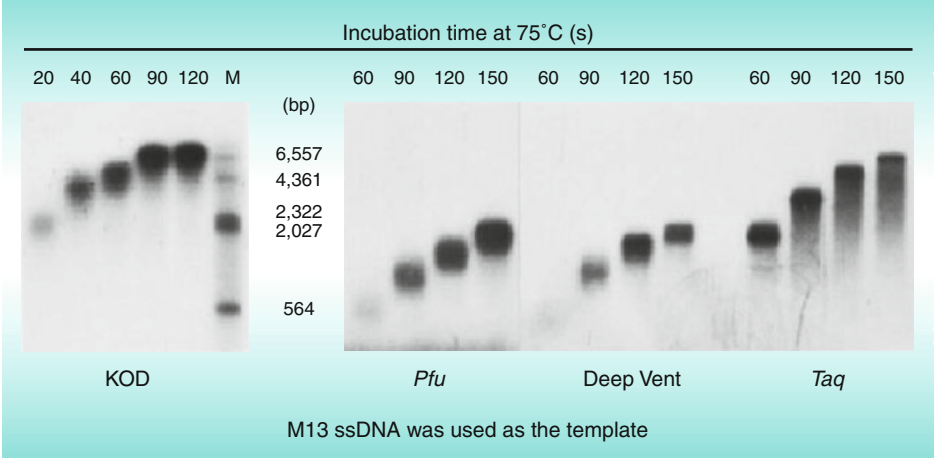


Fig. 4.4.2

Comparison of the elongation rates of various DNA polymerases

Table 4.4.2

Properties of themostable DNA polymerases

Property	Value for indicated DNA polymerase			
	KOD	*Pfu*	Deep Vent	*Tag*
Deduced molecular mass (KDa)	90	90.1	90.6	93.9
Optimum temp[a](°C)	75	75	75	75
Optimum pH at 75°C[a]	6.5	6.5	7.5	8–8.5
Thermostability (half-life)[a]	95°C, 12 h; 100°C, 3 h	95°C, 6 h; 100°C, 2.9 h	95°C, 13.5 h; 100°C, 3.4 h	95°C, 1.6 h
3′-5′ exonuclease activity	+	+	+	–
Fidelity[b]	3.5×10^{-3}	3.9×10^{-3}	ND[c]	1.3×10^{-2}
Terminal transferase activity	–	–	–	+
Processivity (bases)[b]	>300	<20	<20	ND
Elomgation rate(bases/s)[b]	106–138	25	23	61

[a]Our experimental results, using KOD DNA polymerase and commercially available DNA polymerases.
[b]Data from this study.
[c]ND, not determined.

times of 5 and 60 s, respectively. DNA polymerases with higher fidelity are not necessarily suitable for amplification of long DNA fragments because of their potentially strong exonuclease activity. Amplification of a longer target DNA sequence (6 kb) by using the KOD DNA polymerase was attempted; the desired DNA fragment could be observed after a 1 min reaction,

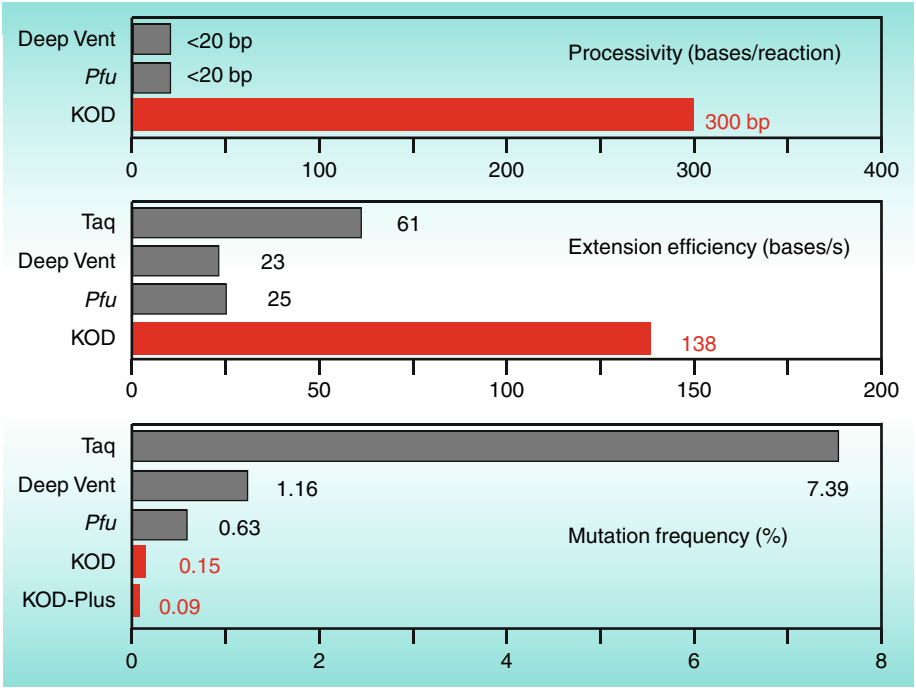


Fig. 4.4.3
Comparison of the characteristics of various DNA polymerases

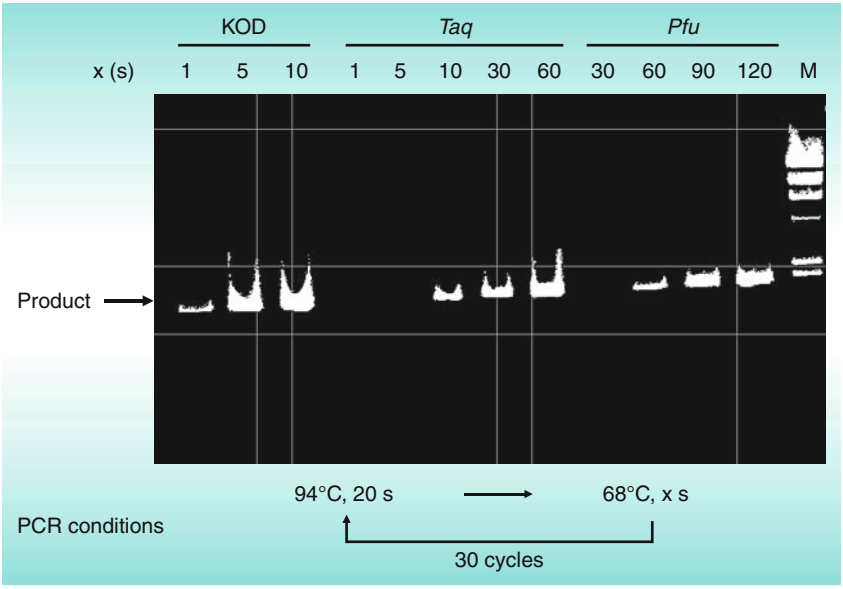


Fig. 4.4.4
Comparison of the elongation speed of various DNA polymerases

whereas 6 min was needed for the *Pfu* DNA polymerase. We also compared the relative fidelity in PCR among KOD, *Pfu,* and *Taq* DNA polymerases. Ratios of the number of mutant colonies to total colonies were 380/13, 531 for KOD DNA polymerase, 269/7, 485 for *Pfu* DNA polymerase, 57/120 for *Taq* DNA polymerase, and 12/10, 320 for spontaneous mutation (background). The mutation frequencies calculated from the first three of these ratios were 2.8%, 3.6%, and 48%, respectively. KOD DNA polymerase showed the lowest mutation frequency in PCR (Takagi et al. 1997).

Mutant DNA Polymerases with Amino Acid Substitution at Exo Regions

As mentioned above DNA polymerase from *T. kodakaraensis* KOD1 is very efficient thermostable PCR enzyme exhibiting higher accuracy and elongation velocity than any other commercially available DNA polymerase. However, when long distance PCR (>5 kbp) was performed with KOD DNA polymerase, amplification efficiency to produce the amount of target sequence becomes lower because of its strong 3′–5′ exonuclease activity for proofreading.

All DNA polymerases with proof reading ability possess three small sequence motifs named Exo I, Exo II, and Exo III (Bernad et al. 1989; Morrison et al. 1991). These three motifs contain respective core sequences, DXE in Exo I, NX2-3 (F/Y) D in Exo II and YX3D in Exo III and these sequences are directly related to the exonuclease activity. In Exo I motif of KOD DNA polymerase (Asp141-Ile-Glu), six site-directed mutageneses for a single amino acid substitution of Ile142 and one mutation for substitutions of both Asp141 and Glu143 residues to Ala (Ala141-Ile-Ala) were designed and their exonuclease activities were examined. Asn210 residue in Exo II, corresponding to Asn420 of Klenow fragment, is considered to have interaction with DNA substrate at upstream region of the 3′ end (Beese and Steitz 1991). The residue was substituted with negatively charged amino acid residue, Asp, in order to interrupt interaction between the Exo II motif and 3′ terminus of the substrate DNA. Tyr311 in Exo III motif, corresponding to Tyr 497 of Klenow fragment, also plays an important role in contact with substrate (Beese and Steitz 1991). Therefore, the residue was substituted with Phe residue.

Nine mutant KOD DNA polymerases were constructed and none of them showed notable difference in thermostability compared with the wild-type KOD DNA polymerase. 3′–5′ exonuclease activity of the mutated KOD DNA polymerases was examined using ^{3}H labeled chromosome of *Escherichia coli* and the results were summarized in *Table 4.4.3* (Nishioka et al. 2001).

PCR Fidelity of Mutated KOD DNA Polymerase

Mutation frequency of several mutated KOD DNA polymerases in PCR was examined and compared with those of the wild-type KOD DNA polymerase and *Taq* DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) as shown in *Table 4.4.3*. Mutation frequency in PCR became higher as 3′–5′ exonuclease activity decreased, showing importance of 3′–5′ exonuclease

Table 4.4.3

Comparison of 3′-5′ exonuclease activity and PCR fidelity amoung wild type and mutated KOD DNA polymerases

DNA polymerase	Relative exonuclease activity (%)	Mutation frequency[a] (%)
WT	100	0.79
I 142N	95	nt[b]
I 142E	76	1.93
I 142Q	64	nt
I 142D	52	3.04
I 142K	30	12.8
I 142R	0	25
Y311F	0.01	nt
N210D	0.1	24.7
D141A/E143A	nt	24.8
Taq	–	28.1

[a]Mutation frequency was measured by the method described in elsewhere (Takagi et al. 1997).
[b]nt indicates "not tested."

activity of KOD DNA polymerase for PCR fidelity. Although decrease in exonuclease activity positively influenced the amount of PCR product, a target length limitation in PCR could not be improved.

PCR by a Mixture of the Mutated and the Wild-Type DNA Polymerases

Because a homogenous DNA polymerase without exonuclease activity was not suitable for a long distance amplification of a target fragment, the combination of the low concentration of wild-type DNA polymerase and the high concentration of exonuclease deficient KOD DNA polymerase was investigated. In this mixed-type DNA polymerase system, DNA elongation is relatively dependent on the exonuclease deficient mutant enzyme and correction of mismatched nucleotides is dependent on the wild-type exonuclease positive DNA polymerase.

In primary screening for a combination of a mutated KOD DNA polymerase and the wild-type KOD DNA polymerase, a combination of N210D and KOD DNA polymerase was chosen because more amount of PCR product was amplified than other combinations. Ratio of mutant and wild-type DNA polymerases (N210D and WT) was varied from 10:1 to 100:1 and efficiency of amplification of DNA fragment with 8.5 kbp (β globin DNA) as a template was compared. In the case of mixing ratios, 40:1, 50:1 and 60:1, efficient DNA amplification could be observed. Therefore, fidelity in PCR was measured as previously reported (Takagi et al. 1997) and values were compared with those of KOD, *Taq*, and LA DNA polymerases (Ex *Taq* DNA polymerase, Takara Suzo Co. Ltd., Tokyo, Japan) (*Table 4.4.4*). The result indicated that two of the mixed-type KOD DNA polymerases showed higher

Table 4.4.4

Comparison of PCR fidelity in the mixture type DNA polymerase system

	Colonies		Mutation frequency (%)
	Total	Mutant	
KOD	37,966	38	0.1
N210D:WT	22,572	500	2.2
40:1			
N210D:WT	27,778	634	2.3
50:1			
N210D:WT	18,886	659	3.5
60:1			
LA(*Ex Taq*)	14,687	406	2.8
Taq	21,128	1,564	7.4

fidelity than *Taq* and LA. The ratio of 40:1 mixture-type KOD DNA polymerase was chosen because of its highest fidelity and the mixture was named KOD-Dash DNA polymerase (Nishioka et al. 2001).

Application of a Mixed-Type KOD DNA Polymerase (KOD-Dash DNA Polymerase) to LA PCR

In order to investigate the availability of KOD-Dash DNA polymerase, ability of DNA polymerization to amplify a long DNA region was compared among KOD-Dash DNA polymerase, *Taq*, and LA DNA polymerase by using λDNA as a template. *Taq* DNA polymerase could amplify only short DNA fragments (<5 kbp). However, both KOD-Dash DNA polymerase and LA DNA polymerase could amplify a long DNA fragments (up to 15 kbp) and also showed no significant difference in amount of DNA amplified, indicating that KOD-Dash DNA polymerase can be applied to long and accurate (LA) PCR (Nishioka et al. 2001).

Neutralizing Monoclonal Antibody Against KOD DNA Polymerase

Although the KOD DNA polymerase is an accurate and time-saving PCR enzyme, nonspecific amplification and primer dimer formation during PCR are still the most serious troubles in amplification of a target DNA especially when only a limited amount of template DNA is available. A most possible reason for low specificity of PCR is undesirable DNA polymerase activity during the first cycle of temperature elevation. In order to inhibit this undesirable DNA polymerase activity at low temperature, several methods of "hot start PCR" were developed (D'Aquila et al. 1991). There are three major ways in hot start PCR. The simplest method is called "manual hot start"; an easy method to apply preheated DNA template and DNA

polymerase into preheated reaction mixture. The second method using solid oil is called "wax method" (Chou et al. 1992). This method is to separate the PCR mixture into two respective fractions of DNA template and DNA polymerase using the solid oil. Disadvantages of these methods are high risk of contamination and complicated manipulation steps.

The third method, a hot start PCR with use of neutralizing monoclonal antibody was developed for *Taq* DNA polymerase (Kellog et al. 1994). This method is based on the principle that the *Taq* DNA polymerase does not have activity until the bound monoclonal antibody is heat-denatured. Indeed this method of the hot start PCR using monoclonal antibody is much easier than other two above-mentioned methods. However, *Taq* DNA polymerase exhibits lower fidelity compared to archaeal family B polymerases. Therefore, the hot start method using neutralizing monoclonal antibody should be applied for the most efficient PCR enzyme like KOD DNA polymerase.

Two mice were immunized by purified KOD DNA polymerase and production of antibody against the DNA polymerase was examined by ELISA. Many positive cells were obtained and 40 clones were established by limiting dilution. Among 40 positive clones, 15 clones were selected and their culture supernatants containing monoclonal antibodies (mAbs) were subjected to KOD DNA polymerase inhibition test. Two monoclonal antibodies exhibiting strong inhibition of the DNA polymerase activity were selected (3G8 and βG1, *Fig. 4.4.5*). Isotypes of these two mAbs were determined and both 3G8 and βG1 belong to IgG1, κ. Each mAbs was purified from the supernatant of their hybridoma cell cultures by a proteinA column and purity was examined by SDS-PAGE. The inhibition of the DNA polymerase activity was confirmed using purified monoclonal antibodies.

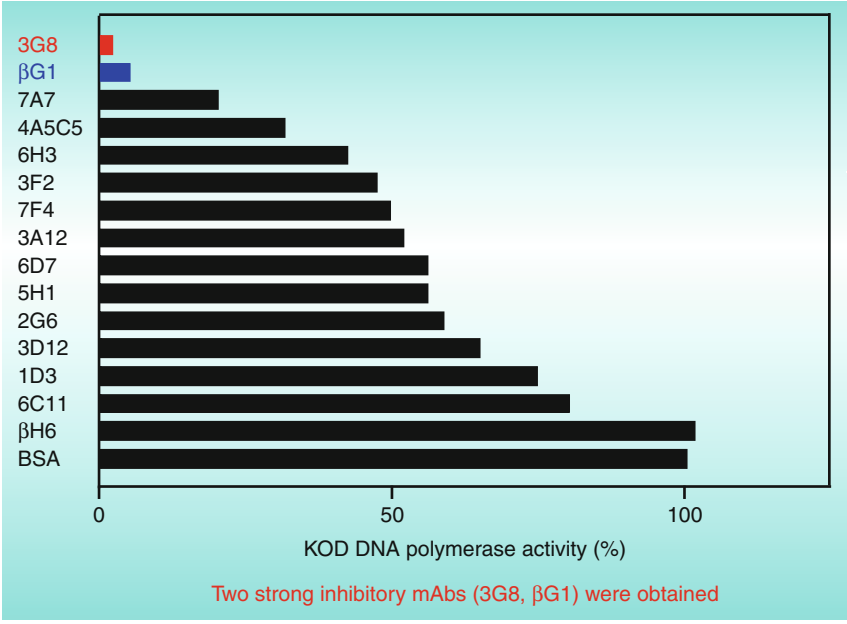


Fig. 4.4.5

Inhibition of KOD DNA polymerase activity by various monoclonal antibodies

Epitope Mapping of mAbs

In order to predict the epitope region of each mAb, inhibition of 3′–5′ exonuclease activity of KOD DNA polymerase by mAbs was examined. When 3G8 was added, remaining exonuclease activity was 18%, while when βG1 was added, the remaining activity was only 4.6%. βG1 could inhibit the exonuclease activity more intensively than 3G8 indicating that epitope of the βG1 might be located in the exonuclease domain of KOD DNA polymerase and epitope region of 3G8 might be located in the other region of the DNA polymerase.

Precise examination of epitope regions for these two mAbs were performed by combinations of partial proteolytic digestion, Western blot analyses followed by N-terminal amino acid sequence determination of the generated peptide fragments. KOD DNA polymerase was digested with V8 protease and trypsin. From the results of Western blotting, protein fragments with molecular weight of 16.5 and 18 kDa were highlighted by βG1 and a fragment with molecular weight of 27 kDa was highlighted by 3G8. Areas of the PVDF membrane containing these highlighted peptide fragments were used for N-terminal amino acid sequence determination. The most possible epitope regions were specified by results of the N-terminal sequencing (Fragments A–C) and molecular size estimation by SDS-PAGE. Fragment A (16.5 kDa) starting from amino acid number 1, and fragment B (18 kDa) starting from amino acid number 134 from N-terminal of KOD DNA polymerase were generated by V8 protease digestion and both fragments were recognized by βG1. Therefore, it was suggested that the epitope of βG1 is located downstream of amino acid number 134 to around 170. This region is located in the conserved region of 3′–5′ exonuclease activity. Indeed βG1 can inhibit exonuclease activity more intensively than 3G8. Amino acid sequences of DNA polymerases from *Pyrococcus furiosus*; *Pfu* polymerase, *T. litoralis*; Vent polymerase, *Pyrococcus* GB-D; Deep Vent polymerase and *Thermus aquaticus*; *Taq* polymerase were compared and dot blot analysis was performed. Interestingly the βG1 interacted with both KOD DNA polymerase and Vent polymerase but not with other DNA polymerases indicating that βG1 recognizes identical amino acid residues of KOD DNA polymerase to Vent polymerase. Amino acid sequence alignment suggested that arginine 169 of KOD DNA polymerase was conserved in amino acid sequence of Vent polymerase but the residue is not conserved in other DNA polymerases. Therefore, the epitope region of βG1 is located in the region including R169 (Mizuguchi et al. 1999).

The protein fragment with molecular weight of 27 kDa (fragment C) generated by V8 protease was recognized by 3G8. The N-terminal amino acid sequence of the fragment C is starting from 385. In order to specify the epitope region for 3G8, a partial peptide region of KOD DNA polymerase (amino acid number 383–423) was expressed as a *trxA* fusion protein using pET-32a expression system and recognition of the fragment by the 3G8 was examined. A positive signal, significantly stronger than the case of *E. coli* lysate, could be detected. When a control experiment using polyclonal antibody (PoAb) was performed, a similar weak signal could be observed for the fusion protein. The possible epitope region for 3G8 (383–423) is located in Region II, which is well conserved among family B DNA polymerases and the region is considered to be essential for catalytic activity of DNA elongation. Therefore, it was suggested that epitope of mAb 3G8 is located in catalytic center of the DNA polymerase (Region II) and the mAb can directly inhibit DNA polymerase activity.

As mentioned above, epitope regions for βG1 and 3G8 are located at different positions. Although epitope for βG1 was shown to be located in exonuclease region, the mAb could inhibit DNA polymerase activity. Bound by βG1, steric hindrance arose between the DNA and

the DNA polymerase around the exonuclease region and eventually DNA polymerase activity as well as exonuclease activity was inhibited (Mizuguchi et al. 1999).

Hot Start PCR with Neutralizing Monoclonal Antipodies

Hot start PCR was attempted with addition of respective monoclonal antibodies, 3G8 and βG1. The result of agarose gel electrophoresis clearly shows that specific amplification of the target IL-6 gene was enhanced by mixing either 3G8 or βG1 monoclonal antibody possibly because these mAbs could inhibit DNA polymerase activity during the time of applying KOD polymerase and first step of heat denaturation.

KOD Dash is a mixture of intact KOD DNA polymerase and its mutant without 3′–5′ exonuclease activity by substituting amino acid residue in exonuclease region (asparagine 210 to aspartate). The enzyme mixture was designed for PCR of longer fragments and similar technique was used previously by mixing family B DNA polymerase (*Pfu*) with 3′–5′ exonuclease activity and *Taq* DNA polymerase without 3′–5′ exonuclease activity. When hot start PCR using monoclonal antibody is attempted, it will be much easier for KOD Dash because the system contains basically single type of DNA polymerase. Therefore, two monoclonal antibodies, 3G8 and βG1 could easily be applied for hot start PCR using KOD Dash. As a control experiment, *Taq* DNA polymerase and monoclonal antibody against *Taq* DNA polymerase were used in the same condition except for reaction temperature (72°C). After PCR, reaction samples were loaded on 1.5% agarose gel electrophoresis. When *Taq* polymerase (without mAb) and Taq polymerase with anti-Taq mAb (Clontech) were used, no clear DNA band around the size of 1.3 kbp corresponding to the amplified DNA products could be detected. When KOD Dash (without mAb) was used, only a little amount of DNA can be amplified. However, when KOD Dash with either 3G8 or βG1 was used, significant amount of amplified DNA fragment with size of 1.3 kbp could be detected. Therefore, it was concluded that hot start PCR of KOD Dash using these two neutralizing mAbs are effective to perform more specific PCR.

Long Distance PCR by Hot Start PCR

PCR would be more useful technique, if amplification of a longer DNA fragment is possible. We attempted amplification of longer DNA fragment (human β-globin gene, 17.5 kbp) using KOD Dash and neutralizing mAbs (3G8 and βG1). Amplification of the target fragment could be detected and especially when both of the two mAbs were used for hot start PCR, total amount of mAbs required for efficient PCR became less (0.1 mg) than the case when one of these two mAbs was used.

This experimental result is consistent with the results of epitope mapping mentioned above. These two monoclonal antibodies can cooperatively inhibit the DNA polymerase by binding at two different epitope regions. Accordingly more efficient hot start PCR could be achieved by addition of two different mAbs (Mizuguchi et al. 1999).

Tertiary Structure of KOD DNA Polymerase

The crystals of KOD DNA polymerase was obtained (Hashimoto et al. 1999) (*Fig. 4.4.6*) and the structure of KOD DNA polymerase was determined (Hashimoto et al. 2001)

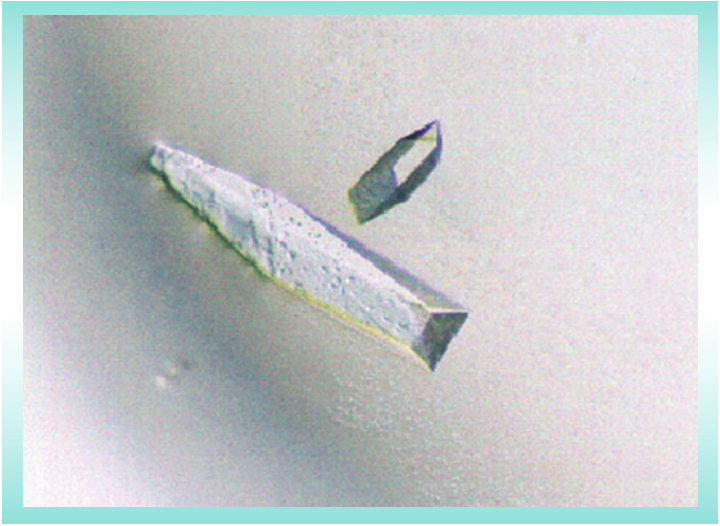


Fig. 4.4.6
Crystal of DNA polymerase from *Thermococcus kodakaraensis* KOD1

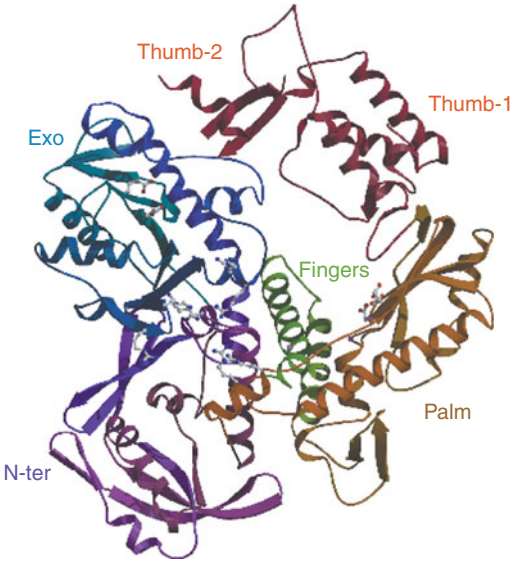


Fig. 4.4.7
Overall structure of KOD DNA polymerases

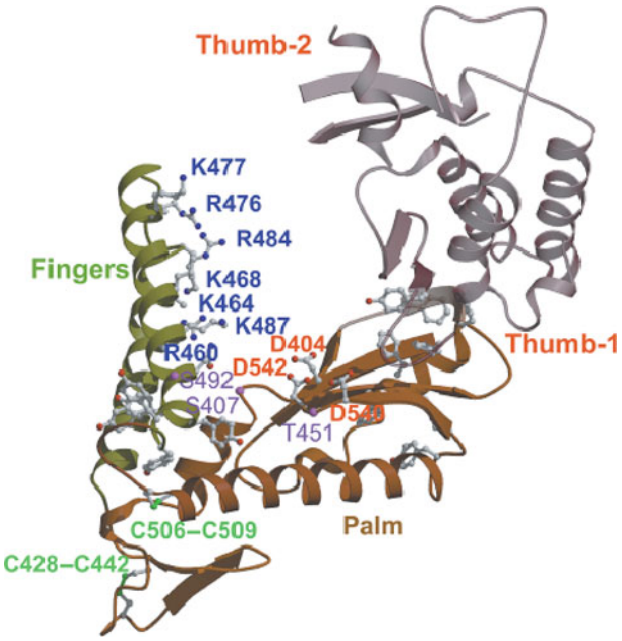


Fig. 4.4.8
Structure of the polymerase domain

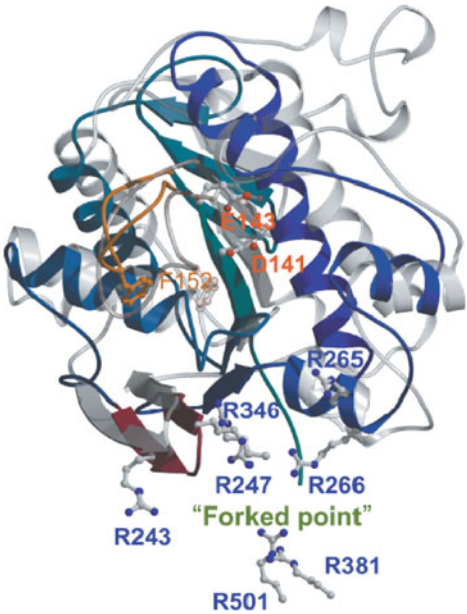


Fig. 4.4.9
Structure of the exonuclease domain

(Fig. 4.4.7). The structural analysis of KOD DNA polymerase was performed in order to clarify the mechanisms of the enzymatic features. Structural comparison of DNA polymerases from hyperthermophilic archaea highlighted the conformational difference in Thumb domains. The Thumb domain of KOD DNA polymerase shows an "opened" conformation. The fingers subdomain possessed many basic residues at the side of the polymerase active site (Fig. 4.4.8). The residues are considered to be accessible to the incoming dNTP by electrostatic interaction. A beta-hairpin motif (residues 242–249) extends from the complex of the RB69 DNA polymerase from bacteriophage RB69. Many arginine residues are located at the forked-point (the junction of the template-binding and editing clefts) of KOD DNA polymerase (Fig. 4.4.9), suggesting that the basic environment is suitable for partitioning of the primer and template DNA duplex and for stabilizing the partially melted DNA structure in the high-temperature environments. The stabilization of melted DNA structure at the forked-point may be correlated with the high PCR performance of KOD DNA polymerase, which is due to low error rate, high elongation rate, and processivity (Hashimoto et al. 2001).

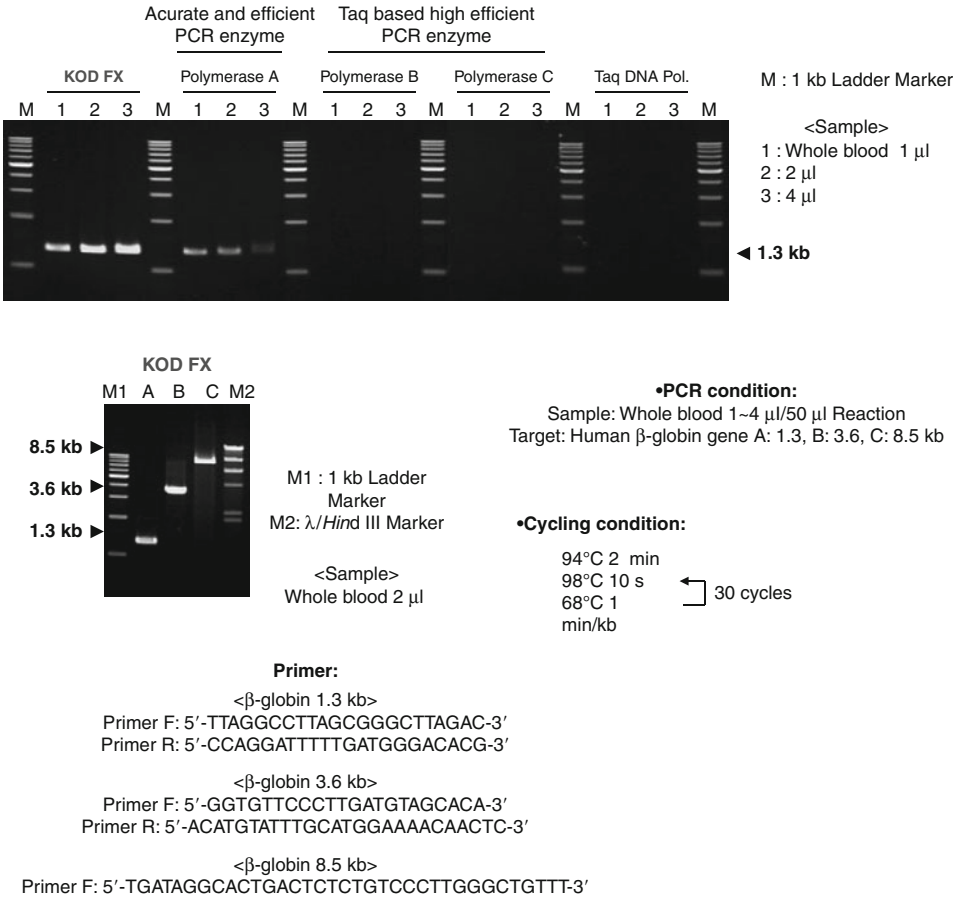
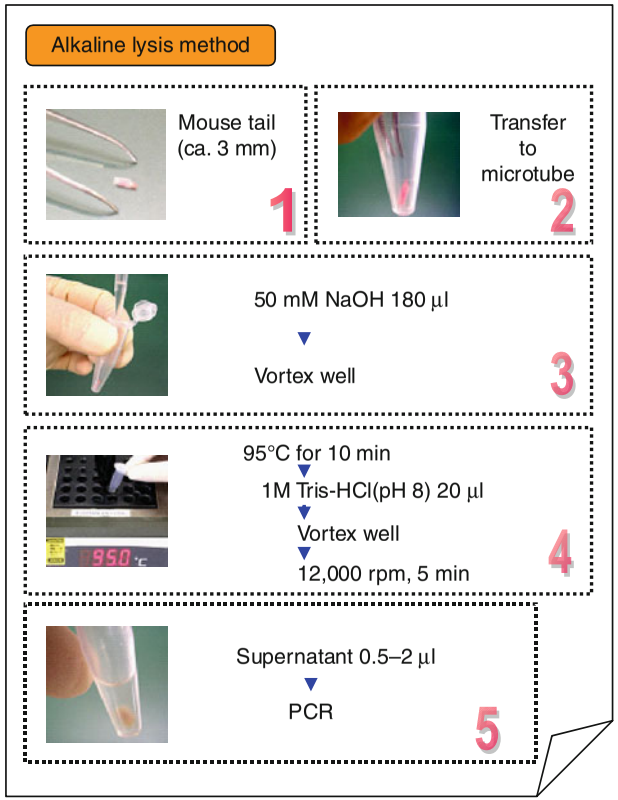


Fig. 4.4.10

PCR for whole blood sample



•PCR condition:

Sample: Supernatant of mouse tail lysates 0.5 µl / 50 µl Reaction
Target: Mouse membrane glycoprotein (Thy-1) gene <M10246> 2.6 kb
Primer: Primer F : 5′-CCACAGAATCCAAGTCGGAACTCTTG-3′ (26 mer)
Primer R : 5′-GTAGCAGTGGTGGTATTATACATGGTG-3′ (27 mer)

•Cycling condition:

94°C 2 min
98°C 10 s
68°C 2.5 min
30 cycles

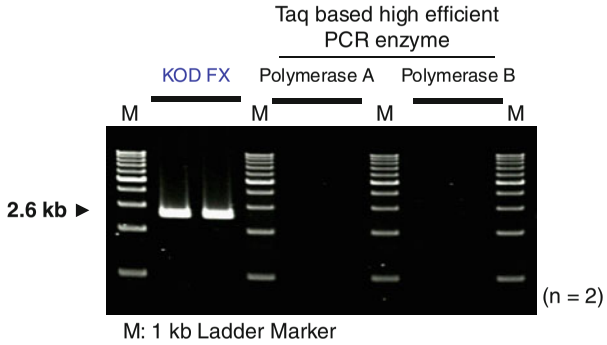
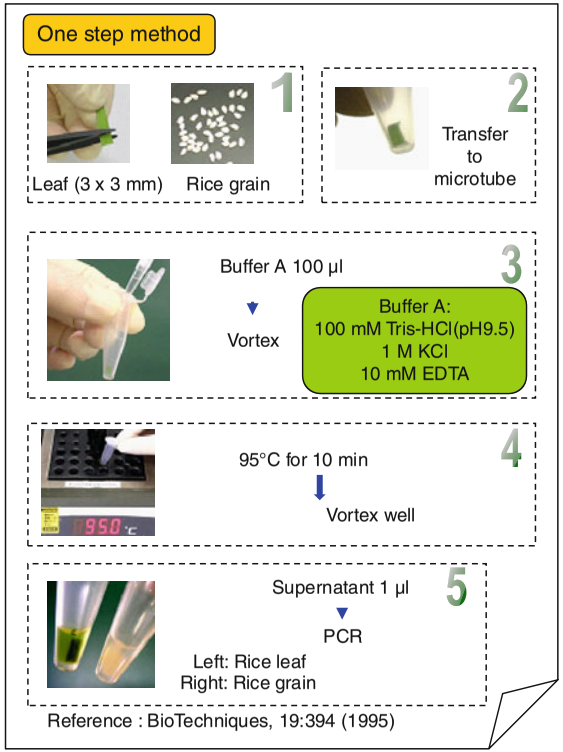


Fig. 4.4.11
PCR for mouse tail lysate sample



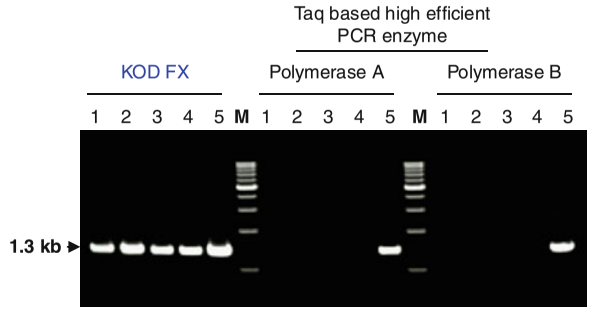
•PCR condition

Sample: Supernatant of plant tissue lysates 1 ml / 50 ml Reaction
Target: rbcL gene 1.3 kb

Primer:
Primer F1: 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ (Tomato&Tobacco)
Primer R1 : 5′-AAGCAGCAGCTAGTTCCGGGCTCCA-3′ (Tomato&Tobacco)
Primer F2 :: ′-ATGTCACCACAAACAGAAACTAAAGC-3′ (Rice)
Primer R2 : 5′-AAGCTGCGGCTAGTTCAGGACTCCA-3′ (Rice)

•Cycling condition:

94°C 2 min
98°C 10 s
68°C 1.5 min ⌐ 30 cycles



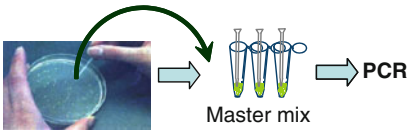
1: Leaf (Tomato)
2: Leaf (Tobacco)
3: Leaf (Rice)
4: Grain (Rice)
5: Purified rice leaf DNA

Fig. 4.4.12
PCR for plant tissue lysate sample

•PCR condition:

Sample : Yeast and Fungus colonies

Protocol:



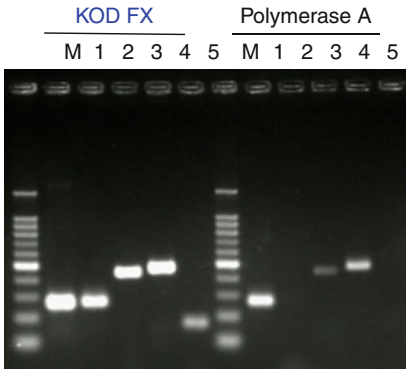
Target: ITS-1 (150–470 bp)

Primer:

Primer F: GTAACAAGGT(T/C)TCCGT
Primer R: CGTTCTTCATCGATC

•Cycling condition:

94°C 2 min
↓
98°C 10 s
50°C 30 s X 30 cycles
68°C 1 min



M: 100 bp DNA Ladder
1 *Aspergillus oryzae*
2 *Aspergillus niger*
3 *Saccharomyces cerevisiae*
5 *Schizosaccharomyces pombe*
6 *Pichia pastris*

Fig. 4.4.13
PCR for yeast and fungus colony samples

High Success-Rate DNA Polymerase, KOD-FX

KOD-FX is based on DNA polymerase from *T. kodakaraensis* KOD1. KOD-FX results in much greater PCR success based on efficiency and elongation capabilities than KOD-Plus or other Taq-based PCR enzymes. KOD-FX enzyme solution contains two types of anti-KOD DNA polymerase antibodies that inhibit polymerase and 3′–5′ exonuclease activities, thus allowing for Hot Start PCR. KOD-FX generates blunt-end PCR products, due to 3′–5′ exonuclease (proofreading) activitiy. Moreover, KOD-FX is effective for the amplification of GC-rich targets and crude samples as shown in *Figs. 4.4.10–4.4.14*. This enzyme enables the following amplifications (Maximum): 40 kbp from lambda DNA, 24 kbp from human genomic DNA, and 13.5 kbp from cDNA. The PCR error ratio of KOD FX is about 10 times less than that of Taq DNA polymerase. Thus, KOD-FX is very useful for automatic PCR system from crude samples such as whole blood sample, mouse tail lysate, crude plant tissue lysate, yeast, and fungus colonies, using disadvantageous primers with high GC content.

Conclusion

The PCR can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. This method of amplifying DNA has numerous

•PCR condition:

Target: NM_002745 Homo sapiens mitogen-activated protein kinase 1 (MAPK1), transcript variant 1
Template: HeLa cDNA (= total RNA 50 ng)/50 ml Reaction
Primer:

Primer F: 5′-ATGGCGGCGGCGGCGGCGGCGGGCGCGGGC-3′ (30 mer) **<GC = 93.3%>**
Primer R: 5′τ-TTAAGATCTGTATCCTGGCTGGAATCTAGC-3′ (30 mer) <GC% = 43.3>
*Start codon : underlined sequence of MAPK1_F, Stop codon : underlined sequence of MAPK1_R

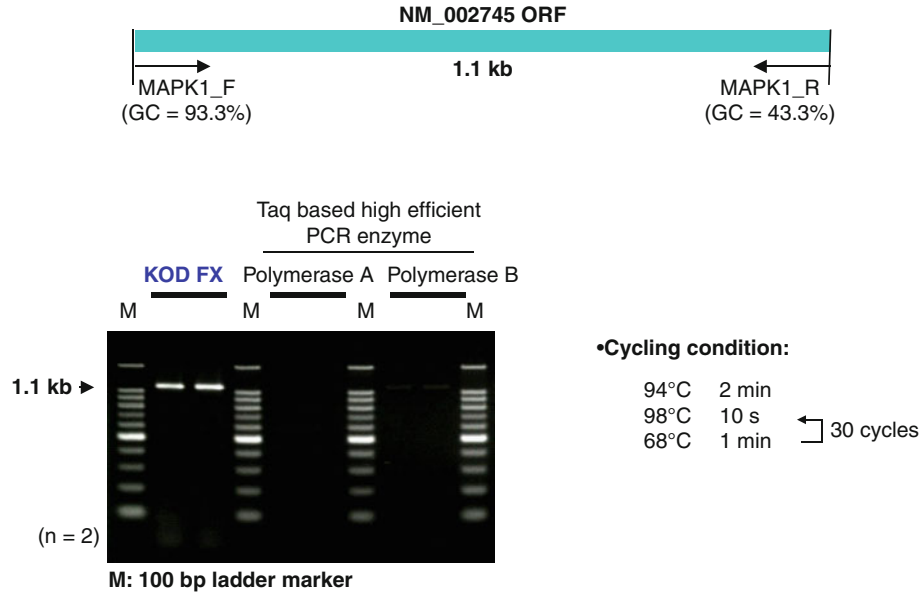


Fig. 4.4.14
PCR for GC-rich target

applications in basic research, human genetic testing, quality control, and forensics. We could develop very sophisticated PCR system based on our original PCR enzyme, KOD DNA polymerase. In 1995, the KOD DNA polymerase and KOD Dash were commercialized in Japan and very widely used in many fields of biotechnology. In 1999, the hot start system was marketed in the USA by GIBCO BRL as well as in Japan. This process of development is also a very good example of enzyme screening and engineering. However, the PCR system is still immature. Its problems in specificity and fidelity are not yet completely solved. Based on our recent result of tertiary structural studies, we are now clarifying the reason of high fidelity and elongation rate more precisely. The information of structure-function relationship will provide better understanding to DNA polymerases and will suggest strategies to engineer better PCR enzymes. Finally, KOD-FX is very useful for automatic PCR system from crude samples, using disadvantageous primers with high GC content.

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4.5 Organic Compatible Solutes of Prokaryotes that Thrive in Hot Environments: The Importance of Ionic Compounds for Thermostabilization

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.5,

Introduction

Many mesophilic organisms use uncharged, small organic compounds, designated compatible solutes (Brown 1976), to preserve cell viability under different stressful environmental conditions, namely to balance fluctuations in the osmotic pressure of the external milieu (Brown 1976; da Costa et al. 1998; Roberts 2005). In some cases, small molecules, like sarcosine and trimethylamine N-oxide, are used to counteract the potentially lethal, denaturing effect brought about by the accumulation of urea in the cell under physiological conditions (Yancey and Somero 1979; Sackett 1997; Yancey 2005; Treberg et al. 2006). Not surprisingly, microorganisms adapted to thrive in hot, marine environments accumulate organic solutes, which are believed to play a role in the protection of cell proteins, and other macromolecules, against structural disruption caused by high thermal energy (Santos et al. 2007a). Several radiation resistant microorganisms accumulate trehalose, an organic solute widespread in the three Domains of Life, which has been proposed as a factor contributing to the remarkable robustness of these cells (Empadinhas et al. 2007; Empadinhas and da Costa 2008a). Also, it is tempting to speculate that cold adapted organisms may use organic solutes to defend the cellular proteome against the deleterious effects of cold denaturation (unpublished results). Thus, it is apparent that small organic solutes are ubiquitous protecting agents utilized by cells to cope with a diversity of aggression factors, their function extending well beyond that of standard osmolytes, whose main role is to balance the osmotic pressure across the cellular membrane and maintain cell turgor.

Given that compatible solutes are implicated in defense against a variety of insults, e.g., too high or too low temperature, low water activity, high level of reactive species, high-dose of ionizing radiation, it is pertinent to question whether specific chemical compounds are better suited to cope with specific damaging factors. Indeed, while many studies claim that trehalose serves as a general stress protector, being implicated in a variety of stress responses (osmotic, heat, acid, acid and oxidative stress), mannosylglycerate, a solute highly associated with (hyper)thermophiles, is far better than trehalose for the preservation of protein structure against thermal denaturation (Ramos et al. 1997; Borges et al. 2002; Faria et al. 2003, 2004, 2008).

The topic "compatible solutes" is of central importance to understand the mechanisms that allow cell survivability under extremely hostile conditions. Despite several decades of research work in this field, many fundamental questions remain unanswered. This is particularly true in respect to the molecular mechanisms underlying the stabilization of proteins by compatible solutes, a topic of great current interest as protein misfolding and aggregation is associated with a number of debilitating diseases, like Parkinson's, Alzheimer's, or Huntington's (Lee and Trojanowski 2006; Skovronsky et al. 2006). Understanding the nature of the molecular interactions, water/solute/protein, that promote the native fold, is a challenging issue that fuels vivid debate in the scientific community and demands powerful cross-disciplinary approaches.

The literature dealing with "compatible solutes" is extremely vast, and different aspects of the theme have been covered in several valuable reviews (Yancey et al. 1982; Galinski 1995; da Costa et al. 1998; Müller et al. 2005; Roberts 2005; Arakawa et al. 2006; Street et al. 2006; Santos et al. 2007a; Empadinhas and da Costa 2008a; Burg and Ferraris 2008; Kurz 2008; Harries and Rösgen 2008; Hu et al. 2009). The present review will focus on the organic compatible solutes typically found in thermophiles and hyperthermophiles, herein designated "thermolytes" for the convenience of a short name. We will consider the nature and distribution of these solutes in the phylogenetic Tree of Life, their biosynthetic pathways and molecular evolution, their

efficacy as stabilizers of model proteins in comparison with mesophilic counterparts, their role as thermoprotectants in vivo, and finally the effect of thermolytes on protein dynamics and evidence for a strong link between rigidification and stabilization.

Distribution of Organic Solutes in (Hyper)thermophiles

Compatible solutes are highly soluble in water and often accumulate to high levels in the cytoplasm, becoming by far the most abundant organic components of a cellular extract, greatly facilitating their identification and structural characterization (Santos et al. 2006). The main difficulty in producing a comprehensive description of solute accumulation in (hyper)thermophiles resides in the poor growth yields characteristic of many of these organisms. Nevertheless, during the last decades, representatives of all known thermophilic branches in the Tree of Life have been examined. The data collected is summarized in *Table 4.5.1*.

Among the variety of organic solutes accumulated by (hyper)thermophiles, some, like di-*myo*-inositol phosphate (DIP) or mannosylglycerate, seem to be restricted, or are mainly found, in thermophiles, while others, like trehalose or α-glutamate, are commonly found in both thermophiles and mesophiles (Santos et al. 2007a). In opposition to the classical definition of a compatible solute (Brown 1976), which states that these compounds should be without charge at physiological pH, the organic solutes typically found in (hyper)thermophiles are negatively charged, a feature which has been linked to their superior ability to act as protein stabilizers (Borges et al. 2002; Faria et al. 2003, 2008).

Chemically, most thermolytes fall into one of two categories: polyol-phosphodiesters and α-hexose derivatives (*Fig. 4.5.1*). As an example of the first category, the distribution of DIP is particularly illustrative since it is widespread in hyperthermophilic archaea and bacteria, and has not been found in organisms with optimal growth temperature below 60°C; moreover, DIP is the only solute accumulating in the most extreme thermophilic organism known to date – *Pyrolobus fumarii* (Gonçalves et al. 2008). The association between DIP and thermophily is further emphasized by the observation that its level generally increases in response to supra-optimal growth temperatures, which has led to the hypothesis of a thermoprotective role for this compound (Santos and da Costa 2002). The mannosylated derivatives of DIP (β-mannosyl-(1→2)-DIP: MDIP and β-mannosyl-(1→2)-β-mannosyl-(1→2)-DIP: MMDIP) have been found only in hyperthermophilic bacteria of the genera *Thermotoga* and *Aquifex* and display a curious behavior: *Aquifex* spp. accumulate MDIP as a response to increased salinity, leaving to DIP the main role in the response at supra-optimal temperatures; on the other hand, MDIP contributes to the heat stress response in *Thermotoga* spp. while the level of DIP is fairly constant (Martins et al. 1996; Lamosa et al. 2006; Rodrigues et al. 2009). The group of polyol-phosphodiesters includes also diglycerol phosphate (DGP), which seems to be restricted to the genus *Archaeoglobus*, and glycero-phospho-inositol (GPI), which has been detected in *Archaeoglobus* spp. and bacteria of the genus *Aquifex* (Gonçalves et al. 2003; Lamosa et al. 2006). GPI is a structural chimera of DGP and DIP, and, while DGP responds primarily to salt stress and DIP to supra-optimal growth temperatures, GPI displays a mixed behavior, accumulating mainly when salt and temperature stresses are applied simultaneously (Lamosa et al. 2006).

Among the sugar derivatives, mannosylglycerate is the most noteworthy of thermolytes. Despite having been found originally in mesophilic red algae (Bouveng et al. 1955), there are no

Table 4.5.1

Organic solute distribution among (hyper)thermophiles

Organisms	$T_{Opt.}$ (°C)	Solutes								Ref
		cBPG	Tre	MG	DIP	α-Glu	β-Glu	Asp	Other	
Archaea										
Pyrolobus fumarii	106				+					1
Pyrodictium occultum	105				+	+				2
Pyrobaculum aerophilum	100		+							2
Pyrobaculum islandicum	100									2
Pyrococcus furiosus	100			↑(S)	↑(T)	+				3
Hyperthermus butylicus	99				+					ur
Pyrococcus horikoshii	98		+	↑(S)	+	+				4
Methanopyrus kandleri	98	+				+				2
Stetteria hydrogenophila	95		+	+	+					5
Aeropyrum pernix	90			+	+					6
Methanotorris igneus	88				↑(T)	↑(S)	↑(S)			7,8
Thermoproteus tenax	88		+							2
Thermococcus stetteri	87			↑(S)	↑(T, S)	+		↑(S)		9
Thermococcus celer	87			↑(S)	↑(T)	+		↑(T)		9
Thermococcus litoralis	85		↑(S)	↑(S)	↑(T)	+		↑(S)	GalHI	9
Thermococcus kodakarensis	85				+	+		+		10
Methanocaldococcus jannaschii	85						↑(S)			8
Palaeococcus ferrophilus	83			↑(T);↑(S)		↑(T)		↑(S)		5
Methanothermus fervidus	83	+				+				2
Archaeoglobus fulgidus VC-16	83				↑(T)	+			DGP ↑(S); GPI ↑(T, S)	2
Archaeoglobus profundus	83			+	+	+				11
Acidianus ambivalens	80		+							2
Archaeoglobus veneficus	75			+	+	+			DGP	11
Thermococcus zilligii	75									9
Sulfolobus solfataricus	75		+							2
Metallosphaera sedula	75		+							2
Methanothermobacter thermoautotrophicus	70	+				+			TCH	12
Methanothermococcus okinawensis	70					+		+		13

Table 4.5.1 (Continued)

Organisms	$T_{Opt.}$ (°C)	Solutes								Ref
		cBPG	Tre	MG	DIP	α-Glu	β-Glu	Asp	Other	
Methanothermobacter marburgensis	65	+				+			TCH	14
Methanothermococcus thermolithotrophicus	65					+	+	+	NAL	8, 15
Thermoplasma acidophilum	60	+								2
Bacteria										
Aquifex pyrophilus	80				↑(T)		↑(S)		GPI ↑(T, S); MDIP ↑(S)	16
Thermotoga maritima	80				↑(S)		↑(S)		MDIP ↑(T)	17
Thermotoga neapolitana	80				↑(S)	+	↑(S)		MDIP ↑(T)	17
Thermosipho africanus	75					+			Pro	17
Thermotoga thermarum	70									17
Marinitoga piezophila	70					+			Pro	13
Fervidobacterium islandicum	70									17
Persephonella marina	70				+		+		GG,GGG	13
Thermus thermophilus	70		↑(S)	↑(S)		+			GB	18
Rhodothermus marinus	65		+	↑(S), ↑(T)		+			MGA ↑(S)	18, 19
Rubrobacter xylanophilus	60		+	+	↑(T)	+			GB, DAGAP	20
Petrotoga mobilis	60					↑(S)	↑(S)		MGG ↑(S)(T), GB	21
Petrotoga miotherma	55					+			MGG, Pro	22

Abbreviations: *cBPG*, cyclic 2,3-bisphosphoglycerate; *Tre*, trehalose; *MG*, mannosylglycerate; *MGA*, mannosylglyceramide; *DIP*, di-*myo*-inositol-1,3′-phosphate; *MDIP*, mannosyl-di-*myo*-inositol-1,3′-phosphate; *DGP*, diglycerol phosphate; *GPI*, glycero-phospho-inositol; *TCH*, 1,3,4,6-tetracarboxyhexane; *α-Glu*, α-glutamate; *β-Glu*, β-glutamate; *Asp*, aspartate; *GalHl*, β-galactopyranosyl-5-hydroxylysine; *GB*, glycine betaine; *Pro*, proline; *NAL*, N^{ε}-acetyl-β-lysine; *GG*, glucosylglycerate; *GGG*, glucosyl-glucosylglycerate; *MGG*, mannosyl-glucosylglycerate; *DAGAP*, di-*N*-acetyl-glucosamine phosphate.

Symbols: The plus sign indicates the presence of the solute in cases for which the response to environmental conditions has not been reported. ↑(S) and ↑(T) indicate that the intracellular level of the solute increases in response to osmotic or heat stress, respectively.

References: 1- Gonçalves et al. 2008; 2- Martins et al. 1997; 3- Martins and Santos 1995; 4- Empadinhas et al. 2001; 5-Neves et al. 2005; 6- Santos and da Costa 2001; 7- Ciulla et al. 1994a; 8- Robertson et al. 1990; 9- Lamosa et al. 1998; 10- Borges et al. 2010; 11- Gonçalves et al. 2003; 12- Gorkovenko et al. 1994; 13- Santos et al. 2007a; 14- Ciulla et al. 1994b; 15- Robertson et al. 1992b; 16- Lamosa et al. 2006; 17- Martins et al. 1996; 18- Nunes et al. 1995; 19- Silva et al. 1999; 20- Empadinhas et al. 2007; 21- Fernandes et al. 2010; 22- Jorge et al. 2007; ur – unpublished results.

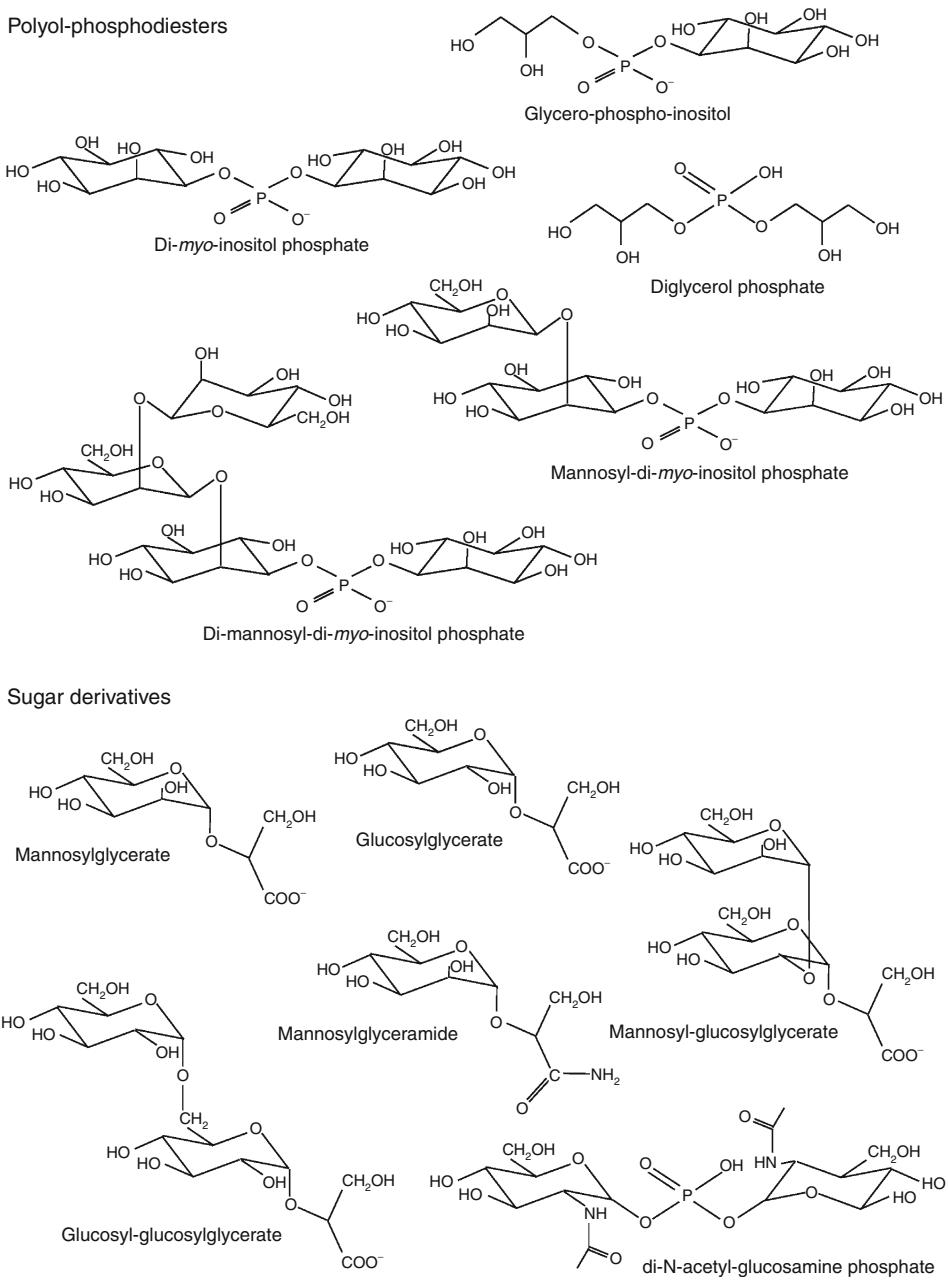

Fig. 4.5.1

Compatible solutes primarily restricted to hyperthermophiles

reports of mannosylglycerate accumulation in mesophilic Bacteria or Archaea. On the contrary, it is relatively common in thermophilic prokaryotes isolated from marine environments (*Table 4.5.1*). Although generally accumulating as a response to the increasing salinity of the growth medium, mannosylglycerate has been suggested to serve also as a thermoprotective compound, with many reports stating its high efficacy as an in vitro protein stabilizer (Ramos et al. 1997; Santos et al. 2007b). The structure of mannosylglycerate comprises one mannose group linked via an alpha glycosidic bond to the hydroxyl group at position 2 of glycerate and serves as lead for several chemical variants. One such variation is mannosylglyceramide, a rare, uncharged derivative, which has been found only in the thermophilic bacterium *Rhodothermus marinus* (Silva et al. 1999). Glucosylglycerate, on the other hand, is primarily associated with mesophilic bacteria and archaea (Kollman et al. 1979; Robertson et al. 1992a; Goude et al. 2004; Empadinhas and da Costa 2008b); thus far, *Persephonella marina* is the only thermophilic organism known to accumulate glucosylglycerate (unpublished results from our team). Mannosyl-glucosylglycerate and glucosyl-glucosylglycerate are two rare derivatives found in *Petrotoga* spp. (Jorge et al. 2007; Fernandes et al. 2010) and *Persephonela marina*, respectively.

Other solutes not falling into these two categories (polyol-phosphodiesters and hexose derivatives) include (see *Table 4.5.1*): β-glutamate, whose occurrence was initially detected in methanogens regardless of their growth temperature (Robertson et al. 1989; Robertson et al. 1990), and later reported in hyperthermophilic bacteria (Martins et al. 1996; Lamosa et al. 2006; Fernandes et al. 2010); aspartate, a negatively charged amino acid highly associated with hyperthermophiles, occurs in *Palaeococcus ferrophilus*, and also in *Thermococcus* spp. (Neves et al. 2005; Lamosa et al. 1998), including the DIP-deficient mutant of *Thermococcus kodakarensis* (see below); cyclic-2,3-bisphosphoglycerate (cBPG), a compound exclusively found in methanogens, and reaching very high levels only in hyperthermophilic members (Kanodia and Roberts 1983; Tolman et al. 1986; Gorkovenko and Roberts 1993; Martins et al. 1997); 1,3,4,6-tetracarboxy-hexane, galactosylhydroxylysine, and di-*N*-acetyl-glucosamine phosphate, rare compounds detected in (hyper)thermophiles; and Nε-acetyl-β-lysine, a solute thus far restricted to the domain Archaea, whose distribution denotes a clear preferential association with mesophiles (Gorkovenko et al. 1994; Lamosa et al. 1998; Martin et al. 1999; Müller et al. 2005; Empadinhas et al. 2007).

Generally, (hyper)thermophiles display a differential pattern of solute accumulation, with the level of specific compounds responding primarily to specific stress factors. In most cases, mannosylglycerate, DGP and charged amino acids show increased levels in response to an increase in salinity of the medium, while DIP or DIP-derivatives tend to accumulate in response to supra-optimal growth temperatures. It is important to consider the dependency between the nature of the solute pool and the composition of the growth medium. In this respect, the hyperthermophile *Archaeoglobus fulgidus* strain 7324 provides a paradigmatic case: when grown on lactate, this archaeon accumulated primarily DGP in response to supra-optimal salinity; however, when starch replaced lactate in the growth medium, DGP was absent, and mannosylglycerate was the sole solute detected (*Fig. 4.5.2*) (Gonçalves 2008). It is interesting that DGP was replaced by a solute also typically involved in the response to increased salinity. The observation of solute replacement is not a rare event. *Thermococcus litoralis* provides another good example of this behavior since different solutes are imported and accumulated intracellularly, depending not so much on their chemical nature as on their availability in the external medium (Lamosa et al. 1998).

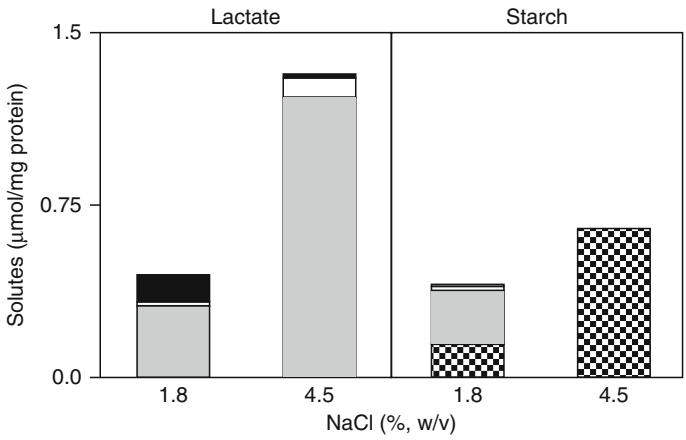

Fig. 4.5.2

Effect of salinity on the accumulation of compatible solutes by *Archaeoglobus fulgidus* 7324, using lactate (*left*) or starch (*right*) as carbon source for growth. Mannosylglycerate (*checkered*), diglycerol phosphate (*gray*), glycero-phospho-inositol (*white*), and di-*myo*-inositol-phosphate (*black*). Data from Gonçalves 2008

Biosynthesis of Thermolytes

Uptake of solutes, such as amino acids, is preferred over the more costly process of de novo synthesis. Transport systems for betaines and trehalose have been characterized in detail in *Archaeoglobus fulgidus* and members of the Thermococcales, respectively (Xavier et al. 1996; Horlacher et al. 1998; Schiefner et al. 2004). However, uptake systems for thermolytes have not been reported. We used ^{14}C-labeled mannosylglycerate to investigate uptake of this solute in a few (hyper)thermophiles: *Thermus thermophilus* showed a very poor transport capacity and no activity has been detected in *Rhodothermus marinus* or *Pyrococcus furiosus* (Sampaio 2005 and our unpublished results).

In contrast with the scarcity of data on solute uptake systems of (hyper)thermophiles, the biosynthetic pathways of several thermolytes have been established, namely cBPG, mannosylglycerate, DIP, GPI, DGP, MDIP, GG, MGG, and Tre. The majority of these solutes are synthesized by two sequential reactions with the involvement of a phosphorylated intermediate that, upon dephosphorylation, is converted to the final product. As a consequence of this dephosphorylating step, the overall synthesis becomes an irreversible process. Therefore, it is plausible to speculate that this two-step pathway was selected during evolution as an efficient strategy to allow accumulation of compatible solutes to high levels in the cell.

The biosynthesis of cBPG, GG, and Tre has been the subject of previous reviews (Elbein et al. 2003; Roberts 2005; Empadinhas and da Costa 2008a, b). In this section, we will review the biosynthesis of mannosylglycerate, MGG, DIP, GPI, DGP, and MDIP.

Mannosylglycerate

There are two pathways for the synthesis of mannosylglycerate (*Fig. 4.5.3*) (Martins et al. 1999). In the single-step pathway, mannosylglycerate synthase (MGS) catalyzes the transference of the mannosyl moiety of GDP-mannose to D-glycerate, yielding mannosylglycerate.

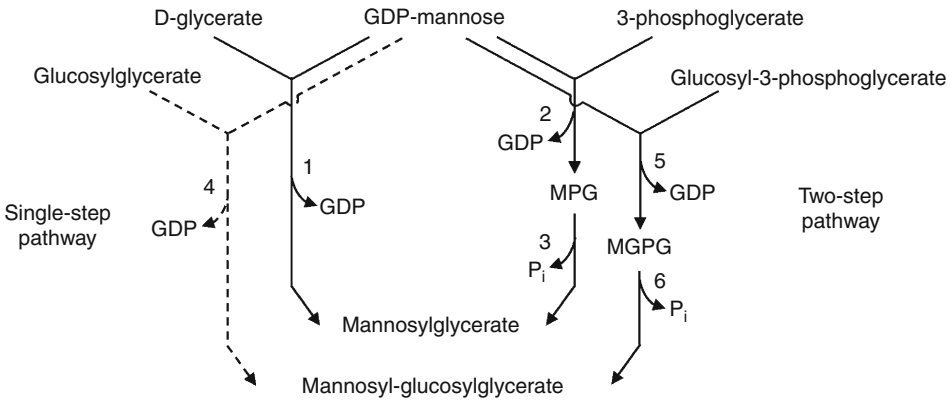

Fig. 4.5.3

Pathways for the synthesis of mannosylglycerate and mannosyl-glucosylglycerate. Enzymes: (1) mannosylglycerate synthase, (2) mannosyl-3-phosphoglycerate synthase, (3) mannosyl-3-phosphoglycerate phosphatase, (4) mannosyl-glucosylglycerate synthase, (5) mannosyl-glucosyl-3-phosphoglycerate synthase, and (6) mannosyl-glucosyl-3-phosphoglycerate phosphatase. The *discontinuous line* indicates that this reaction has not been firmly established. MPG, mannosyl-3-phosphoglycerate, and MGPG, mannosyl-glucosyl-3-phosphoglycerate. Data from Martins et al. 1999 and Fernandes et al. 2010

In the two-step pathway, mannosylglycerate is synthesized via two sequential reactions, which are catalyzed by mannosyl-3-phosphoglycerate synthase (MPGS) and mannosyl-3-phosphoglycerate phosphatase (MPGP). In the first reaction, the mannosyl moiety of GDP-mannose is transferred to 3-phosphoglycerate to form a phosphorylated intermediate, mannosyl-3-phosphoglycerate (MPG), which is hydrolyzed to mannosylglycerate in the second reaction.

The single-step pathway has been detected in the thermophilic bacterium *Rhodothermus marinus* (Martins et al. 1999) and in the mesophilic red algae *Caloglossa leprieurii* (our unpublished results). Moreover, MGS homologues have been identified in the moss *Physcomitrella patens* subsp. *patens* and also in the mesophilic alga *Griffithsia japonica* (NCBI database). The MGS from *Rhodothermus marinus* has been characterized in detail and the three-dimensional structure is known (Martins et al. 1999; Flint et al. 2005).

The two-step pathway was initially detected in cell extracts of the thermophilic bacterium *Rhodothermus marinus*, and later shown to be present in several other bacteria and archaea adapted to hot environments: *Thermus thermophilus*, *Rubrobacter xylanophilus*, *Pyrococcus horikoshii*, *Palaeococcus ferrophilus*, *Thermococcus litoralis*, *Archaeoglobus profundus*, and *Archaeoglobus veneficus* (Martins et al. 1999; Empadinhas et al. 2001; 2003; Neves et al. 2005; Empadinhas and da Costa 2008b; our unpublished results). The genes coding for the synthase (*mpgS*) and phosphatase (*mpgP*) have been identified in *Pyrococcus horikoshii* and *Rhodothermus marinus* by reverse genomics (Empadinhas et al. 2001; Borges et al. 2004); genes with high sequence homology are present in several other organisms (*Fig. 4.5.4*) with the *mpgS* gene always located immediately upstream of the *mpgP* gene. In members of the order Thermococcales (*Pyrococcus* spp., *Palaeococcus ferrophilus*, *Thermococcus litoralis*, *Thermococcus barophilus*, and *Thermococcus* AM4), the *mpgS* and *mpgP* genes are organized in an operon-like structure with the genes encoding phosphomannose mutase and

phosphomannose isomerase/mannose-1-phosphate guanylyl transferase, the two enzymes catalyzing the synthesis of GDP-mannose.

The X-ray structure of *Pyrococcus horikoshii* MPGP has been disclosed (Kawamura et al. 2008) and preliminary crystallographic analyses of the MPGSs from *Thermus thermophilus* and *Rubrobacter xylanophilus* are available in the literature (Sá-Moura et al. 2008; Gonçalves et al. 2009). The structural analysis of the *Thermus thermophilus* MPGS in complex with GDP-mannose:Mg^{2+} revealed the existence of a second metal ion, whose involvement in catalysis

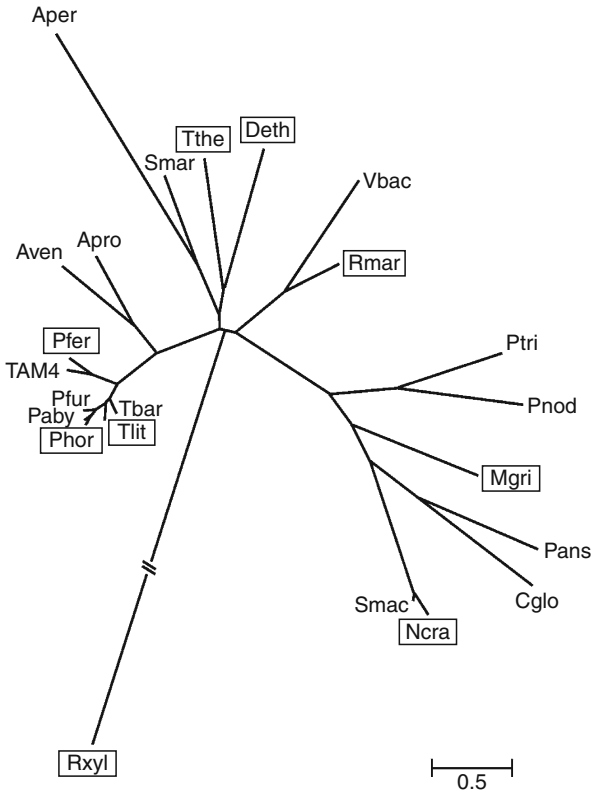

Fig. 4.5.4

Unrooted phylogenetic tree based on amino acid sequences of mannosyl-3-phosphoglycerate synthase. Open boxes indicate proteins with confirmed MPGS activity. MEGA 4.1 software (Tamura et al. 2007) was used for sequence alignment and to generate the phylogenetic tree using the neighbor-joining method. Bar, 0.5 change per site. Abbreviations: Aper, *Aeropyrum pernix*; Pfer, *Palaeococcus ferrophilus*; Pfur, *Pyrococcus furiosus*; Paby, *Pyrococcus abyssi*; Phor, *Pyrococcus horikoshii*; Apro, *Archaeoglobus profundus*; Aven, *Archaeoglobus veneficus*; Tlit, *Thermococcus litoralis*; Tbar, *Thermococcus barophilus*; TAM4, *Thermococcus* AM4; Vbac, *Verrucomicrobiate bacterium*; Rxyl, *Rubrobacter xylanophilus*; Rmar, *Rhodothermus marinus*; Tthe, *Thermus thermophilus* HB27; Smar, *Staphylothermus marinus*; Deth, *Dehalococcoides ethenogenes*; Mgri, *Magnaporthe grisea*; Ncra, *Neurospora crassa*; Cglo, *Chaetomiun globosum*; Pnod, *Phaeosphaeria nodorium*; Pans, *Podospora anserina*; Ptri, *Pyrenophora tritici-repentis*; Smac, *Sordaria macrospora*

was proven by site-directed mutagenesis (Gonçalves et al. 2010). The biochemical properties of MPGS and MPGP have been reviewed elsewhere (Santos et al. 2007a). Interestingly, the MPGS from *Rubrobacter xylanophilus*, a thermophilic bacterium known to accumulate mannosylglycerate, has no significant sequence homology with known MPGSs, and accordingly does not cluster together with typical MPGSs (Fig. 4.5.4) (Empadinhas and da Costa 2008b). This unique MPGS shows a peculiar substrate specificity, being able to use GDP-mannose as well as GDP-glucose as glycosyl donors for 3-phosphoglycerate, hence yielding mannosyl-3-phosphoglycerate and glucosyl-3-phosphoglycerate, respectively. Empadinhas and da Costa (2008b) speculated that this bifunctional MPGS/GPGS could be the ancestral protein from which all known MPGSs and GPGSs evolved.

The regulation of mannosylglycerate synthesis was studied in *Rhodothermus marinus* (Borges et al. 2004). This thermophilic bacterium is the only organism known to possess two pathways for mannosylglycerate synthesis: the single-step pathway is induced in response to heat stress, whereas the level of the synthase in the two-step pathway increases during osmoadaptation. Therefore, the two pathways play specialized roles in thermoadaptation and osmoadaptation of *Rhodothermus marinus*.

Inspection of public databases resulted in the identification of MPGS homologues in the mesophilic bacteria *Dehalococcoides ethenogenes*, and *Verrucomicrobiae bacterium*, and also in several fungi (Fig. 4.5.4). The MPGS activity of the respective gene products has been confirmed in *Neurospora crassa* and *Dehalococcoides ethenogenes* (Empadinhas et al. 2004; Empadinhas 2005). Therefore, the synthesis of mannosylglycerate seems not restricted to (hyper)thermophiles, but its physiological role in algae is unknown, and the accumulation of this solute in other mesophiles awaits demonstration. It is expected that the investigation of the molecular evolution of mannosylglycerate synthesis will shed light on the origin and dissemination of this trait in the Tree of Life.

Mannosyl-glucosylglycerate

Thus far mannosyl-glucosylglycerate (MGG) has been detected only in species of the genus *Petrotoga* (Table 4.5.1); the biosynthesis of MGG was studied in *Petrotoga mobilis* (Fernandes et al. 2010). Interestingly, the biosynthetic scheme closely resembles that of mannosylglycerate, namely in regard to the presence of two alternative pathways (Fig. 4.5.3). In the two-step pathway, glucosyl-3-phosphoglycerate (GPG), the product of glucosyl-3-phosphoglycerate synthase, is condensed with GDP-mannose, yielding mannosyl-glucosyl-3-phosphoglycerate (MGPG) in a reaction catalyzed by mannosyl-glucosyl-3-phosphoglycerate synthase. Subsequently, MGPG phosphatase hydrolyzes the phosphorylated intermediate into MGG. The recombinant mannosyl-glucosyl-3-phosphoglycerate synthase and the partially purified native mannosyl-glucosyl-3-phosphoglycerate phosphatase were characterized biochemically (Fernandes et al. 2010). Alternatively, the synthesis of MGG was proposed to occur in a single-step reaction catalyzed by mannosyl-glucosylglycerate synthase (MGGS). The firm identification of the gene encoding this activity in *Petrotoga mobilis* was not possible, but the authors characterized a homologous protein in *Thermotoga maritima* (37% of identity), which used glucosylglycerate (GG) and GDP-mannose to produce MGG. The presence of this activity in *Thermotoga maritima* is rather intriguing since neither MGG nor GG have been detected in the solute pool of this organism (Martins et al. 1996; Rodrigues et al. 2009).

Polyol-phosphodiesters

The bioynthesis of DIP was initially investigated in the archaea *Methanotorris igneus* (Chen et al. 1998) and *Pyrococcus woesei* (Scholz et al. 1998). These independent studies led to distinct proposals for the biosynthesis of DIP. Chen et al. (1998) proposed the involvement of two intermediates, CDP-inositol and *myo*-inositol, but failed to prove the existence of CDP-inositol; on the other hand, Scholz et al. (1998) proposed that the synthesis of DIP occurred without the formation of intermediate metabolites. Both teams confirmed the activity of *myo*-inositol 1-phosphate synthase (IPS), the enzyme that catalyzes the conversion of glucose 6-phosphate into L-*myo*-inositol 1-phosphate.

Recently, we investigated the synthesis of DIP in *Archaeoglobus fulgidus*, and established the following biosynthetic pathway (*Fig. 4.5.5*): (1) glucose 6-phosphate is converted into L-*myo*-inositol 1-phosphate by IPS, a well known enzyme (Chen et al. 2000; Neelon et al. 2005); (2) L-*myo*-inositol 1-phosphate is activated to CDP-inositol at the expense of CTP; (3) CDP-inositol is coupled with L-*myo*-inositol 1-phosphate to yield a phosphorylated intermediate, 1,3′-di-*myo*-inosityl-phosphate 1-phosphate (DIPP); (4) finally, DIPP is dephosphorylated into DIP by the action of a phosphatase (Borges et al. 2006; Rodrigues et al. 2007). The authors provided NMR structural data to prove the existence of the intermediate metabolites CDP-inositol and DIPP.

Definitive evidence for the occurrence of this pathway was provided recently by the identification of the genes encoding CTP:inositol 1-phosphate cytidylyltransferase (IPCT), the enzyme that catalyzes the condensation of CTP and L-*myo*-inositol 1-phosphate into CDP-L-*myo*-inositol, and CDP-inositol:inositol 1-phosphate transferase (DIPPS) that produces phosphorylated DIP from CDP-L-*myo*-inositol and L-*myo*-inositol 1-phosphate (Rodionov et al. 2007; Rodrigues et al. 2007). Roberts's group proposed that the final step in DIP synthesis (the dephosphorylating step) is catalyzed by inositol monophosphatase (IMPase), a nonspecific phosphatase known to use L-*myo*-inositol 1-phosphate and fructose-1,6-bisphosphate as substrates (Stec et al. 2000; Rodionov et al. 2007).

In *Thermotoga maritima* the genes encoding IPS, IPCT, DIPPS, and IMPase are arranged in a gene cluster and the predicted activities were confirmed by functional expression in *E. coli* (Rodionov et al. 2007). The predicted activities of IPCT and DIPS were also confirmed by functional expression in *E. coli* of the putative genes from several (hyper)thermophiles, *Archaeoglobus fulgidus*, *Pyrococcus furiosus*, *Thermococcus kodakarensis*, *Aquifex aeolicus*, and *Rubrobacter xylanophilus* (Rodrigues et al. 2007). Furthermore, the stereochemistry of DIP and of all intermediate metabolites in DIP synthesis was established by NMR analysis, resorting to specific labeling of L-*myo*-inositol 1-phosphate with carbon 13 (Rodrigues et al. 2007). It was proven that the two inositol molecules in DIP have different stereochemical configurations, i.e., DIP is synthesized as the L,D stereoisomer. The correct structure is represented in *Fig. 4.5.1* and can be designated as L,D-di-*myo*-inositol-1,1′-phosphate, or L,L-di-*myo*-inositol-1,3′-phosphate if the stereo-specific numbering convention is used instead.

Given the large number of hyperthermophilic organisms with sequenced genomes, several homologues of IPCT/DIPPS were found in public databases. Interestingly, in most cases, these two activities (IPCT and DIPP synthase) are fused in a single gene product, yet separate genes are present in a few hyperthermophiles, the crenarchaeota *Aeropyrum pernix* and *Hyperthermus butylicus*, and several *Thermotoga* species (*Fig. 4.5.6*). Homologues of IPCT/DIPPS are found in various (hyper)thermophilic, marine organisms belonging to the *Euryarchaeota* (Thermococcales, Archaeoglobales, and Methanococcales), the *Crenarchaeota*

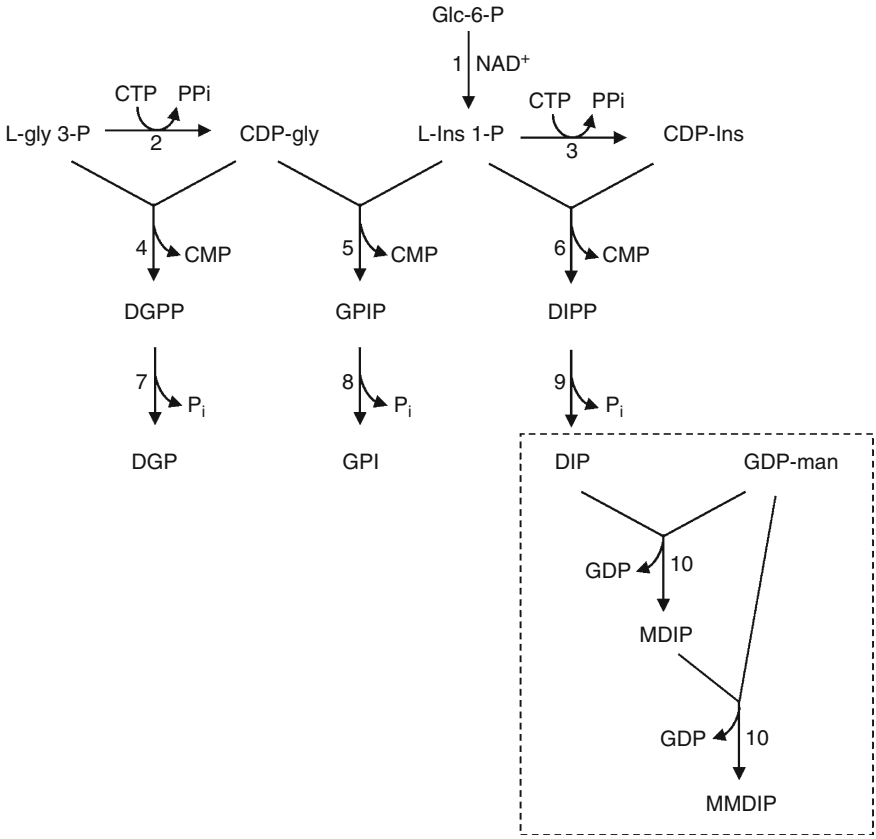

Fig. 4.5.5

Pathways for the synthesis of DGP, GPI, and DIP in *Archaeoglobus fulgidus* and for the synthesis of MDIP and MMDIP in *Thermotoga maritima* (dashed box). Enzymes: (1) L-*myo*-inositol 1-phosphate synthase, (2) CTP:glycerol 3-phosphate cytidylyltransferase, (3) CTP:L-*myo*-inositol 1-phosphate cytidylyltransferase, (4) DGPP synthase, (5) GPIP synthase, (6) DIPP synthase, (7) DGPP phosphatase, (8) GPIP phosphatase, (9) DIPP phosphatase, and (10) MDIP synthase. DGP, diglycerol phosphate; DGPP, 1X,1′X-diglyceryl phosphate 3-phosphate; DIP, di-*myo*-inositol phosphate, DIPP, 1,3′-di-*myo*-inosityl phosphate 1′-phosphate (DIPP); GPI, glycero-phospho-inositol; GPIP, 1-(1X-glyceryl)-*myo*-inosityl phosphate 3-phosphate; MDIP, mannosyl-di-*myo*-inositol-1,3′-phosphate; MMDIP, di-mannosyl-di-*myo*-inositol-1,3′-phosphate; Glc 6-P, glucose 6-phosphate; L-Ins 1-P, L-*myo*-inositol 1-phosphate; gly, glycerol; Ins, *myo*-inositol; and L-gly 3-P, glycerol 3-phosphate. Data from Borges et al. 2006 and Rodrigues et al. 2009

(Thermoproteales and Desulfurococcales), the *Korarchaeota*, and *Bacteria* (Thermotogales, Aquificales, and Rubrobacterales). A phylogenetic tree based on the sequences of the IPCT/DIPPS proteins displays three groups: one refers to the organisms in which IPCT and DIPPS are encoded by separated genes, a second one comprises the members of the Aquificales and the *Euryarchaeota*, and the third group comprises fused IPCT/DIPPS with an extra C-terminal tail (*Fig. 4.5.6*). Interestingly, this topology is unrelated with that of the phylogenetic tree

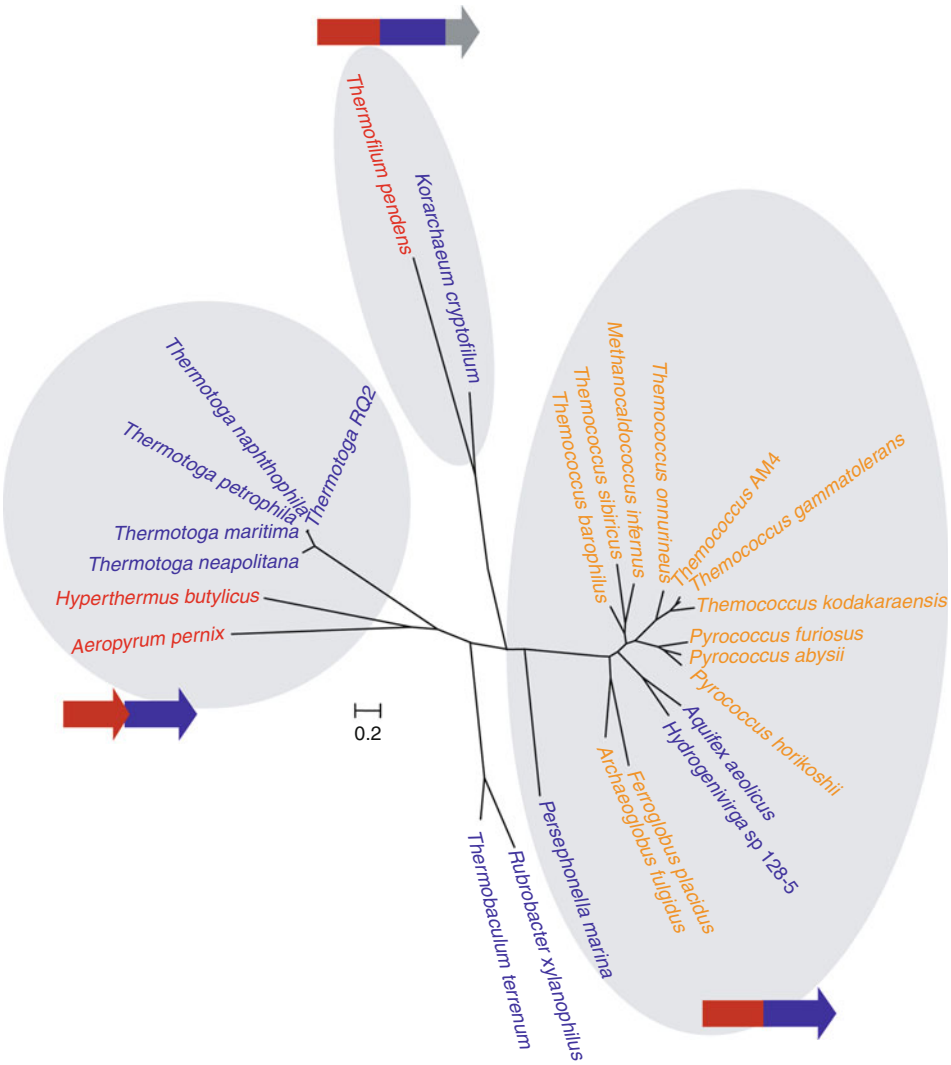

Fig. 4.5.6

Unrooted phylogenetic tree based on available amino acid sequences of IPCT/DIPPS. Amino acid sequences of IPCT and DIPPS were artificially fused in the cases where the activities are encoded in different genes. Homologues of IPCT/DIPPS were retrieved from the existing protein databases (GenBank) using BLAST (Altschul et al. 1997). MEGA 4.1 software (Tamura et al. 2007) was used for sequence alignment and to generate the phylogenetic tree using the neighbor-joining method. Bar, 0.2 change per site. The bacterial IPCT/DIPPS are depicted in blue, the euryarchaeotal in orange, crenarchaeotal in red, and korachaeotal in purple. The IPCT and DIPPS domains are depicted in red and blue, respectively, and indicate that the IPCT and DIPPS genes are separated and fused, respectively. The genes containing IPCT, DIPPS, and an extra domain are indicated by

based on 16S RNA sequences (Yarza et al. 2008), suggesting a complex evolutionary process, and the occurrence of Lateral Gene Transfer events.

The synthesis of two other polyol-phosphodiesters, diglycerol phosphate (DGP) and glycerol-phospho-inositol (GPI), accumulating in a restricted number of hyperthermophiles, proceeds via the condensation of CDP-glycerol with the respective phosphorylated polyol, glycerol 3-phosphate for DGP, and L-*myo*-inositol 1-phosphate for GPI. The synthesis of DGP and GPI also proceeds via the formation of phosphorylated intermediates, which are subsequently dephosphorylated to form the final products (*Fig. 4.5.5*). It is known that the IPCT/DIPPS involved in the synthesis of DIP is also able to synthesize GPI; thus far our attempts to identify the gene encoding DGP synthase were unsuccessful (Rodrigues et al. 2007).

Mannosyl-di-*myo*-inositol phosphate (MDIP) and di-mannosyl-di-*myo*-inositol phosphate (MMDIP) have been recently identified in *Thermotoga maritima* (*Fig. 4.5.5*). The synthesis of MDIP involves the transfer of the mannosyl group from GDP-mannose to DIP in a single-step reaction, catalyzed by a unique β-1,2-mannosyltransferase. The stereochemistry of MDIP has been established: the compound is composed by two stereochemically distinct inositol moieties with the mannosyl residue (β-configuration) attached to the inositol moiety in the L-configuration (Rodrigues et al. 2009).

The novel β-1,2-mannosyltransferase is unrelated with known glycosyltransferases and it is restricted to members of the two deepest lineages, i.e., the Thermotogales and the Aquificales within the domain Bacteria. Interestingly, the enzyme can use MDIP as an acceptor of a second mannose residue, yielding the di-mannosylated derivative of DIP (MMDIP) (Rodrigues et al. 2009).

Do Compatible Solutes from Hyperthermophiles Play a Physiological Role in Thermoprotection?

Many marine (hyper)thermophiles accumulate organic solutes in response to supra-optimal growth temperatures, an observation that suggests their role in thermoprotection (Santos et al. 2007a). However, accumulation of organic solutes is by no means essential for life at high temperature; indeed, hyperthermophiles isolated from non-saline environments, such as *Thermotoga thermarum, Hydrogenobacter islandicum, Thermococcus zilligii*, and *Pyrobaculum islandicum*, do not accumulate organic solutes (Martins et al. 1996, 1997; Lamosa et al. 1998). Therefore, the postulated contribution of thermolytes to in vivo protection of macromolecules and other cellular components against high temperature lacks definitive proof. Progress towards this end has been hampered by unavailability of genetic tools for manipulation of (hyper)thermophiles. Fortunately, an efficient gene disruption system has been developed for *Thermococcus kodakarensis* (Sato et al. 2005) and we took advantage of this tool to investigate the physiological role of DIP in the adaptation of that archaeon to heat stress. The gene encoding the key activities implicated in DIP synthesis (IPCT/DIPPS) was disrupted and the resulting mutant examined in respect to its ability to cope with heat stress (Borges et al. 2010). Unexpectedly, the growth rate of the deficient mutant was similar to that of the parental strain, which accumulated considerable amounts of DIP, especially under heat stress. Analysis of the solute pool in the mutant showed that DIP was substituted by aspartate (*Fig. 4.5.7*). These results indicate that DIP is part of the strategy used by *Thermococcus kodakarensis* to cope with heat stress, and aspartate can be used as an alternative solute of similar efficacy (Borges et al. 2010). Although thermolytes are not essential for life at high temperature, there is some evidence that they play a role in the adaptation of these organisms to supra-optimal temperatures.

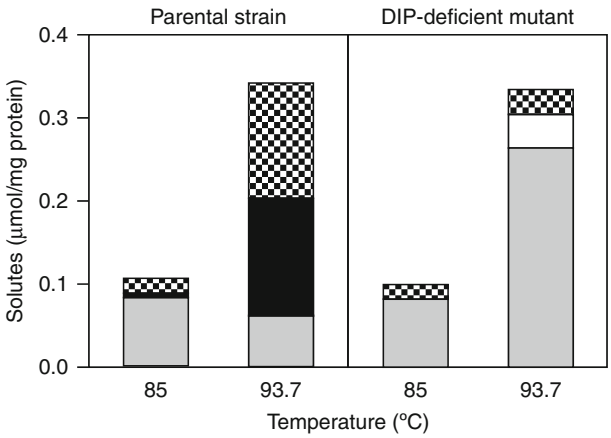

Fig. 4.5.7

Effect of the growth temperature on the accumulation of α-glutamate (*checkered*), DIP (*black*), *myo*-inositol-phosphate (*white*), and aspartate (*gray*) in *Thermococcus kodakarensis* parental strain and DIP-deficient mutant. Data from Borges et al. 2010

Performance of Thermolytes as Stabilizers of Model Enzymes

Organisms with optimal growth temperatures above 60°C accumulate primarily negatively charged organic solutes, like DIP, mannosylglycerate, DGP, aspartate, and glutamate, whereas mesophilic organisms typically use neutral or zwitterionic compounds, like trehalose, proline, ectoines, and betaines. The in vivo protecting function of charged solutes has not yet been fully demonstrated, but their effect on protein stability in vitro is profusely documented (Ramos et al. 1997; Shima et al. 1998; Lamosa et al. 2000; Borges et al. 2002; Santos et al. 2007b; Faria et al. 2008; Longo et al. 2009). In particular, a recent comprehensive study compared the effectiveness of thermolytes with that of compatible solutes from mesophiles for the protection of model enzymes against heat-induced denaturation, aggregation, and inactivation (Faria et al. 2008). Also, newly synthesized organic compounds, inspired by mannosylglycerate, were included in this study with the two-fold purpose of discovering better stabilizers and understanding the molecular basis for protein stabilization by thermolytes.

Charged compounds were consistently more effective for stabilization of protein structure against thermal denaturation. For example, the addition of 0.5 M mannosylglycerate resulted in an increase of 8.5°C in the melting temperature (T_m) of pig heart mitochondrial malate dehydrogenase (MDH), while the same concentration of glycerol produced an increment in *Tm* of only 1°C. Even very high concentrations of glycerol (2.5 M) were unable to produce an increase in *Tm* greater than 3°C. This trend is clearly apparent from the set of increment values in *Tm* observed for two model enzymes (*Fig. 4.5.8*), which shows that the ionic compounds (except for the control KCl) cluster in the upper-right corner, while neutral solutes, such as trehalose, mannosylglyceramide, glycerol, and ectoine, appear in the lower-left corner. Although the superiority of negatively charged compounds is patent, the scatter in this data subset reflects a degree of solute/protein specificity that is not negligible. For example, mannosyl-lactate is the best stabilizer of staphylococcal nuclease, but it has only a moderate effect on malate dehydrogenase, and the converse relationship holds for DIP. On the other hand, the different degrees of stabilization rendered by mannosylglycerate and

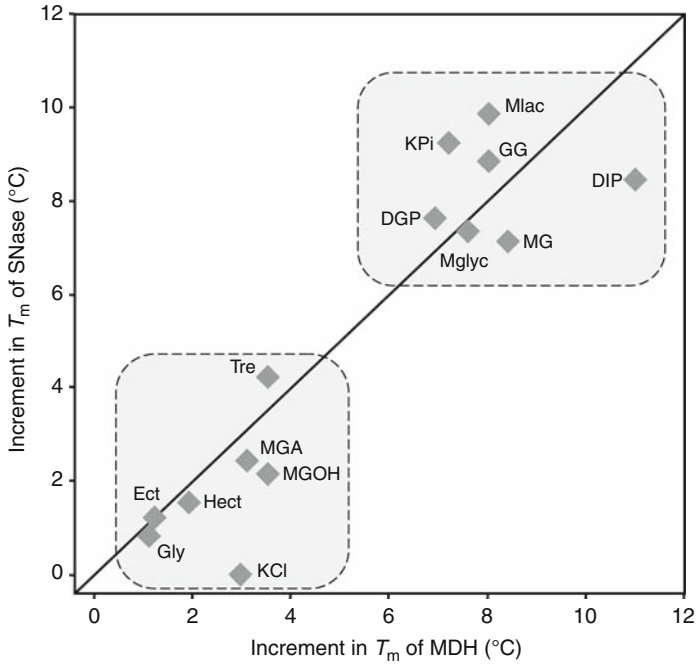

Fig. 4.5.8

Effect of solutes on the melting temperature of staphylococcal nuclease (SNase) and pig heart malate dehydrogenase (MDH). Solutes at 0.5 M concentration. In the case of ionic solutes, potassium was used as counterion. Gly: glycerol; Tre: trehalose; Ect: ectoine; Hect: hydroxyectoine; DIP: di-*myo*-inositol-1,3′-phosphate; DGP: diglycerol phosphate; MG: mannosylglycerate; MGA: mannosylglyceramide; GG: glucosylglycerate; Mlac: mannosyl-lactate; Mglyc: mannosyl-glycolate; MGOH: mannosyl-glycerol; KPi: potassium phosphate; KCl: potassium chloride. Data from Faria et al. 2008

glucosylglycerate are difficult to rationalize in view of the similarity of the molecular structures of these two glycosides.

There is also a correlation between charged solutes and the greater ability to prevent protein aggregation as evaluated by light scattering experiments, but the pattern is not as clear-cut as in the case of thermodynamic stabilization. For example, mannosylglyceramide has an effect comparable to that of charged solutes, such as mannosylglycerate, whereas mannosyl-lactate has a poor performance, which is comparable to that of glycerol (Faria et al. 2008). In respect to protection against inactivation, the general association between the negative charge and greater protection appears to hold true, but di-*myo*-inositol phosphate is a notable exception, showing unexpected destabilizing effects upon malate dehydrogenase and also lactate dehydrogenase (Borges et al. 2002).

We are still far from being able to predict which solute will render best protection to a given enzyme, but the amount of data available corroborates the conclusion that charged solutes are clearly better to oppose thermal unfolding of protein structures. In respect to protection of enzymes against inactivation the pattern is less defined, and DIP, in particular, shows aberrant, puzzling behavior. In contrast with the unreliable performance of DIP, mannosylglycerate is the best wide-ranged protector of enzymes.

Thermolytes and Protein Dynamics: The Link Between Stabilization and Rigidification

It is generally accepted that compatible solutes confer extra stability to proteins without affecting significantly their native structure or function (Foord and Leatherbarrow 1998; Lamosa et al. 2003); however, clear alterations in protein dynamics have been observed by us and by others (Wang et al. 1995; Lamosa et al. 2003; Doan-Nguyen and Loria 2007; Pais et al. 2009). Therefore, it is reasonable to propose that modulation of protein internal dynamics is part of the mechanism of protein stabilization by organic solutes. In this context, several questions immediately come to mind: is there a correlation between protein stabilization and rigidification? Is this rigidification selective for specific regions of the protein rather than being a global event? Are motional restrictions associated with specific dynamic regimes?

Protein dynamics cover a wide range of motions, from small vibrational fluctuations of bond lengths (pico-nanosecond time scale) to translations of atom groups (micro-millisecond) to motions of whole structural elements (second to minute). NMR spectroscopy can probe motions at all these time scales and therefore is a powerful methodology to address the questions stated above.

To ascertain whether the stabilization rendered by solutes is associated with changes in internal mobility, a thorough characterization of the effect of mannosylglycerate on the internal motions of a model protein, staphylococcal nuclease (SNase), was performed in our lab, using various NMR experiments to access multiple times scales (Pais et al. 2009). In parallel experiments, the structural stability of SNase as a function of mannosylglycerate concentration was assessed by measurements of T_m values using differential scanning calorimetry. This allowed for characterization of the dynamic properties of the protein as its stability was varied. A strong correlation was observed between the stability of SNase in each of the conditions studied and the respective generalized order parameter S^2, an indicator of the mobility of the protein backbone in the pico-nanosecond time scale, with greater values of S^2 denoting higher rigidity. In particular, a positive linear correlation was found between S^2 values and the T_m at increasing concentrations of mannosylglycerate, meaning that the amplitude of the backbone fluctuations was progressively restrained in the presence of the solute (*Fig. 4.5.9*). This correlation was further validated by the observation that the addition of urea, a chaotropic agent, or an increase in the experimental temperature, resulted in a decrease of S^2. Curiously, the addition of 0.35 M mannosylglycerate (at 37°C) resulted in a restriction of fast motions in SNase comparable to that brought about by a 5°C reduction in the working temperature. In summary, we were able to establish a strong link between restriction of the high frequency motions and the increased stability of SNase as a function of mannosylglycerate concentration, and this was not a consequence of an increase of the ionic strength or the viscosity of the solution (Pais et al. 2009).

A similar methodology, NMR spin-relaxation measurements and model-free analysis, has been used to show that the addition of trimethylamine N-oxide to ribonuclease A, destabilized by the presence of 0.7 M guanidine hydrochloride, caused a significant decrease in the amplitude of backbone fluctuations (Doan-Nguyen and Loria 2007). Although the very limited number of studies prevents a firm conclusion, it appears that protein rigidification, in the fast dynamic regime, could be a general phenomenon associated with stabilization by compatible solutes, regardless of their charged or non-charged nature.

Very slow protein motions, on the minute time scale, can be probed by measuring the rates of exchange of backbone amide protons with the solvent (Krishna et al. 2004). The effect of

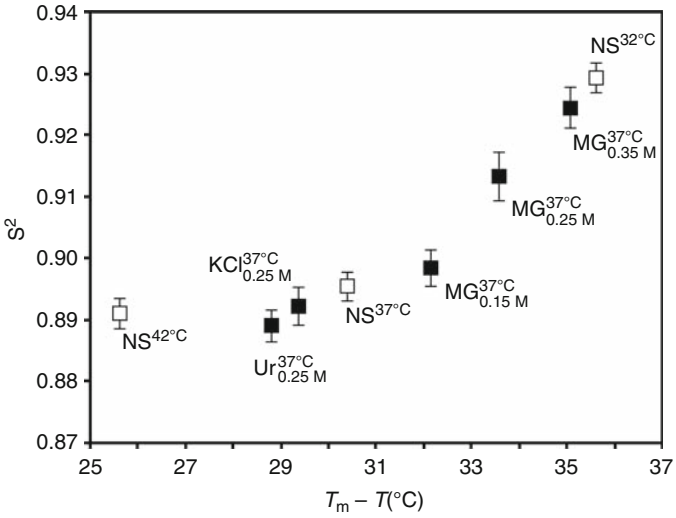

Fig. 4.5.9

Plot of the average S^2 values for staphylococcal nuclease as a function of the shift away from the melting temperature. The (Tm-T) parameter is the difference between the temperature at which relaxation values were measured and the Tm of the protein in the presence of a given solute, NS (no solute), KCl (potassium chloride), Ur (urea), MG (mannosylglycerate, potassium salt). The subscripts give the concentration of the solute (M) and the superscripts are the experimental temperatures (°C). The error bars represent the error of the average calculated from the individual uncertainties on the order parameter S^2. Data from Pais et al. 2009

compatible solutes and various salts on the exchange rates is very dependent on the solute and on the specific location of the amide proton (Wang et al. 1995; Christoffersen et al. 1996; Foord and Leatherbarrow 1998; Knubovets et al. 1999; Lamosa et al. 2003; Tadeo et al. 2007). Nevertheless, a general trend is that stabilizing solutes decrease to a larger extent the exchange rates of the most slowly exchanging protons, i.e., those which are normally more buried in the protein structure. These protons are only expected to exchange when the protein undergoes wide opening motions, taking the structure close to the unfolded state. Thus, stabilizing solutes seem to oppose these motions and promote protein tightness.

In conclusion, by an as yet undetermined mechanism, the ensemble of protein conformations becomes tighter in the presence of compatible solutes; we speculate that this added tightness promotes the optimization of the network of hydrophobic interactions, hydrogen bonds, salt bridges, and Coulomb forces involved in protein structure, thereby shifting the equilibrium towards the native state.

Concluding Remarks

Prokaryotes adapted to hot environments use a portfolio of compatible solutes, comprising several unique negatively charged organic solutes, which accumulate not only to maintain cell volume when the external osmolarity fluctuates, but also in response to heat stress. This shapes the basis for the hypothesis that these solutes were selected through evolution to render

maximal protection to macromolecules against thermal denaturation in vivo. Mutants of *Thermococcus kodakarensis* disrupted in the synthesis of di-*myo*-inositol phosphate, the most common solute of hyperthermophiles, showed growth profiles under thermal stress similar to those of the parental strain; interestingly, the lack of di-*myo*-inositol phosphate was offset by the accumulation of aspartate, a negatively charged amino acid apparently with equivalent performance. This provides some evidence for the involvement of ionic organic solutes in thermoprotection, but a definitive demonstration of the physiological role of these compounds is still missing. However, the superior ability of charged organic compounds to act as protectants of protein structure in vitro is well established. We showed that thermolytes were often better stabilizers than the inorganic ions placed at the top of the Hofmeister series, i.e., phosphate and sulfate. Therefore, it appears that prokaryotes adapted to grow at limit temperatures evolved the synthesis and accumulation of ionic organic solutes as the best stabilizers available. The routes for the synthesis of these novel compounds have been identified in the last decade, but there are many unanswered questions in respect to the thermosensing and regulation mechanisms underlying the final step in the adaptation response, i.e., the accumulation of a specific solute. The availability of genetic tools to manipulate hyperthermophiles isolated from marine habitats is expected to lead to rapid progress in the area.

The perspectives for the application of compatible solutes in biotechnology and medicine are very promising, particularly, their chaperone features that enable the rescue of misfolded proteins. These properties have been reported both for neutral and charged solutes, but the importance of the potential applications in the treatment or prevention of several diseases attract a great research effort to this subject. Finally, perhaps the most challenging and appealing issue, at least for biophysicists, is to decode the network of molecular interactions that are responsible for the beneficial impact of compatible solutes on macromolecule stability. Information on this fundamental matter will cast light on the mechanisms of protein folding/unfolding and eventually lead to the rational design of efficient stabilizers, tailored for relevant applications.

Acknowledgments

This work was funded by the Fundação para a Ciência e a Tecnologia (FCT) and Programa Operacional Ciência e Inovação (POCTI), Portugal, PTDC/BIA-MIC/71146/2006, and PTDC/BIO/70806/2006. L.G. Gonçalves, T. Pais and M.V. Rodrigues acknowledge grants from FCT, Portugal (SFRH/BPD/26905/2006, SFRH/BD/42210/2007, and SFRH/BD/25539/2005, respectively). The NMR spectrometers are part of The National NMR Network (REDE/1517/RMN/2005), supported by "Programa Operacional Ciência e Inovação (POCTI) 2010" and Fundação para a Ciência e a Tecnologia (FCT).

Cross-References

3.3 Osmoadaptation in Methanogenic archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1
3.6 Adapting to Changing Salinities: Biochemistry, Genetics, and Regulation in the Moderately Halophilic Bacterium *Halobacillus halophilus*
4.1 History of Discovery of Hyperthermophiles

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4.6 Metalloproteins from Hyperthermophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.6,

Abstract: Metalloproteins play critical roles in living cells. There are more than a dozen metals with known or suspected biological functions, which include determining structure, electron transfer, and catalysis. The binding of a metal cofactor can generate beautifully colored proteins, but, more importantly, these metalloproteins function in essentially every metabolic pathway, greatly extending the chemistry past those that are available to a living cell with only the side groups of the 20 amino acids. The number and types of metalloproteins in cells remain surprisingly underappreciated, and only recently, with the advent of coupled purification and detection techniques, such as capillary electrophoresis and inductively coupled plasma emission mass spectrometry, is the true extent of the metalloproteome being revealed. Proteins from hyperthermophilic microorganisms are of particular utility for studying metalloproteins due to their extreme stability and relative ease of purification, both from native and recombinant sources. Herein we focus on the metalloproteins that have been characterized from some model hyperthermophilic systems with an emphasis on their properties and the roles that they play in primary metabolism.

Introduction

Metalloproteins play essential roles in almost every aspect of cellular function (Lippard and Berg 1994; Bertini et al. 2001; Finkelstein 2009). Proteins utilize inorganic cofactors from the alkali and alkaline metals (Groups 1 and 2 of the Periodic Table), for example, K^+ and Mg^{2+} (Cowan 2002; Page and Di Cera 2006; Sissi and Palumbo 2009), many of the transition metals (Groups 5–12), for example, Fe^{2+} and Zn^{2+} (Theil and Goss 2009; Maret and Li 2009), and some metalloids (in Groups 13–15), for example, boron and silicon (Sumper and Brunner 2008; Nielsen 2009; Hansch and Mendel 2009). It is estimated that as many as half of all proteins may contain some type of metal cofactor (Holm et al. 1996; Degtyarenko 2000; Castagnetto et al. 2002; Andreini et al. 2009a). Such cofactors can play roles in maintaining structure (Opella et al. 2002; Barondeau and Getzoff 2004; Meyer 2008), functions such as enzymatic catalysis (Rees 2002; Williams 2003; Barondeau and Getzoff 2004; Meyer 2008; Moura et al. 2008), and electron transfer (Richter and Ludwig 2009). In some organisms, some transition metals are used as terminal electron acceptors for disposing of electrons generated by metabolism (Lovley 1993), while in others, certain metals can be extremely toxic to various cell types under certain conditions (Summers 2009). However, herein we focus on transition metal cofactors that form stable metal-protein complexes such that these metalloproteins can be purified and characterized.

Hyperthermophilic microorganisms are particularly useful as model systems for studying the structure and functions of metalloproteins. Hyperthermophiles are defined as organisms with an optimal growth temperature of at least 80°C (Stetter 2006), while thermophiles are usually regarded as organisms with optimal growth temperatures in the range 45–80°C (Brock 1995); see Chap. 4.1 History of Discovery of Hyperthermophiles, hyperthermophiles are also of interest as they are among the most deeply rooted organisms in the tree of life (House 2009) and, while somewhat controversial, it is increasingly thought that the Last Common Ancestor of extant life was a hyperthermophile (Schwartzman and Lineweaver 2004). Proteins from hyperthermophiles are extremely stable (Unsworth et al. 2007), and this allows both ease of purification and manipulation (room temperature instead of 4°C). The majority of hyperthermophiles are members of the Archaea, although there are bacterial phyla such as Aquificales (Coenye and Vandamme 2004) and Thermotogae (Conners et al. 2006).

The majority of hyperthermophiles are strictly anaerobic, but some, such as the bacterial genus *Aquifex*, are microaerophilic, and the archaeal genus *Sulfolobus* are aerobes; see Chaps. 4.1 History of Discovery of Hyperthermophiles and 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles. Therefore, metalloproteins representing members of virtually all energy-conserving pathways can be found in the hyperthermophilic organisms. The extreme thermostability of proteins from these organisms not only facilitates their purification, it makes them ideal for many types of structural and functional studies (Unsworth et al. 2007; Luke et al. 2007; Greaves and Warwicker 2009).

Metalloproteins from *Pyrococcus furiosus*

Pyrococcus furiosus is perhaps the best studied of the hyperthermophilic archaea; see Chaps. 4.1 History of Discovery of Hyperthermophiles and 7.1 Microbiology of Volcanic Environments. It was first isolated in 1986 (Fiala and Stetter 1986) and since then a large number of its proteins, both native and recombinant, have been purified and characterized, and many of these are metal-containing proteins. *P. furiosus* is a strict anaerobe that grows by fermentation of peptides or carbohydrates. While it preferentially reduces elemental sulfur (S°) to H_2S to dispose of excess reductant (Schut et al. 2007), in the absence of S^o it produces H_2. The metalloproteins that have been obtained from *P. furiosus* are summarized in *Table 4.6.1*. These were obtained either by direct purification from native biomass or as a recombinant form by heterologous expression in *Escherichia coli* of a *P. furiosus* gene. As might be expected, many of these are involved in the primary metabolic pathways of the organism. We briefly describe below, the metalloproteins involved in those pathways.

The metalloenzymes involved in the production of acetate, CO_2, and H_2 from carbohydrate growth substrates by *P. furiosus* are shown in *Fig. 4.6.1*; see Chap. 4.1. The organism uses a broad range of both simple and complex sugars and the conversion of starch is catalyzed by a zinc-containing α-amylase (Savchenko et al. 2002); see Chap. 4.2 Carbohydrate-Active Enzymes from Hyperthermophiles: Biochemistry and Application. The glucose that is produced is oxidized by an unusual Embden-Meyerhof glycolytic pathway, (Verhees et al. 2003) in which the expected glyceraldehyde-3-phosphate dehydrogenase is replaced by glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR). This is significant since GAPOR contains both Fe as a [4Fe-4S] cluster and tungsten (W) as a W-pterin cofactor. The enzyme uses another Fe-containing protein, ferredoxin (Fd) as the electron acceptor. This small (67 residue) protein contains a single [4Fe-4S] cluster (Aono et al. 1989). The second oxidation step in the conversion of glucose to acetate is catalyzed by another Fe-containing and Fd-utilizing enzyme, pyruvate:ferredoxin oxidoreductase (POR) (Blamey and Adams 1993). This oxidizes pyruvate to acetyl CoA and CO_2 and contains multiple [4Fe-4S] centers (see below). The other metalloenzyme in the glycolytic pathway is phosphoglucose isomerase (PGI), which has been shown to require a metal, thought to be Fe in vivo, but Co gives very high activity (Berrisford et al. 2003).

In the absence of S°, reducing equivalents in the form of reduced Fd are disposed of as H_2 gas (*Fig. 4.6.1*). This is accomplished by another metalloenzyme, the membrane-bound respiratory hydrogenase (MBH), which both reduces protons (to H_2) and pumps them across the membrane to generate a proton motive force (Sapra et al. 2003); *P. furiosus* also contains two other hydrogenases, both cytoplasmic, and these are thought to recycle the H_2 produced by the membrane-bound enzyme for biosynthesis (Ma and Adams 2001; Jenney and Adams

Table 4.6.1

***Pyrococcus furiosus* metalloproteins purified and metal content confirmed[a]**

Enzyme/Protein	Gene(s)	Metal(s)	Reference
Purified from *Pyrococcus furiosus*			
Ribonucleotide reductase	PF0440	Co	Riera et al. (1997)
Cobalt-activated carboxypeptidase	PF0456	Co	Cheng et al. (1999)
Aminoacylase*[b]	PF0597	Co	Story et al. (2001)
Phosphoglucose isomerase (PGI)*	PF0196	Fe, Co	Yoon et al. (2009)
Indolepyruvate ferredoxin oxidoreductase (IOR)	PF0533–0534	Fe, Co	Mai and Adams (1994)
Pyruvate ferredoxin oxidoreductase (POR)	PF0965–0967, PF0971	Fe	Blamey and Adams (1993)
2-ketoisovalerate ferredoxin oxidoreductase (VOR)	PF0968–0971	Fe	Heider et al. (1996)
Superoxide reductase (SOR)*	PF1281	Fe	Jenney et al. (1999)
Rubredoxin (Rd)*	PF1282	Fe	Blake et al. (1991)
Rubrerythrin (Rr)*	PF1283	Fe	Weinberg et al. (2004)
Ferredoxin NADPH oxidoreductase (FNOR)	PF1327–1328	Fe	Ma and Adams (1994)
Ferredoxin (Fd)*	PF1909	Fe	Aono et al. (1989)
Sulfide dehydrogenase	PF1910–1911	Fe	Hagen et al. (2000)
Hydrogenase I	PF0891–0894	NiFe	Bryant and Adams (1989)
Hydrogenase II	PF1329–1332	NiFe	Ma et al. (2000)
Membrane-bound hydrogenase (MBH)[c]	PF1433–1434	NiFe	Sapra et al. (2000)
Aldehyde:ferredoxin oxidoreductase (AOR)	PF0346	W, Fe	Mukund and Adams (1991)
Glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR)	PF0464	W, Fe	Mukund and Adams (1995)
Formaldehyde ferredoxin oxidoreductase (FOR)	PF1203	W, Fe	Roy et al. (1999)
Formaldehyde ferredoxin oxidoreductase (WOR5)	PF1480	W, Fe	Bevers et al. (2005)
Formaldehyde ferredoxin oxidoreductase (WOR4)	PF1961	W, Fe	Roy and Adams (2002)
Proline dipeptidase*	PF1343	Zn, Co	Ghosh et al. (1998)
Lysyl aminopeptidase*	PF1861	Zn, Co	Story et al. (2005)
Recombinant forms			
Chaperonin	PF1974	Co, Mn	Hongo et al. (2006)
Aconitase	PF0201	Fe	van Vugt-Lussenburg et al. (2009)
Hybrid cluster protein (HCP)	PF0692	Fe	Overeijnder et al. (2009)
Ferritin	PF0742	Fe, Zn	Tatur et al. (2006)
Dps-like protein	PF1193	Fe	Ramsay et al. (2006)

Table 4.6.1 (Continued)

Enzyme/Protein	Gene(s)	Metal(s)	Reference
Fumarase	PF1754–1755	Fe	van Vugt-Lussenburg et al. (2009)
Cystathione-beta-synthase domain protein	PF1953	Fe	Proudfoot et al. (2008)
Phosphomannose isomerase	PF0589	Mg	Mizanur and Pohl (2009)
RNAse HII	PF1781	Mn, Mg	Kitamura et al. (2010)
Tungstate transport protein A (WtpA)	PF0080	WO_4, MoO_4	Bevers et al. (2006)
Disulfide oxidoreductase	PF0094	Zn	Ren et al. (1998)
Alpha amylase	PF0477	Zn	Savchenko et al. (2002)
eIF2b	PF0481	Zn	Sokabe et al. (2006)
Novel winged helix-turn-helix protein	PF0610	Zn	Wang et al. (2007)
Threonine dehydrogenase	PF0991	Zn	Machielsen and van der Oost (2006)
Rad50	PF1167	Zn	Hopfner et al. (2002)
RPP21 PNAse P subunit	PF1613	Zn	Amero et al. (2008)
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	PF1690	Zn, others	Schofield et al. (2004)
DNA directed RNA polymerase subunit	PF2009	Zn	Reich et al. (2009)

[a] *Table 4.6.1* shows the *P. furiosus* proteins that have been purified either from native cell material or in recombinant form, or both, as indicated. The gene indicates the unique identifier in the *P. furiosus* genome, and the metals that have been demonstrated to either copurify with the protein, or to stimulate activity of the protein are indicated. Proteins that purify as heteromeric complexes are shown as multiple genes, for example, PF0891–PF0894 for hydrogenase I. Some proteins (e.g., PF0742, ferritin) have been purified from native biomass (Tatur et al. 2006) but in quantities too small for metal analysis. The recombinant form, which contains Fe, is listed here.

[b] Both native and recombinant forms have been purified.

[c] A catalytically active sub-complex was solubilized from cell membranes and purified but that contained only two of the fourteen subunits that the native complex is predicted to contain (Sapra et al. 2000).

2008). The catalytic site of all three of the hydrogenases consists of a nickel and an iron atom and they also contain multiple iron-sulfur (FeS) clusters, like many other enzymes of this type (Vignais and Colbeau 2004). The complex roles that metals play in these metabolic conversions are illustrated in *Fig. 4.6.2*, which depicts the oxidation of pyruvate to acetyl CoA and H_2 by a combination of POR, Fd, and MBH. POR is an octomer $[(\alpha\beta\gamma\delta)_2]$ and Fd is reportedly a dimer (Hasan et al. 2002). MBH contains 14 different subunits but their stoichiometry is not known. As shown in *Fig. 4.6.2*, the production of H_2 from pyruvate by a transient POR-Fd-MBH complex would involve, at a minimum, 14 [4Fe-4S] clusters, in addition to the NiFe-center at the catalytic site of the MBH.

Proteins containing iron-sulfur, nickel, tungsten, zinc, and cobalt are therefore intimately involved in starch to H_2 conversion by *P. furiosus*, and a similar suite of metals are involved in peptide fermentation. As shown in *Table 4.6.1*, several proteolytic enzymes have been characterized from *P. furiosus* that contain iron, zinc, and/or cobalt, and these convert peptides to the amino acids (*Fig. 4.6.3*). The corresponding 2-keto acids are produced by glutamate

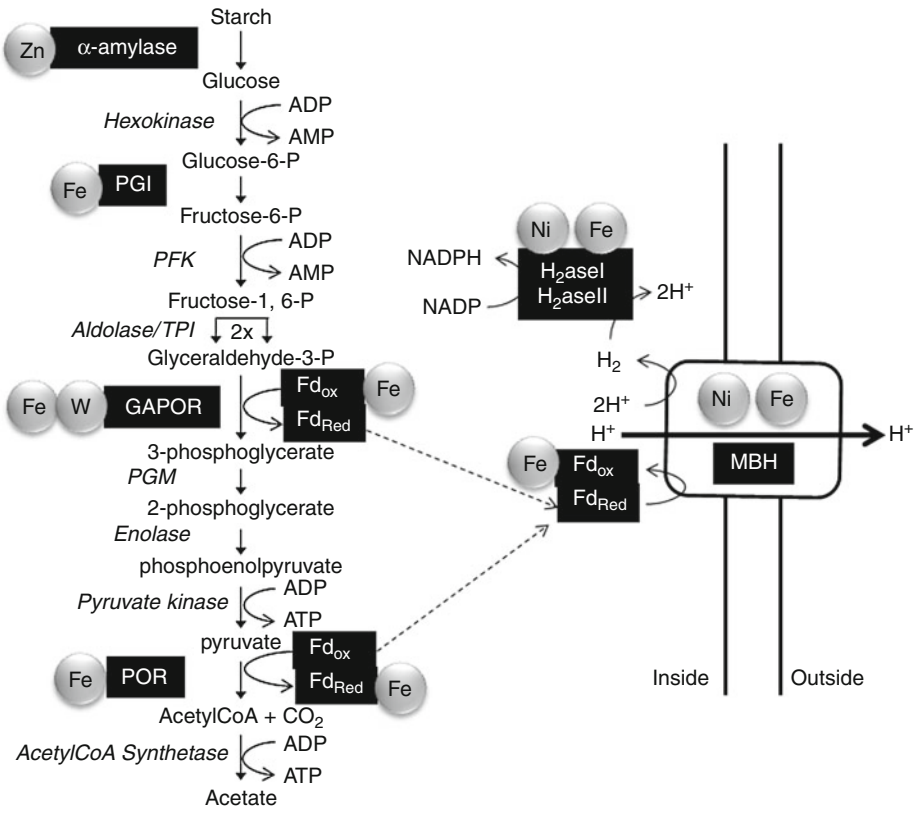

Fig. 4.6.1

The glycolytic pathway of *P. furiosus* and the associated metabolic route for H_2 metabolism. The abbreviations are: ACS, acetyl CoA synthetase; Fd_{Ox}, oxidized ferredoxin; Fd_{Red}, reduced ferredoxin GAPOR, glyceraldehyde-3-phosphate ferredoxin oxidoreductase; H_2ase I and II, soluble hydrogenases I and II; MBH, membrane-bound hydrogenase; PFK, Phosphofructokinase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; POR, pyruvate ferredoxin oxidoreductase; TPI, triosephosphate isomerase. See text for details

dehydrogenase and aminotransferases, none of which are metal containing (Robb et al. 1992; Ward et al. 2002). However, the 2-ketoacid oxidoreductases that produce the corresponding CoA-derivatives, of which POR is a member, are FeS-containing, Fd-dependent enzymes. Aldehydes are also produced during 2-ketoacid oxidation, and these are oxidized by another W-containing enzyme, aldehyde ferredoxin oxidoreductase (AOR) (Mukund and Adams 1991). AOR is the prototypical member of the so-called AOR family of W-containing enzymes in *P. furiosus* of which there are five members, including GAPOR (see *Table 4.6.1*). The reduced Fd that is generated by 2-ketoacid oxidation can be used to reduce NADP to NADPH for biosynthesis by another FeS-containing enzyme, ferredoxin NADP oxidoreductase (FNOR). Energy is conserved from this catabolic pathway in the form of ATP by a group of

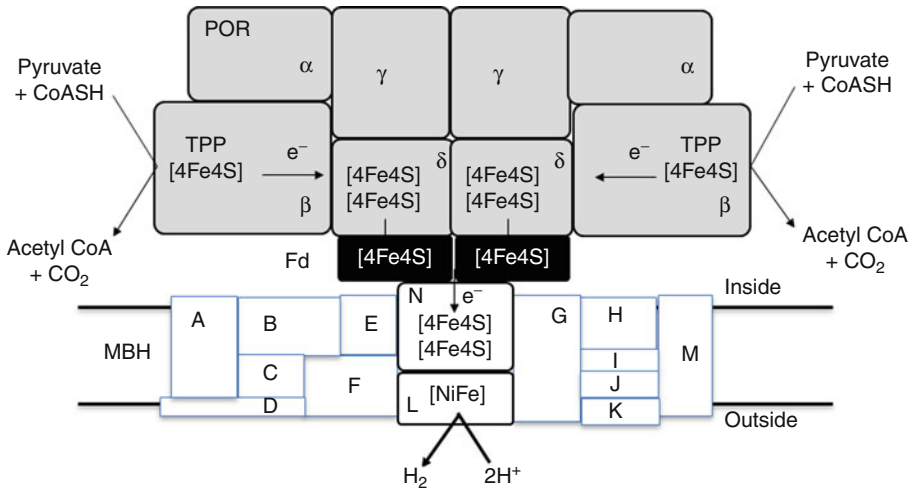

Fig. 4.6.2

Electrons from the oxidation of pyruvate are ultimately disposed of by a membrane-bound hydrogenase in *P. furiosus*. The abbreviations are: CoASH, coenzyme A; Fd, Ferredoxin (black boxes); MBH, membrane-bound hydrogenase (white boxes); POR, pyruvate Fd oxidoreductase (gray boxes); TPP, thiamine pyrophosphate. See text for details

enzymes termed acyl CoA synthetases (ACS: see *Fig. 4.6.3*), but these do not contain transition metal cofactors (Mai and Adams 1996a).

Like many strict anaerobes, *P. furiosus* does not contain any heme-based or cytochrome-type proteins. However, it does contain representatives of another group of proteins that contain iron but lack FeS clusters, the so-called non-heme iron-containing proteins (Neidig et al. 2005; Kurtz 2006; Andreini et al. 2007). Three of these have been purified from *P. furiosus* and they are involved in the response of the organism to oxidative stress; see Chaps. 4.5 Organic Compatible Solutes of Prokaryotes that Thrive in Hot Environments: The Importance of Ionic Compounds for Thermostabilization and 7.1 Microbiology of Volcanic Environments. As shown in *Fig. 4.6.4*, superoxide is reduced by superoxide reductase (SOR), which contains a single Fe atom coordinated by four approximately planar histidinyl nitrogens and an axial cysteine and glutamate (Jenney et al. 1999; Yeh et al. 2000; Dey et al. 2007), while hydrogen peroxide is reduced by rubrerythrin (Rr), which contains a mononuclear Fe center coordinated by four cysteinyl residues and a binuclear Fe-center coordinated primarily by glutamate residues (Weinberg et al. 2004; Kurtz 2006; Riebe et al. 2009). The electron donor for both SOR and Rr is rubredoxin (Rd), a small 54 residue protein containing a single iron atom also coordinated by four cysteinyl sulfurs (Jenney and Adams 2001; Vondrasek et al. 2007). SOR, Rd, and Rr are believed to represent the mechanism by which anaerobic and microaerophilic organisms protect themselves from reactive oxygen species (Jenney et al. 1999; Kurtz 2006; Riebe et al. 2009).

Metal cofactors therefore play critical roles in the primary metabolism and the primary response mechanism of *P. furiosus*. Below, we discuss the approaches that were used to obtain this and related information using this model organism, and briefly survey similar work involving metalloproteins in other hyperthermophiles.

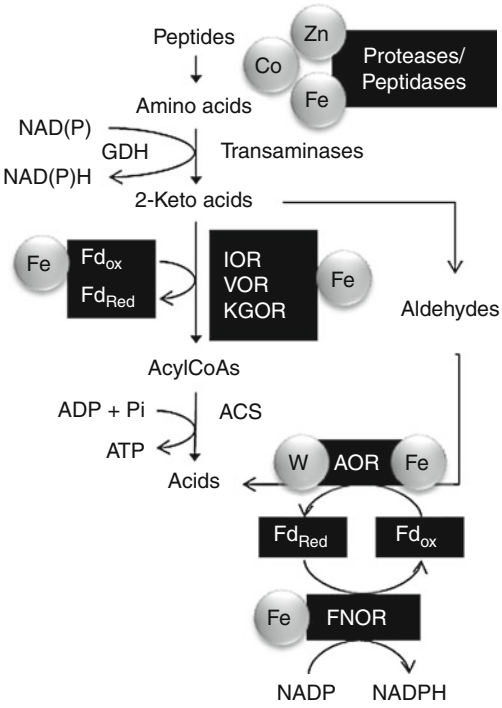

Fig. 4.6.3

The peptidolytic pathway of *P. furiosus*. The abbreviations are: ACS, acetylCoA synthetase; AOR, aldehyde Fd oxidoreductase; Fd_{Ox}, oxidized ferredoxin; Fd_{Red}, reduced ferredoxin; FNOR, Fd:NADP oxidoreductase; GDH, glutamate dehydrogenase; IOR, indolepyruvate Fd oxidoreductase; KGOR, 2-ketoglutarate Fd oxidoreductase; VOR, 2-ketoisovalerate Fd oxidoreductase. See text for details

Culture and Growth Conditions

Anaerobic hyperthermophiles such as *P. furiosus* are relatively simple to cultivate in a high-temperature incubator (small-scale), in fermenters, and even in ingenious home-made devices such as a modified residential water heater (Worthington et al. 2003); see Chap. 4.1. They may be grown either in batch (Blamey et al. 1999; Verhagen et al. 2001; Schut et al. 2003) or continuous culture (Brown and Kelly 1989; Pysz et al. 2001). Such organisms are remarkable in that vegetative cultures can even survive autoclaving in some cases (Stetter 1999), and liquid cultures can be stored routinely at room temperatures for extended periods of time (months) and maintained by regular transfers. For *P. furiosus*, the growth medium is typically artificial seawater supplemented with a carbon source (peptides or maltose) and a low concentration of yeast extract, although some defined media have been reported (Rinker and Kelly 1996; Godfroy et al. 2000). The addition of S^o is also required for significant growth on peptides (Verhagen et al. 2001). *P. furiosus* has been grown up to the 600L scale with cell growth yields as high as ~4 g/L wet weight (Verhagen et al. 2001). The closely related *Thermococcus kodakaraensis* (Atomi et al. 2004) has been extensively cultured on solid media as well using 1% w/v Gelrite as the solidifying agent and polysulfide instead of S^o as the source of elemental sulfur (Sato et al. 2003).

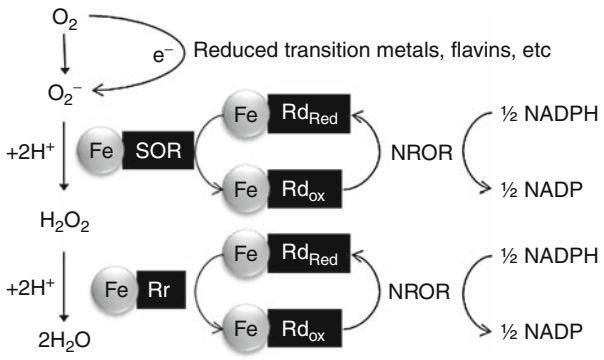

Fig. 4.6.4

Non-heme iron proteins mediate protection against reactive oxygen species in anaerobes such as *P. furiosus*. The abbreviations are: NROR, NADPH rubredoxin oxidoreductase; Rd_{Ox}, oxidized rubredoxin; Rd_{Red}, reduced rubredoxin; Rr, rubrerythrin; SOR, superoxide reductase. See text for details

Metalloprotein Purification

Many metalloproteins, and particularly those from strictly anaerobic organisms such as *P. furiosus*, are sensitive to oxygen. This means they lose significant amounts of activity and/or their metal cofactor(s) is modified or lost from the protein after exposure to O_2 (Mai and Adams 1996b; Ma and Adams 1999; Jenney and Adams 2008; Mettert et al. 2008). Consequently, all metalloproteins from *P. furiosus* are routinely purified under anaerobic conditions, using buffers that are extensively degassed, flushed with Ar, and the oxygen-scavenger sodium dithionite is added (Verhagen et al. 2001; Menon et al. 2009; Yan and Kiley 2009). Some metalloenzymes have been shown to be irreversibly inactivated by dithionite (Mukund and Adams 1995; Iwasaki et al. 2002), and in these cases dithiothreitol is added (although this is a poor oxygen scavenger; Roy and Adams 2002). Column chromatography fractions are collected into sealed serum bottles that have been degassed and flushed with Ar, and fractions can be concentrated using standard positive pressure ultrafiltration systems, or by centrifugation in an anaerobic chamber (Verhagen et al. 2001; Menon et al. 2009; Evans et al. 2009). Samples may be frozen anaerobically by dropping from a syringe into a small plastic scintillation vial suspended in liquid nitrogen, and stored under liquid nitrogen. Oxygen-sensitive metalloenzymes can be stored indefinitely under such conditions without significant losses in catalytic activity (Verhagen et al. 2001; Menon et al. 2009; Yan and Kiley 2009).

Identification and Characterization

The prediction of metalloproteins based on primary sequence data remains very difficult (Castagnetto et al. 2002; Kasampalidis et al. 2007; Shu et al. 2008). There are certain common motifs which can be found in many proteins, such as CXXC (where C = cysteine, X = any amino acid). Such a motif coordinates in part the Fe in Rd and the FeS clusters in small redox proteins like Fd as well as more complex FeS-containing enzymes, but this motif also coordinates other metal ions such as zinc (Blokesch et al. 2004). In general, for any motif of this type,

the number of residues between multiple motifs, the amino acids making up the motifs, and even the number of residues between the metal binding residues within a motif can be highly variable (Kasampalidis et al. 2007; Shu et al. 2008; Frederiksen and Piccirilli 2009). Motifs such as CXXC can thus suggest that a particular protein is a metalloprotein, but this needs to be experimentally verified. Clues can also be provided by structures of even distantly related homologs (Levy et al. 2009), but purification of the protein is required for confirmation.

The traditional techniques of protein purification can be applied to oxygen-sensitive metalloproteins as long as precautions are taken to exclude air. The metalloprotein is monitored typically by a specific enzymatic activity or immunoanalysis (Regnier 1987; Hearn and Anspach 1990; Mai and Adams 1994; Asenjo and Andrews 2009; Linn 2009). Some metalloproteins are conveniently identified simply by their color, which arises from charge-transfer bands between the metal cofactor and the amino acid ligands, such as sulfur to iron (McMillin 1978; Moura et al. 1991; Fu et al. 1992; Clay et al. 2002; Solomon et al. 2006). Iron sulfur center-containing proteins will be brown (by eye), regardless of oxidation state, with a broad absorbance maximum at ~390 nm (Brereton et al. 1998; Kim et al. 2001; Lill 2009). Non-heme, non-FeS proteins may also be colored but only in a given oxidation state. For example, the oxidized forms of Rd (Jenney and Adams 2001) and Rr (Weinberg et al. 2004) are red, while that of SOR is blue (Jenney et al. 1999), but all of these proteins are colorless in the reduced state. Consequently, they cannot be identified in this manner during an anaerobic purification using dithionite-containing buffers. There are colorimetric chemical assays for certain metals that can be used for detecting metalloproteins, including iron (Lovenberg et al. 1963) and zinc (Makino 1999) as well as Ni, Co, Mn, and W (Agnihotri and Mehta 2004; Huang et al. 2009; Goodarzi et al. 2009; Reboucas et al. 2009; Sabel et al. 2010). The spectroscopic methods for determination of metals in biological samples are atomic absorption spectroscopy (AAS) and inductively coupled plasma emission mass spectrometry (ICPMS) (Szpunar 2005; Lobinski et al. 2006; Becker and Jakubowski 2009; Careri et al. 2009). ICPMS is extremely sensitive and can simultaneously measure 50 or more metals (and metalloids) in biological samples (Szpunar 2005; Caruso et al. 2006; Timerbaev 2007; Li et al. 2008; Mounicou et al. 2009). It is of interest to note that such speciation is suggested to be an important environmental factor affecting epigenetic expression of genes in many human diseases (Wrobel and Caruso 2009). There are other techniques for determination of specific metals, including laser ablation from solid materials (typically linked to ICP-MS (Ma et al. 2004; Szpunar 2005; Becker and Jakubowski 2009)), and spectroscopic techniques, such as x-ray absorption spectroscopy (XAS) (George et al. 1998; Strange and Feiters 2008), which have the added advantage of indicating types of the metal ligands. However, these techniques often require relatively large amounts of pure metalloprotein. Protocols are being developed to increase the throughput and the sensitivity of these techniques, thus lowering the sample volume requirements, making these more viable for rapid detection of metals in small samples (Scott et al. 2005; Ascone and Strange 2009). These high-throughput (HTP) techniques are being developed due to the rising interest in elucidation of the cell "metallome."

The Metallome and Metalloproteomics

The complete collection of metals found in a living cell, the so-called metallome (Williams 2001; Szpunar 2004) and its corollary, the metalloproteome (Scott et al. 2005; Thierse et al. 2008) has only been considered relatively recently (Shi and Chance 2008; Thierse et al. 2008;

Andreini et al. 2009b; Manley and Gailer 2009). This interest has been focused mainly on understanding the contents of human plasma (Manley and Gailer 2009; She et al. 2003) and from the perspective of structural genomics projects (Scott et al. 2005; Jenney et al. 2005). The increasing number of HTP protocols for detection of metalloproteins from chemical assays (Hogbom et al. 2005; Atanassova et al. 2008) to attempts at annotation based on structural data (Shi et al. 2005), to those performed by ICPMS (Manley and Gailer 2009), are allowing more complete identification of the metallome and a glimpse of the metalloproteome. These protocols begin from proteomics, separating and identifying the individual proteins of the organism, then attempting to define the metalloprotein classes within the proteome (Kulkarni et al. 2006; Shi and Chance 2008; Gonzalez-Fernandez et al. 2009; Mounicou et al. 2009). These protocols are heavily dependent on ICPMS, and coupled separation/detection techniques are becoming better able to completely isolate and identify metalloproteins (Li et al. 2008; Becker and Jakubowski 2009; Mounicou et al. 2009; Wrobel and Caruso 2009; Manley and Gailer 2009; Gonzalez-Fernandez et al. 2009).

Biophysical Characterization

Approximately 14% of the nonredundant reported structures in the Protein Database contain stoichiometric amounts of one or more transition metal. For example, from the hyperthermophile *P. furiosus*, structures from natively purified metalloproteins include rubredoxin and superoxide reductase (non-heme Fe) (Blake et al. 1992a; Yeh et al. 2000), ferredoxin (4Fe4S cluster) (Nielsen et al. 2004), rubrerythrin (diiron center and rubredoxin-like center) (Tempel et al. 2004), aldehyde ferredoxin oxidoreductase and formaldehyde ferredoxin oxidoreductase (tungsten) (Chan et al. 1995; Hu et al. 1999), and some recombinant proteins such as prolidase (Co, Zn) (Maher et al. 2004a). Frequently, metal cofactors appear in unexpected places, even in homologs of proteins that have been well characterized for many years. A good example from the hyperthermophile field is the RNA polymerase of *Sulfolobus solfataricus*. This was shown to possess an extra domain that contained a [4Fe-4S] cluster. Of unknown function, this domain is not conserved in all archaea and some polymerases contain the domain with one or two FeS centers (Hirata and Murakami 2009). This example demonstrates the need to analyze all novel proteins for the possible presence of a metal cofactor. Proteins containing metal centers that absorb in the visible region of the spectrum and/or are redox active can be characterized by a number of different spectroscopic techniques such as UV/visible and near infrared absorption, magnetic circular dichroism (MCD) (Kirk and Peariso 2003), electron paramagnetic resonance (EPR) (Ubbink et al. 2002), and x-ray absorption spectroscopy (XAS) (Strange and Feiters 2008) to determine the magnetic and electronic properties of the metal center (Staples et al. 1997; Lehnert et al. 2001; Clay et al. 2002; Dey et al. 2007; Solomon et al. 2008). Many *P. furiosus* metalloproteins have been so characterized, for example, AOR (Mukund and Adams 1990), Fd (Brereton et al. 1999), Rd (van Elp et al. 1993) and Ni-substituted Rd (Huang et al. 1993), and SOR (Clay et al. 2002). Rd has been particularly useful for XAS studies where metals such as nickel are substituted for the iron (George et al. 1992).

The reduction potential of a metal center can be determined by any technique that can distinguish one or more redox state, such as visible and EPR spectroscopy (Staples et al. 1997; Lindahl 2002), and cyclic voltammetry (Hill and Hunt 1993; Brereton et al. 1998; Armstrong 2005). *P. furiosus* proteins provide excellent examples of use of these techniques, such as cyclic voltammetry to study ferredoxin (Brereton et al. 1999), and EPR titration to study AOR

(Mukund and Adams 1990). Rubredoxin from *P. furiosus* has also been used extensively to analyze the basis of the extreme thermostability of proteins from hyperthermophiles by circular dichroism (Cavagnero et al. 1998), by NMR (Hiller et al. 1997; Hernandez et al. 2000), and mutagenesis (Eidsness et al. 1997); see Chap. 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles.

Structural analyses of metalloproteins by x-ray crystallography can reveal the presence of a previously unknown metal center, and collection of anomalous scattering data can allow identification of the metal in some cases (Sommerhalter et al. 2005; Lee et al. 2009). Conversely, paramagnetic metal cofactors can be a distinct hindrance in determining structural features using nuclear magnetic resonance (NMR) (Machonkin et al. 2004; Bertini et al. 2008). However, in some cases, metal cofactors can be replaced with non-paramagnetic metals such as zinc or gallium to facilitate NMR studies (Johnson et al. 2001). For example, the NMR structure of *P. furiosus* Fe-containing Rd was determined using the Zn-substituted form (Blake et al. 1992a). NMR can be a very useful tool in determining which ligands in a protein interact with a metal cofactor, particularly loosely bound ones by looking for chemical shifts upon metal interaction (Jensen et al. 2007). Freeze-trapping of reaction intermediates for both x-ray crystallographic (Wilmot and Pearson 2002; Ellis et al. 2008) as well as EPR (Bollinger and Krebs 2006) studies has proven very useful for elucidating enzymatic mechanisms of metalloproteins. The presence of a metal cofactor in otherwise uncharacterized proteins can also be used as a guide to function, and this was utilized to predict the substrate for one of the W-containing AOR family members of *P. furiosus* (Roy and Adams 2002). It is important to note that the AOR from *P. furiosus* was the very first hyperthermophilic enzyme structure determined, as well as the first for a W- or pterin-containing protein (Chan et al. 1995).

Characterization of Metal Centers

More in-depth studies of the electronic structure of metal centers in proteins are a very active field of research. For the simpler centers containing a single metal or a single metal cluster, the redox proteins of *P. furiosus* have been of enormous utility in providing stable, robust samples that in some cases have allowed the pioneering of a particular technique. For example, the single iron atom of Rd can be substituted by a number of other transition metals such as Zn (Blake et al. 1991), Co and Ni (Moura et al. 1991), Cd (Bonomi et al. 2002), Hg (Maher et al. 2004b), and Ga and Ge (LeMaster et al. 2006). The acid-denatured *P. furiosus* protein spontaneously refolds in the presence of many types of metal ions, and even the apoprotein has structure (Zartler et al. 2001; Bonomi et al. 2008). This also allows substitution of ^{57}Fe isotope for Mössbauer spectroscopy (Dunham et al. 1993) as well as for nuclear resonance vibrational spectroscopy (NRVS) (Scheidt et al. 2005) and resonance Raman spectroscopy (Xiao et al. 2005; Tan et al. 2007). The very first crystal structure of a hyperthermophilic protein was that of *P. furiosus* rubredoxin, and it is still being used to pioneer newer techniques such as neutron diffraction (Bau 2004) and to complement NMR techniques (Blake et al. 1992b; Tian et al. 2000), including substitution of the Fe for Cd and Hg (Blake et al. 1994). In general Rd from *P. furiosus* has been heavily studied by a number of structural techniques, x-ray crystallography (Day et al. 1992), NMR (Blake et al. 1992a), and neutron diffraction (Kurihara et al. 2004).

Pyrococcus furiosus Fd has also proved useful due to the presence of a single FeS cluster, which facilitates spectroscopic interpretations, its stability, and the ability to generate

a recombinant form that is indistinguishable from the native form (Kim et al. 2001). Like Rd, the Fd can be chemically denatured, and isotopic substitutions can be made (including ^{36}S for the inorganic sulfur) (Fee et al. 1971; Meyer et al. 1986; Menon et al. 1998). The Fd has another advantage in that, for unknown biological reasons, its [4Fe-4S] cluster is coordinated by only three cysteinyl sulfurs with an aspartate residue replacing the expected fourth cysteine. This greatly facilitates removal of one Fe atom to generate a [3Fe-4Fe] cluster (Conover et al. 1990), and many metals can replace this single iron creating an [M3Fe-4S] cluster including nickel, zinc (Srivastava et al. 1993), thallium, cesium (Fu et al. 1994), cobalt, manganese (Finnegan et al. 1995), cadmium, copper, and chromium (Staples et al. 1997). All four metal atoms in the cluster have also been replaced by gallium to give a [4Ga-4S] center (Johnson et al. 2001). *P. furiosus* Fd has also been intensively investigated by NMR for structure (Busse et al. 1992; Teng et al. 1994) and time-resolved studies of the redox state (Gorst et al. 1995), EPR (Telser et al. 1998a), and electron-nuclear double resonance (ENDOR) (Telser et al. 1998b) spectroscopies for structural analysis of the metal center. Another area of interest is other ligands, which can interact with the [4Fe-4S] center of *P. furiosus* ferredoxin including, for example, cyanide (Telser et al. 1995).

There have also been many studies to investigate the extreme stability of these metalloproteins. For example, *P. furiosus* Rd is estimated to have a melting temperature near 200°C, and it has a half-life of several days at 100°C; thus, even if its stability is drastically reduced by mutagenesis, it is still stable for significant periods at 100°C (Hiller et al. 1997; Zartler et al. 2001); see Chaps. 4.5 Organic Compatible Solutes of Prokaryotes that Thrive in Hot Environments: The Importance of Ionic Compounds for Thermostabilization and 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles. Despite this extreme stability, x-ray crystallography data indicate that the overall structure is very similar to mesophilic homologs; for example, the crystal structures of *P. furiosus* (T_{opt} 100°C for growth) and *Clostridium pasteurianum* (T_{opt} 37°C) Rds are virtually superimposable (Eidsness et al. 1997; Zartler et al. 2001). This is another property of hyperthermophilic metalloproteins, which makes them ideal models for study. Mutagenesis has been used extensively to study the ligands and thermodynamic properties of *P. furiosus* ferredoxin (Calzolai et al. 1997; Brereton et al. 1998; Brereton et al. 1999; Duderstadt et al. 1999a, b; Hasan et al. 2006), as well as for structural studies (Wang et al. 1999; Sham et al. 2002; Nielsen et al. 2004), and this approach has also generated novel types of [3Fe-4S] clusters (Duderstadt et al. 1998).

Recombinant Expression and Purification

For the past decade or so, the goal of structural genomics (SG) initiatives has been to determine the three-dimensional structures of representatives of all protein families through the development of high throughput techniques to clone genes, obtain recombinant proteins and their crystal (and in some cases NMR) structures. A number of these projects have targeted proteins from hyperthermophilic organisms including *P. furiosus* (Mallick et al. 2000; Adams et al. 2003; Lee et al. 2003; Robinson-Rechavi and Godzik 2005; Unsworth et al. 2007; Keller et al. 2009), and have demonstrated that, perhaps surprisingly, their genes can be expressed in mesophilic hosts like *E. coli*, and the resulting proteins fold correctly into functional, thermostable forms. However, it should be noted that there have been few direct comparisons of the native and recombinant forms. In fact, one involving a *P. furiosus* enzyme, the lysine specific aminopeptidase, showed that the recombinant version, while still relatively thermostable, was much less

stable than the native (Story et al. 2005). There is another special consideration for recombinant metalloproteins, however, and that is specificity of the metal cofactor that is inserted into the recombinant protein. In some cases, the recombinant protein may contain the wrong metal (if the native metal is known) or no metal cofactor at all (Eidsness et al. 1992; Jenney and Adams 2001; Jenney et al. 2005). It is believed that most cells do not contain pools of free metal ions and that there are specific storage proteins and chaperones for metal ions to prevent incorrect metal incorporation (Tottey et al. 2005; Pordea et al. 2008; Chen and He 2008). If the recombinant host organism lacks the appropriate chaperones or metal-processing proteins of the organism from which a given gene was obtained, the corresponding heterologously produced recombinant protein may well incorporate the incorrect metal(s).

The tremendous scope of SG projects (Banci and Rosato 2003; Hasnain 2004; Conners et al. 2006; Murillo et al. 2007; Joachimiak 2009; Dessailly et al. 2009) has greatly exceeded the pace of biochemical characterization of purified native proteins (Singh and Mozzarelli 2009; Baran et al. 2009; Griffiths and Wang 2009; Hrmova and Fincher 2009; Steuart 2010). One cannot assume that the metal cofactor used by a specific enzyme in one organism will be the same in other organisms, despite homologous sequences (Nguyen et al. 2007). Of course, in terms of metal cofactors, a commonly used tool for recombinant protein production is the presence of an affinity tag that can be used for protein purification. One of the most widely used is the polyhistidine-tag. This enables immobilized metal affinity chromatography (IMAC) purification, which involves the use of metal ions such as Ni, Co, or Zn (Arnau et al. 2006; Block et al. 2009). However, we were able to show by the purification of several hundred His-tagged recombinant *P. furiosus* proteins that there was no significant nonspecific binding of metals during in vitro manipulation (Jenney et al. 2005; Sugar et al. 2005). These data also indicate that the His-tag does not bind metal in vivo, presumably because of the lack of availability of free metal ions as discussed above.

While a remarkable number of recombinant proteins are functionally folded in the bacterial host *E. coli*, and in some cases thermophilic proteins have been successfully expressed in *E. coli* at higher growth temperatures (Koma et al. 2006), recombinant production of a hyperthermophilic archaeal protein in an archaeal host would be (presumably) more likely to incorporate the correct metal cofactor. Such expression systems have been developed for a thermophilic bacterial system, *Thermus thermophilus* (Cava et al. 2009) (see ❯ Chap. 4.7 Genetics of Thermophiles), and also for several archaeal systems including both mesophiles such as *Methanococcus maripaludis* (Gardner and Whitman 1999) (see ❯ Chap. 3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1) and *Haloferax volcanii* (perhaps the most advanced) (Allers et al. 2010) (see ❯ Chap. 3.2 Diversity of Halophiles), and hyperthermophiles such as *Sulfolobus* spp. (Albers et al. 2006; Wagner et al. 2009), and *T. kodakarensis* (Santangelo et al. 2008; Borges et al. 2010). Cell-free translation systems using extracts from the hyperthermophile *T. kodakarensis* are also being developed (Endoh et al. 2008); see ❯ Chap. 4.8 Genetic Tools and Manipulations of the Hyperthermophilic Heterotrophic Archaeon Thermococcus kodakarensis.

There is significant interest in the biotechnological and industrial use of enzymes from hyperthermophiles (see ❯ Chap. 4.1 History of Discovery of Hyperthermophiles), perhaps the most obvious being DNA polymerase from *P. furiosus* (Pfu polymerase) for polymerase chain reaction amplification of DNA (Lundberg et al. 1991); see ❯ Chap. 4.4 Enzymes Involved in DNA Amplification (e.g. Polymerases) from Thermophiles: Evolution of PCR Enzymes. A large number of enzymes are of potential interest due to their high thermal stability, including some metal-containing proteases (Conners et al. 2006; de Miguel Bouzas et al. 2006;

Unsworth et al. 2007) (see Chap. 4.2 Carbohydrate-Active Enzymes from Hyperthermophiles: Biochemistry and Application); however, the full potential of these enzymes for industrial use has yet to be realized. There is also great interest in the rational design of proteins, particularly metalloproteins (Ueno et al. 2006; Lu et al. 2009), and understanding stability of hyperthermophilic metalloproteins is a key aspect of this design process (Iwasaki et al. 2005; Renugopalakrishnan et al. 2005; Dudev and Lim 2008). There are also applications of hyperthermophilic metalloproteins, for example, in use of *P. furiosus* ferritin for environmental remediation of phosphate (Jacobs et al. 2010); see Chap. 4.1 History of Discovery of Hyperthermophiles. The true potential of hyperthermophilic metalloproteins is only slowly being realized, but the promise is tremendous, in terms of understanding the fundamental roles metals play in protein structure and function in all cells, as well as in terms of engineering and utilization for biotechnological and industrial purposes.

Acknowledgments

Research in the authors' laboratories was supported by the Department of Energy, the National Science Foundation, and the National Institutes of Health.

Cross-References

- 3.2 Diversity of Halophiles
- 4.1 History of Discovery of Hyperthermophiles
- 4.2 Carbohydrate-Active Enzymes from Hyperthermophiles: Biochemistry and Application
- 4.3 Lignocellulose Converting Enzymes from Thermophiles
- 4.4 Enzymes Involved in DNA Amplification (e.g. Polymerases) from Thermophiles: Evolution of PCR Enzymes
- 4.7 Genetics of Thermophiles
- 4.9 Thermophilic Protein Folding Systems
- 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles
- 6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens
- 7.1 Microbiology of Volcanic Environments
- 8.2 Distribution and Ecology of Organic Solvent Tolerant Microorganisms

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4.7 Genetics of Thermophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.7,

Introduction

Increasing numbers of genomic sequence of thermophiles have been reported so far. In the so-called post-genomic era, much effort has been made for uncovering biological meaning hidden in the genomic context, which is a great challenge in computational biology. In addition to the computer-based analysis, a lot of gene products are analyzed in vitro with the recombinant proteins produced in a mesophile such as *Escherichia coli* taking advantage of the available nucleotide sequence information. The recombinant proteins from thermophiles can easily be purified by heat treatment to remove the heat-labile mesophilic proteins, followed by column chromatography if necessary. Many of the crystal structures of proteins revealed to date are derived from thermophiles.

Sometimes, however, it is difficult to predict a gene function within a cell by in silico analysis. Genetic manipulation system for the native thermophile host is one of the valuable methods to overcome the problem. In particular, a gene targeting system, which relies on the genomic integration of a selectable marker, is a powerful tool to investigate in vivo function. Shuttle vectors, which consist of both *E. coli* and thermophile replicons as well as selection markers in each host, are also important tools to express a gene of interest in the native host for several reasons. First, post-translational modification in a heterologous expression system is sometimes different from that produced in the native cell. Second, genetically encoded tagging of a protein is beneficial to determine the localization of a protein in the native host as well as for purification of the protein and in vitro biochemical analysis. Third, some genes cannot be expressed in the functional form with mesophilic systems, especially for proteins composed of multiple different subunits and membrane-embedded proteins. A system for overproduction in a thermophile may be needed for protein preparation. Finally, gene cloning in a thermophile specifically permits directed evolution for thermoadaptation of mesophilic gene products, which is important from a biotechnological point of view.

In this review, we focus on the genetic manipulation systems for *Thermus thermophilus*, *Sulfolobus* species and *Thermococcus kodakaraensis*, which have been established as reliable and widely available systems.

T. thermophilus

The type species of the genus *Thermus* is *T. aquaticus* which was isolated from a hot spring in Yellowstone National Park, USA (Brock and Freeze 1969). The type strain of *T. thermophilus*, strain HB8, was isolated from Mine hot spring, Japan (Oshima and Imahori 1974). *T. thermophilus* is a rod shaped, aerobic, gram-negative, non-spore forming, and extremely thermophilic eubacterium (not an archaeon). The G+C content of the chromosomal DNA is 69%. Strain HB8 can grow in a temperature range of 50–85°C and in neutral pH. Both strain HB8 and HB27 can grow in a synthetic medium (Oshima 1983).

The complete nucleotide sequences of two strains of *T. thermophilus*, HB8 and HB27, have been revealed (Henne et al. 2004; Bruggemann and Chen 2006; Also see http://www.srg.harima.riken.go.jp/h_db/index.html). In the case of strain HB8, the chromosomal DNA consists of 1,849,742 nucleotide base pairs and 1,973 open reading frames were identified. In addition, DNAs from two plasmids carry 14 and 252 structural genes, respectively.

A wealth of gene disruption and shuttle vectors for functional gene expression has been reported for *T. thermophilus*. The genetic manipulation system for *T. themrophilus* is the most sophisticated one among all thermophiles' systems and it serves as a useful reference for other thermophiles. It is not difficult to express the genes of *Thermus* species in *E. coli*. In addition, specially designed plasmid vectors for the expression of high GC genes in *E. coli* were constructed (Ishida and Oshima 2002) and the plasmids are available from a commercial company (http://www.nippongene.com/pages/products/clomod/dna_vec/plead/index.html). In addition, plasmid vectors for the expression of about 1,700 *T. thermophilus* HB8 genes in *E. coli* is also available upon request from Prof. S. Kuramitsu, the Department of Biology, Osaka University, Suita, Osaka, Japan. Chromosomal DNA can be purchased from Takara Bio Inc. (see http://www.takara-bio.co.jp/). Riken will distribute the plasmids for construction of gene knockout mutants of *T. thermophilus* (about 1,000 genes are available to date including functionally unidentified genes) (see http://www.brc.riken.jp/).

Like proteins from other extreme thermophiles and hyperthermophiles, recombinant proteins coded by *T. thermophilus* genome and expressed in *E. coli* can be easily purified by heat treatment at 70–80°C followed by ion-exchange column chromatographic purification, and often easily crystallized. The heat stable enzymes are easy to handle and to determine their basic parameters such as optimal conditions for catalytic function, k_{cat} and K_m. Taking these advantages, *T. thermophilus* is one of the well-characterized model organisms of the structural genomics project (Yokoyama et al. 2000) and "Thermus Whole Cell Project" is going on (visit http://www.thermus.org/e_index.htm). In the whole cell project, 2,059 among total 2,226 genes (including those carried on plasmids) in *T. thermophilus* genome were cloned into *E. coli* expression plasmids, 944 recombinant proteins were purified, and 3D structures of 466 proteins have been solved to date.

Mobile Elements

Conjugation has been also demonstrated (Ramirez-Arcos et al. 1998). The *nar* operon encoding a respiratory nitrate reductase gene cluster could be transferred to an aerobic strain by conjugation, allowing the exconjugant to grow anaerobically.

Many insertion sequences or transposases have been found in *Thermus* species (Utsumi et al. 1995; Ashby and Bergquist 1990; Henne et al. 2004; Bruggemann and Chen 2006). A φIN93 derivative harboring one of the insertion sequences was isolated during the propagation (Matsushita and Yanase, 2009). This IS will be a useful genetic mobile tool.

A phage is a potential vehicle for gene transfer as widely found in mesophiles. Several *Thermus* phages have been described, including the contractile-tailed icosahedral dsDNA ϕYS40, which infects *T. thermophilus* HB8 (Sakaki and Oshima 1975), the filamentous phage PH75 (Pederson et al. 2001), which is a useful model for structural analysis of the phage virion, and TS2126 (Blondal et al. 2005) that infects the thermophilic eubacterium *T. scotoductus*. The whole genomic sequence of ϕYS40 was revealed (Naryshkina et al. 2006) and the transcriptional regulation of the phage was analyzed (Sevostyanova et al. 2007). 115 *Thermus* phages belonged to the *Myovidae, Siphoviridae, Tectiviridae,* and *Inoviridae* families were collectively described (Yu et al. 2006). More recently, a lysogenic phage called φIN93 was isolated and the gene encoding a unique lysozyme was found (Matsushita and Yanase 2008). None of these phages has been developed as a genetic tool.

DNA Uptake

The pioneering findings that *T. thermophilus* has the ability to uptake extracellular free DNA with no sequence specificity (Koyama et al. 1986) in a constitutive fashion (Hidaka et al. 1994) has led to the major source of success for developing reliable and widely available genetic manipulation system for *T. thermophilus*. Kinetic study of DNA uptake revealed that the rate is 40 kb/s and the process is energy dependent (Schwarzenlander and Averhoff 2006). Many of the host strains for transformation are HB27 or its derivatives because of the absence of a cryptic plasmid and higher transformation efficiency than other strains (Koyama et al. 1986) as well as seemingly less modification-restriction system (de Grado et al. 1999).

DNA uptake is mediated by a macromolecular transport machinery comprising of many different proteins related to type IV pili, which is evolutionarily related to type II secretion apparatus. A hypothetical model of the DNA translocator in *T. thermophilus* (Averhoff 2004, 2009) has been presented based on genetic studies (Friedrich et al. 2001, 2002, 2003), bioinformatics, subcellular localization of competence proteins (Rumszauer et al. 2006), and DNA binding and DNA uptake studies (Schwarzenlander et al. 2009). Although the essential feature of DNA translocating system seems to be similar to that of other gram-negative bacteria, several non-conserved proteins including *comZ* (Friedrich et al. 2003) and PilW (Friedrich et al. 2002) were found in *T. thermophilus*.

Selection Markers

For complementation of auxotrophs the *trpB* (Koyama et al. 1990a), the *leuB* (Tamakoshi et al. 1995), and the *pyrE* genes are available (Tamakoshi et al. 1997, 1999). A uracil auxotrophic *pyrE* mutant can be selected in the presence of 5-fluoroorotic acid (5-FOA) (Tamakoshi et al. 1997, 1999). This property allows the selection of sequential disruption of multiple genes by performing the following two steps successively. Step 1 is replacement of a target gene with the *pyrE* gene in a Δ*pyrE* strain, which is selected without uracil. Step 2 is regeneration of the Δ*pyrE*, which is selected with 5-FOA and uracil. The strain MT111 or TTY1 has been used as a starting Δ*pyrE* strain (Tamakoshi et al. 1999).

The *mdh* gene encoding malate dehydrogenase was used as a selection marker (Kayser and Kilbane 2001), but the system seems to be difficult to deal with due to the small-colony phenotype of the *mdh* strains.

Several combinations of antibiotics and the cognate inactivating genes used for *T. thermophilus* genetic manipulation have been reported including streptomycin (Koyama et al. 1986), kanamycin (Matsumura and Aiba 1985; Liao et al. 1986; Hoseki et al. 1999), bleomycin (Brouns et al. 2005) and hygromycin B (Nakamura et al. 2005). Among these markers kanamycin resistance has been most widely used. All of them except for streptomycin are thermostabilized with evolutionarily technique described in Directed Evolution of Thermostability of Proteins section. Thermally stabilized kanamycin and hygromycin B resistance genes can be obtained by contacting with Prof. Seiki Kuramitsu of Osaka University, Department of Biology, 1-1 Yamadaoka, Suita, Osaka 565-0871, Japan, and Prof. Akira Nakamura of Tsukuba University, Graduate School of Life and Environmental Sciences, 1-1 Tennodai, Ibaraki 305-8571, Japan, respectively.

The *rpsL1* allele, which encodes K47R/K57E double mutant S12 ribosomal protein, conferred a streptomycin-dependent phenotype to *T. thermophilus* in the presence of the wild-type

rpsL allele (Blas-Galindo et al. 2007). This dominant character allows positive selection of a transformant in both introduction and deletion of the mutant allele, similar to the principle with the *pyrE* gene marker described above. This strategy was applied to isolation of Δ*narC* mutants (Blas-Galindo et al. 2007).

Replicative Shuttle Vectors

Most of the autonomously replicative plasmid vectors used for *T. thermophilus* are derived from either two cryptic plasmids: one is pTT8 from *T. thermophilus* HB8 (Koyama et al. 1990a, b) and the other is from *Thermus* sp. ATCC27737 (de Grado et al. 1998) or its derivative, pMK18 (de Grado et al. 1999). The latter can transform *Thermus* species more efficiently and uses the minimal replicon. Another cryptic plasmid, pNHK101, from *Thermus* sp. TK10, was used to express the *crtB* gene involved in the carotenoid biosynthesis (Kobayashi et al. 2005). The transformation efficiency was low, but this plasmid could coexist with pTT8 within a cell, which expands the versatility of the *Thermus* genetic manipulation system.

Gene Targeting or Insertional Mutagenesis for Functional Analysis

The early descriptions of the usefulness of insertional mutagenesis in *T. thermophilus* were the construction of the *slpA* (Lasa et al. 1992) and its transcriptional regulators (Fernandez-Herrero et al. 1997) mutant strains, whose genes were insertionally inactivated by kanamycin resistance marker gene.

As a unique system for gene inactivation, the use of an antisense RNA strategy (Moreno et al. 2004) was reported for Mn-catalase by cloning the cat gene in the opposite direction to the genetic marker gene HTK under the strong promoter for the *slpA* gene based on pMK18.

Every gene cannot necessarily be inactivated by insertional mutagenesis if the gene is lethal for growth. It is required that the gene is conditionally inactivated to prove the essentiality. In order to overcome the problem, it is necessary to develop a conditional gene inactivating system. Expression of an essential gene under a strictly regulatable promoter followed by knockout of the native gene seems to be a promising strategy, which has not been demonstrated to date in *T. thermophilus*.

Many gene disruption experiments for functional analysis have been reported so far. A limited number of these are presented below.

The reverse genetic studies of the *speA*, *B*, *D*, and *E* genes (*speC* homolog is missing in *T. thermophilus* genome) suggested a novel metabolic pathway for biosynthesis of polyamines in *T. thermophilus* with N^1-aminopropylagmatine as an intermidate (Ohnuma et al. 2005; Oshima 2007). It was suggested that the new metabolic pathway is distributed in other microorganisms such as a hyperthermophilic archaea *Pyrococcus furiosus* (Cacciapuoti et al. 2007) and *Thermococcus kodakaraensis* (Morimoto et al. 2010).

Disruption of some *lys* genes and *arg* genes revealed that in *T. thermophilus* lysine is synthesized via α-aminoadipic acid pathway, not the diaminopimelic acid commonly found in prokaryotes, and the pathway is functionally and evolutionary related with the arginine biosynthesis (Kosuge and Hoshino 1998; Kobashi et al. 1999; Miyazaki et al. 2001, 2002, 2003; Fujiwara et al. 2006), which is widely distributed in the genus *Thermus* (Kosuge and Hoshino 1999).

Lon protease is known to contribute to protein quality control and the *lon* strain of *E. coli* BL21 is frequently used for overproduction of the heterologous gene product. In the same way, Lon protease-deficient strain of *T. thermophilus* was constructed, which improved production of heterologous proteins from thermophiles and hyperthermophiles although other proteases seem to be responsible for degradation of heterologous proteins (Maehara et al. 2008).

A specific post-transcriptional modification of thermophile tRNA contributes to stabilization of the structure in high temperature environment. The genes responsible for the modification in *T. thermophilus* have been identified by insertional mutagenesis (Shigi et al. 2006a, b, 2008).

Disruption of a gene identified several transcriptional regulators including a cAMP receptor protein as a global transcriptional regulator (Shinkai et al. 2007a), RNA polymerase σ^E (Shinkai et al. 2007b) and its regulator, anti-σ^E (Sakamoto et al. 2008), and the stationary phase-specific regulator (Agari et al. 2008).

Denitrification for an anaerobic respiration process in *T. thermophilus* has been extensively investigated by Berenguer's group using many gene disruptants. It is helpful to refer to a review article (Cava et al. 2009) as well as the latest report (Cava et al. 2008b).

Conditional Expression

Several plasmid constructs with inducible promoters for *T. thermophilus* have been described (Kayser et al. 2001; Park and Kilbane 2004). The most prominent inducibility reported to date is the *nar* promoter (Moreno et al. 2003), in which the transcription was activated by the addition of KNO_3 and the simultaneous arresting of the shaker, leading 200-fold increase of the β-galactosidase as a cytoplasmic reporter and about 20-fold increase of the alkaline phosphatase as a periplasmic reporter. This promoter was used for expression of His-tagged Tth DNA polymerase in *T. thermophilus* (Moreno et al. 2005).

Reporter System for Transcription of a Gene and Localization of a Protein

Although gene chip technology is a powerful method for global transcriptional analysis in *T. thermophilus* (Shinkai et al. 2007a, b), a conventional reporter system is still useful for a specific gene analysis.

The first report about quatifying promoter activity in *T. thermophilus* described the use of the kanamycin resistance marker as a reporter gene, in which randomly sheared DNA fragments were searched for promoter activity (Maseda and Hoshino 1995). The genes encoding a β-galactosidase from *Thermus* strain T2 (Koyama et al. 1990b) and an alkaline phosphatase from *T. thermophilus* HB8 (Castan et al. 2002) were used as cytoplasmic and periplasmic reporters, respectively (Moreno et al. 2003). The β-galactosidase gene from *Thermus* sp. A4 was used for transcriptional regulation analysis of two inducible promoters PdnaK (regulating the DnaK heat shock-inducible protein) and Parg (regulating expression of an arginine-inducible protein) and a carbon-regulated promoter Pscs-mdh (regulating expression of succinyl-CoA and malate dehydrogenase) (Park and Kilbane 2004). Gene transcription level for several genes encoding DNA repair system, including *uvrA*, *uvrB*, *uvrC*, *ruvA*, *ruvB*, *ruvC*, and *recA*, was monitored by β-glycosidase reporter assay with 2-nitrophenyl-β-D-glucopyranoside as a substrate using a δ*bgl* strain, JOS9, as a parental strain (Ohta et al. 2006). The same group

also analyzed temperature dependence of two uracil-DNA glycosylase genes expression (Sakai et al. 2008). The *agaA* gene encoding alpha-glycosidase was used as a reporter to analyze a transcriptional attenuation of lysine biosynthetic gene expression (Tsubouchi et al. 2005) and a repression by arginine (Fujiwara et al. 2006).

A genetic tag for protein localization studies was developed. Superfolder GFP (sGFP), a variant of the green fluorescent protein that folds efficiently, was functional in vivo at 70°C in *T. thermophilus* and permitted the use of the protein as a localization tag in vivo using confocal microscopy using GroES, NarC, and PhoA as model proteins (Cava et al. 2008a).

Tagged Proteins

T. thermophilus is one of the well-characterized model organisms of the structural genomics project (Yokoyama et al. 2000) taking advantage of the protein's thermostability, rendering easy purification of recombinant proteins expressed in *E. coli* and crystallization. The three-dimensional structures of several hundred gene products have been determined to date (http://www.thermus.org/). However, several thermophilic proteins requiring cofactors, appropriate post-translational processing, or specific components during multisubunit assembly cannot be overproduced in an active form in such mesophilic systems. Moreover, membrane proteins are generally difficult to express in a heterologous host. Therefore, a homologous thermophilic host system appears to be appropriate for the expression of such proteins. In addition to proteins, homologous expression system is indispensable for RNA study. A tRNA with a post-transcriptional modifiication for thermostability and its mutants were successfully expressed in *T. thermophilus* (Shigi et al. 2002), identifying the determining residues for the modification.

V_0V_1-ATPase is a proton-translocating ATPase responsible for acidification of eukaryotic intracellular compartments and for ATP synthesis in archaea and some eubacteria including *T. thermophilus*. Because the V_0, the membrane portion of *T. thermophilus* V-ATPase, was not expressed in *E. coli*, an integration vector system of the thermophile was applied to prepare the several recombinant V-ATPases, which have been used for biochemical, biophysical, and structural analysis.

A mutant *atpA* gene encoding N-terminus His_8-tagged A subunit was cloned to construct an integration vector (Yokoyama et al. 2003b), which was used to transform *T. thermophilus* TTY1 (Δ*pyrE*) (Tamakoshi et al. 1999). The transformant selected on the minimal medium plate without uracil expressed the His-tagged A subunit instead of the wild type. The recombinant V-ATPase was purified by Ni-NTA and anion exchange column chromatography from the membrane of the transformant. Typically, over 30 mg of V_0V_1-ATPase was obtained from 200 g of the recombinant cells and the yield was improved later to about 50 mg. The highly purified V-ATPase was sensitive to *N*, *N*′-dicyclohexylcarbodiimide (DCCD), showing that the reconstituted enzyme was fully functional and it revealed each subunit arrangement within the enzyme (Yokoyama et al. 2003a). This recombinant enzyme was subjected to two-dimensional crystallization (Gerle et al. 2006).

Single molecular rotation analysis of the enzyme had to be performed with a mutant V-ATPase with lower sensitivity to Mg-ADP inhibition during ATP hydrolysis. For the analysis another integration vector was constructed (Yokoyama et al. 2003b). Although the additional mutation sites were about 700 bp away from the genetic selection marker gene, the gene replacement between the mutant and the wild-type gene occurred in two out of six

clones randomly selected transformed cells without uracil. The successful introduction of the mutations is probably because the homologous region was long enough to recombine the mutation sites.

In the essentially same way Glu23 of the L subunit was replaced with Cys for biotinylation, which was linked to a streptavidin-coated bead for observation of the rotation assay, with HTK gene as a genetic marker (Yokoyama et al. 2003b). The His_8-tag added to the N termini of the A subunits was used to immobilize the enzyme to the Ni^{2+}-NTA-coated glass surface.

A His_3-tag was introduced to the C-termini of the c subunit for purification of V_0, which separates from V_0V_1 during anion exchange column chromatography, and immobilization to Ni^{2+}-NTA-coating glass surface. Single molecule analysis with the reconstituted enzyme showed a stepwise rotation, pausing every 120°, and ATP synthesis after reconstitution (Nakano et al. 2008).

Another example is overproduction of His-tagged Tth DNA polymerase (Moreno et al. 2005), which was expressed by the *narp* promoter under a semi-anaerobic condition with nitrate. The inducible transcription is a highly regulated. The DNA polymerase was purified by Ni-NTA column chromatography as an active form capable of PCR as the commercial enzyme produced in *E. coli*.

A tagged protein expressed in *T. thermophilus* has contributed to elucidate gene expression control of lysine biosynthesis (Tsubouchi et al. 2005). The His_8-tagged *hcs* leader peptide-α-galactosidase fusion protein whose expression was controlled by the *hcs* promoter was purified, indicating that the *hcs* leader peptide is actually translated in a Shine-Dalgarno sequence-independent manner and functions in attenuation control of the expression of lysine biosynthetic genes.

Directed Evolution of Thermostability of Proteins

Thermophilic hosts are very attractive for selection of thermoadapted proteins originally shown by *Geobacillus stearothermophilus* (Matsumura and Aiba 1985; Liao et al. 1986), in which variants of thermostabilized kanamycin nucleotidyltransferase were selected. Such functional selection methods were applied to a metabolic enzyme in *T. thermophilus*. A chimeric *leuB* gene, which consists of *T. thermophilus* and *Bacillus subtilis leuB* genes encoding 3-isopropylmalate dehydrogenase (IPMDH) in the leucine biosynthetic pathway, was expressed instead of the wild-type *leuB* gene in the thermophile and mutant strains were selected at higher temperatures on the minimal medium without leucine (Tamakoshi et al. 1995; Kotsuka et al. 1996). One of the mutations found within the thermophilic part was introduced into the wild-type thermophilic *leuB* gene, resulting in a creation of a more thermostable enzyme than the wild-type ThIPMDH (Kotsuka et al. 1996), which is a suppressor mutation method. Step-by-step directed thermostabilization of *B. subtilis* IPMDH (Akanuma et al. 1998) and *Saccharomyces cerevisiae* IPMDH (Tamakoshi et al. 2001) were demonstrated; α-glycosidase was thermostabilized with the essentially same strategy (Fridjonsson et al. 2002).

Selection of antibiotic resistance genes encoding mutant thermostabilized enzymes in a thermophile is easier than that of a metabolic enzyme because of no need for disruption of the gene of interest. In *T. thermophilus* a hyperthermostable kanamycin nucleotidyltransferase, HTK, was selected (Hoseki et al. 1999) using the variant thermostabilized in *G. stearothermophilus* as a starting material in combination with DNA shuffling method for an efficient

mutagenesis. This mutant gene HTK has been widely used as a selection marker in *T. thermophilus*. Thermostablized variants of bleomycin-binding protein from *Streptoalloteichus hindustanus* were selected in *T. thermophilus* HB27 with an error-prone PCR library (Brouns et al. 2005), providing bleomycin resistance to *T. thermophilus* transformants up to 77°C. Thermostabilized hygromycin B phosphotransferases were also selected, by which the transormant could be selected up to 65°C (Nakamura et al. 2005).

The activity of a gene product cannot be necessarily linked to the growth of the thermophile. An activity-independent selection system of protein thermostabilization, which is based on a fusion construct comprising the protein of interest and HTK has been developed, in such a way that thermostable mutants provide increased kanamycin resistance (Chautard et al. 2007). The same strategy might be available with sGFP as a fusion partner for directed thermostabilization.

Sulfolobus Species

Sulofolobus species are the model systems belonged to Crenarchaeota in Archaea. The whole genomic sequences of more than ten *Sulfolobus* representative strains have been completed, including *S. solfataricus* (She et al. 2001), *S. acidocaldarius* (Chen et al. 2005) and S. *islandicus* whose genetic manipulation systems are described in this review. Among the genetic tools developed in recent years, the most frequently used system is the one for *S. solfataricus*.

Replicons for Shuttle Vectors

Replicons for the *Sulfolobus-E. coli* replicative shuttle vectors constructed so far fall into three elements: the virus SSV1, the plasmid-virus hybrid pSSVx together with the helper virus SSV2, and the cryptic plasmid pRN1.

A *S. shibatae* (DSM 5389) virus particle, *Sulfolobus* spindle shaped virus 1 (SSV1), which was originally isolated as a 15 kbp plasmid from *S. shibatae* (Schleper et al. 1992), infects the closely related strain *S. solfataricus*. Upon infection, SSV1 DNA integrates site-specifically into the 3′ end of a tRNA gene in the host genome, but it is also detected as a plasmid. This virus-based vector widely used so far is pMJ03 (Jonuscheit et al. 2003) or pMJ05 (Albers et al. 2006), which has both the *pyrEF* and *lacS* genes for stable vector maintenance. The virus-based vector spreads efficiently throughout the culture, thus circumventing the need for an efficient transformation procedure.

pSSVx harbored by *S. islandicus*, which was assigned to the pRN family, is also capable of spreading in the cell cultures of *S. solfataricus* in the presence of SSV1 or SSV2 as helpers (Arnold et al. 1999). The pSSVx-derived shuttle vectors for the *lacS* gene transfer was constructed (Aucelli et al. 2006). A new isolate, pSSVi, from *S. solfataricus*, which resembles the pRN plasmid family, is capable of spreading in the presence of the helper virus SSV1 or SSV2 (Wang et al. 2007).

pRN1, a relatively small size (5.4 kb) cryptic plasmid (Keeling et al. 1996), was used to construct the shuttle vectors with the *pyrEF* genes and the *lacS* gene for complementation of *S.acidocaldarius* and *S. solfataricus* mutanat strains (Berkner et al. 2007). The regions important for replication or maintenance was delineated by the series of the vectors resulted from random insertion into pRN1. These constructs are stable and do not integrate or rearrange. These vectors are introduced in the recipient cells by electroporation (Schleper et al. 1992).

Selection Markers

The *adh* gene encoding alcohol dehydrogenase conferring resistance to butanol and benzyl alcohol in transformed cells was used as a selection marker (Aravalli and Garrett 1997). Hygromycin phosphotransferase conferring resistance to hygromycin B was thermostabilized in *S. solfataricus* by an evolutionary method (Cannio et al. 1998, 2001). Both of the selection methods seem to have problems concerning reproducibility (Berkner and Lipps 2008).

More reliable and widely used selection markers for *Sulfolobus* species are the *pyrEF* genes and the *lacS* gene. The *pyrE* and *pyrF* genes encode orotate phosphoribosyl transferase and orotidine-5′-monophosphate decarboxylase, respectively. Mutants with these genes require uracil for growth and the absence of the genes can be selected with 5-fluoroorotic acid. The genes have been used as selection markers for *S. solfataricus* (Jonuscheit et al. 2003), *S acidocaldarius* (Reilly and Grogan 2001; Kurosawa and Grogan 2005), and *S. islandicus* (Deng et al. 2009). Mutants with the *lacS* gene encoding β-glycosidase are unable to grow on a medium containing only lactose as sole carbon and energy source. This selection has been used to complement the *lacS* strain of *S. solfataricus* (Jonuscheit et al. 2003) and *S. islandicus* (Deng et al. 2009).

Insertional Mutagenesis for Functional Analysis

Until recent years, gene knockout method for *Sulfolobus* species has been restricted to *S. solfataricus*, using the *lacS* gene encoding β-glycosidase which is essential for growth of *S. solfataricus* on lactose minimal medium and therefore can be used as a selectable marker. An optimization of the method for integration of exogenous DNA into *S. solfataricus* was described (Albers and Driessen 2008), showing that the 10-min post-electroporational treatment of the cells in water at 75°C before transfer to liquid medium was critical for selecting gene disruptants.

Gene knockout schemes for *S. islandicus* (Deng et al. 2009) and *S. acidocaldarius* (Wagner et al. 2009) have been described.

The *amyA* gene encoding α-amylase was insertionally inactivated by transformation of a *lacS* mutant strain, in which the native *lacS* gene had been disrupted by spontaneous transposition of IS1217 (Worthington et al. 2003). Growth of the mutant strain in various organic compounds indicated that the α-amylase was essential for growth on starch, glycogen, and pullulan.

The *merA* gene encoding mercuric reductase confers mercury resistance. The gene expression is controlled by a transcriptional regulator, MerR. The disruption mutant of the *merA* gene constructed by the same system as the *amyA* gene exhibited greater sensitivity than the wild-type strain (Schelert et al. 2004). The *merR* disruption mutant whose parental strain, PBL2025, harbors a large chromosomal deletion spanning the *lacS* and the flanking regions exhibited increased resistance to mercuric chloride (Schelert et al. 2004). The *merRA* double mutant and *merR* operator mutants were created (Schelert et al. 2006).

The *flaJ* gene encoding *S. solfataricus* polytopic membrane protein required for biogenesis of the flagellar was disrupted by the *lacS* gene as a selectable marker (Szabo et al. 2007). The mutant strain showed no swimming motility.

Disruption of the genes required for the functional assembly of sugar binding proteins on the cell surface of *S. solfataricus*, termed bindosome, resulted in severe defect of the cells to grow on sugar substrates and to uptake glucose (Zolghadr et al. 2007).

The expression of SSO0120 gene encoding type IV-like pili was induced by UV light treatment on the cell surface of *S. solfataricus*, leading to cell aggregation, which was confirmed by the gene replacement by the *lacS* gene and complementation of the mutant strain with a virus-based shuttle vector based on pMJ05 (Frols et al. 2008).

The gene encoding a leucine-responsive regulatory protein family, Ss-LrpB, was inactivated with the *lacS* gene, demonstrating that the transcriptional regulator acts as an activator of its three neighboring target genes unlike the bacterial Lrp-like regulators (Peeters et al. 2009).

Reporter System

The only reporter gene used to quantify transcriptional level so far in *Sulfolobus* is the *lacS* gene encoding a β-glycosidase, which also shows β-galactosidase activity. The expression of the β-galactosidase gene under the control of heat-inducible promoter increased after heat shock (Jonuscheit et al. 2003; Aucelli et al. 2006). Regulation of the *araS* gene, which encodes for the arabinose binding protein, was monitored by cloning the *lacS* gene behind the upstream region of the *araS* gene, showing the high level expression of the gene only when cells were grown in the presence of arabinose (Lubelska et al. 2006). This inducible promoter was used to express the recombinant tagged proteins as described below.

Heterologous and Homologous Gene Expression

The *adh* gene encoding alcohol dehydrogenase from *G. stearothermophilus* was heterologously expressed in *S. solfataricus* cells by a hybrid transcriptional apparatus consisting of the *S. solfataricus adh* promoter and aspartate aminotransferase terminator (Contursi et al. 2003).

Heterologous and homologous genes encoding a His-tagged, a Strep-tagged, or both tagged proteins were expressed, which were controlled under either the heat-inducible promoter of the α subunit of the chaperonin TF55 or an arabinose-inducible promoter (Albers et al. 2006). The yields ranged from 0.5 to 1 mg/l of culture after affinity chromatography. One of the tagged proteins FlaI protein produced in *S. solfataricus* could only be detected in the membrane fraction in contrast to soluble cytoplasmic fraction expressed in *E. coli*, suggesting a presence of an associate molecule in the *Sulfolobus* membrane fraction, which could not be noticed only by heterologous expression in *E. coli*. The same strategy to produce a His_8 and Strep tagged protein in *S. solfataricus* was applied to ABCE1, a requirement for translation initiation and/or ribosome biosynthesis (Barthelme et al. 2007).

About tenfold increase of a/eIF2-γ production in *S. solfataricus* under control of the *araS* promoter resulted in stabilization of bulk mRNA, showing that the initiation factor in translation has a dual function: one is requirement for binding of initiator tRNA to the ribosome and the other is counteraction against 5′→3′ directional mRNA decay (Hasenohrl et al. 2008).

Thermococcus kodakaraensis KOD1

Thermococcus kodakaraensis KOD1, which was previously reported as *Pyrococcus kodakaraensis* KOD1 (Atomi et al. 2004), belongs to Euryarchaeota in Archaea. The complete nucleotide

sequence of the *T. kodakaraensis* genome was determined and annotated (Fukui et al. 2005). The *Thermococcus* strain has emerged in the recent years as the best-studied model organism, partly thanks to the versatile genetic manipulation system.

Gene Transfer and Homologous Recombination

Originally, the transformation of *T. kodakaraensis* was performed by a high concentration of $CaCl_2$ (Sato et al. 2003). It has been revealed, however, that *T. kodakaraensis* exhibits a natural competence (Sato et al. 2005), that is, it incorporates extracellular DNA without any treatment, which was distinct from that of the closely related archaeon *Pyrococcus abyssi* (Lucas et al. 2002). Whether the added DNA is circular or linear did not seriously affect the transformation efficiency (Sato et al. 2005). The efficiency depends on the length of homologous regions: the maximum efficiency (10^2/μg DNA) and a lower efficiency (10^1/μg DNA) were achieved with more than 1,000 bp and 500 bp, respectively, but 100 bp was too short to promote effective homologous recombination (Sato et al. 2005). This dependency is similar to that found in *T. thermophilus*. For easy serial use of the *pyrF* gene as a genetic marker as described for yeast (Alani et al. 1987) pop-out recombination vectors with tandem repeats flanking the marker genes were constructed (Sato et al. 2005).

Selection Markers

The *pyrF* and the *trpE* genes encoding orotidine-5′-monophosphate decarboxylase and the large subunit of anthranilate synthase, respectively, have been used for complementation of the cognate auxotroph mutants (Sato et al. 2003, 2005). A strain deficient in the *pyrF* gene can be selected in the presence of 5-FOA as described in *T. thermophilus* and *Sulfolobus*.

In addition to complementation of auxotrophic mutants, antibiotic selection system with the use of mevinolin (Santangelo et al. 2008b) or its analog simvastatin (Matsumi et al. 2007), both of which are specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an essential enzyme for the formation of mevalonate in the archaeal membrane lipid biosynthesis, was developed. The *hmg* gene encoding HMB-CoA reductase from *T. kodakaraensis* or *P. furiosus* was used as the selection marker (Santangelo et al. 2008b; Matsumi et al. 2007).

Vectors

Most studies with *T. kodakaraensis* gene manipulation system reported to date use integration vectors for gene disruption, which are composed of *E. coli* vector sequence and the selection marker gene *pyrF* or *trpE* flanked by the sequence of interest. In addition, an autonomously replicative shuttle vector was constructed based on pTN1 (Soler et al. 2007) from *T. nautilus* and an *E. coli* commercial vector, pCR2.1-TOPO. Three copies of the recombinant plasmid were present in a *T. kodakaraensis* cell (Santangelo et al. 2008b). The transformation efficiency was about $1/10^8$ recipient cells when transformed with 1 μg DNA (Santangelo et al. 2008b).

Gene Disruption

Gene disruption system was constructed for the thermophile using uracil-auxotrophic mutants in the *pyrF* gene encoding orotidine-5′-monophosphate decarboxylase with mutations isolated

by positive selection using 5-fluoroorotic acid (Sato et al. 2003). In order to demonstrate the usefulness of the system, the *trpE* gene was disrupted, in which the *pyrF* mutant strains were transformed with the knockout vectors for homologous double-crossover event at the *trpE* locus. The host strains used were KU25 with a single nucleotide deletion by UV mutagenesis (Sato et al. 2003) and then the improved version KU216 with an almost complete deletion of the *pyrF* gene to avoid unintended recombination between the *pyrF* gene and the mutated allele on the chromosome (Sato et al. 2005). With the resultant strain KUWH1 (Δ*pyrF* Δ*trpE*) the *trpE* gene was used as another genetic marker to construct Δ*hisD* strain (Sato et al. 2005).

For gene functional analysis, this system was successfully used to disrupt the genes for gluconeogenesis (Sato et al. 2004), biosynthesis of ribulose monophosphate to build the pentose moiety of nucleotides (Orita et al. 2006), prefoldings (Danno et al. 2008), chaperonins (Fujiwara et al. 2008), a transcriptional regulator controlling for glycolytic and gluconeogenic pathways (Kanai et al. 2007), RNA polymerase subunits (Hirata et al. 2008), glycolytic modified Embden-Meyerhof pathway (Imanaka et al. 2006), Archaeal type III RuBisCOs (Sato et al. 2007), amylopullulanase and sugar transporters (Matsumi et al. 2007), polyamine biosynthesis (Fukuda et al. 2008), and a putative manganese-dependent transcription regulator (Louvel et al. 2009). This system was also used to repair a frameshift in the ferric uptake regulator homolog (Louvel et al. 2009).

Reporter System

A gene reporter system with TK1761 encoding *T. kodakaraensis* beta-glycosidase was constructed (Santangelo et al. 2008a), in which a fragment to be quantified for promoter activity was positioned upstream of the reporter gene on the chromosome. The β-glycosidase activity present in the transformant lysate was determined by monitoring the change in absorbance of a chromogenic substrate, o-ntirophenyl-β-D-glucopyranoside or o-nitrophenyl-β-D-mannopyranoside. This system was applied to reveal nonsense-codon-mediated polarity (Santangelo et al. 2008a). The reporter gene was used to investigate the transcriptional and translational levels of expression of Fe(II) uptake regulation protein with the replicative shuttle vector (Louvel et al. 2009).

Tagged Proteins

A hemagglutinin (HA) epitope-tagged or HA-his_6-tagged RNA polymerase was expressed with the replicative shuttle vector described above that resulted in an eightfold higher abundnace than chromosome expression, and functional RNA polymerase was purified with a Ni^{2+}-charged chelating column chromatography (Santangelo et al. 2008b). Relative amounts of HA-tagged RpoLThe HA whose gene's unite with nucleotide replacements in the ribosome site or with alternative initiation codons were assessed with western blot analysis with anti-HA antibodies (Santangelo et al. 2008b).

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4.8 Genetic Tools and Manipulations of the Hyperthermophilic Heterotrophic Archaeon *Thermococcus kodakarensis*

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.8,

Abstract: The robust and rapid growth of heterotrophic hyperthermophilic *Thermococcales* in laboratory culture has resulted in their extensive study. As experimental systems, they are used to investigate archaeal biochemistry, molecular biology, and hyperthermophily. This has led to much of our current understanding of *Archaea*, the discovery of novel metabolic pathways and biocatalysts, commercially useful heat-stable enzymes, and biotechnology applications. Six genome sequences have been established, but genetic techniques and tools that make novel strain constructions and in vivo probing of gene functions have been developed only for *Thermococcus kodakarensis*. Here we describe the development and current status of *T. kodakarensis* genetics, and provide detailed descriptions of the procedures, strains, and tools available for genetic research with this heterotrophic archaeal hyperthermophile.

Introduction

The *Thermococcales* are anaerobic heterotrophic *Euryarchaea*. They are globally distributed in marine and fresh water environments, and most of the species studied in detail are hyperthermophiles (Garrity and Holt 2002; Lepage et al. 2004; Teske et al. 2009). The genome sequences of six species are publicly available (Kawarabayasi et al. 1998; Robb et al. 2001; Cohen et al. 2003; Fukui et al. 2005; Lee et al. 2008; Zivanovic et al. 2009). They are all relatively small (~2.5 Mbp) and comparisons reveal that although the majority of the ~2,000 genes present are conserved, gene deletions, additions, and genome rearrangements have occurred that presumably provide individual species with ecological growth and survival advantages. In contrast to many hyperthermophiles, the *Thermococcales* grow quickly (doubling times of ~40 min) and to high cell densities (~10^9 cells/ml) in laboratory culture. They are therefore attractive vehicles for experimental research, and studies of *Pyrococcus furiosus* over the past ~25 years have resulted in much of our current understanding of archaeal physiology, molecular biology, and hyperthermophily (Cavicchioli 2007; Garrett and Klenk 2007). More recently investigations have also been focused on additional pyrococcal and thermococcal species, and since the report of transformation of *T. kodakarensis* (Sato et al. 2003), this species has become the subject of much research interest. Without genetics, research with the *Thermococcales* was seriously limited, but with the discovery that *T. kodakarensis* is naturally competent for DNA uptake and chromosomal transformation, this limitation was removed. Here we describe the development and the availability of genetic procedures and tools now for research with *T. kodakarensis*.

The wild-type strain, originally named *Pyrococcus kodakaraensis* KOD1 and then *Thermococcus kodakaraensis* KOD1, was isolated from a shallow marine solfatara near Kodakara Island in Japan (Morikawa et al. 1994; Atomi et al. 2004). The genome sequence of this isolate, now more simply designated *Thermococcus kodakarensis*, was published ~10 years later (Fukui et al. 2005). *T. kodakarensis* is an anaerobic heterotroph that grows optimally at ~85°C on peptides, starch, or chitin. Energy is gained using a modified Embden–Meyerhof pathway with S° as the preferred terminal electron acceptor generating H_2S. In the absence of S°, *T. kodakarensis* produces hydrogen and the use of *T. kodakarensis* as a hyperthermophilic system to produce high value products, include renewable energy, is now an area of intense research interest (Kanai et al. 2005; Murakami et al. 2006). Approximately 90% of the circular *T. kodakarensis* genome (2.03 Mbp; 52% G+C) is coding sequence, with ~2,300 open reading frames (ORF) annotated and ~50% predicted to be within multi-gene operons (Fukui et al. 2005). There is a single origin of DNA replication with an adjacent Cdc6 gene, one 16S-23S

rRNA operon, two unlinked 5S-, and one 7S RNA-encoding gene. The *T. kodakarensis* genome encodes readily identifiable DNA replication, repair and recombination enzymes, two archaeal histones, a 12-subunit DNA-dependent RNA polymerase (RNAP), RNA modification and processing enzymes, translation factors, ribosomal proteins, protein modification, secretion and degradation systems. Similarly, based on sequence homologies, genes encoding many metabolic enzymes have also been identified but there is much still to be discovered; ~50% of the *T. kodakarensis* ORFs encode proteins whose functions cannot be confidently predicted.

In parallel with the development of genetics, several complementary and essential technologies have also been established that facilitate comparative and confirmatory in vitro and in vivo research with *T. kodakarensis*. Very robust in vitro transcription and in vitro translation systems are available (Santangelo et al. 2007; Endoh et al. 2007), and DNA microarrays have been constructed and used to characterize and quantify the expression of all ORFs in *T. kodakarensis* cells grown under a variety of conditions and with specific genetic changes (Kanai et al. 2007, 2010). *T. kodakarensis* enzymes have been generated as recombinant proteins in heterologous hosts, and several characterized in detail (e.g., Ezaki et al. 1999; Rashid et al. 2002; Murakami et al. 2006; Orita et al. 2006; Sato et al. 2007). Strong constitutive archaeal promoters have been identified and so, with *T. kodakarensis* genetics established, *T. kodakarensis* proteins have also now been produced in their native environment (Yokooji et al. 2009), and proteins from other hyperthermophiles can be generated as recombinant proteins in this hyperthermophilic expression system.

Transformation of *T. kodakarensis*

T. kodakarensis grows in laboratory culture as quasi-spherical, motile, planktonic cells, and grows on media with a defined composition facilitating the isolation and use of auxotrophs in genetics. Transformation of *T. kodakarensis* is remarkably simple (Sato et al. 2003, 2004). Incubation of cells with DNA that has sequences homologous to the *T. kodakarensis* chromosome results in DNA uptake and integration into the chromosome. By flanking any gene with chromosomal sequences, the gene will be integrated into the *T. kodakarensis* chromosome by recombination in the homologous flanking sequences. Unlike most transformation protocols, there is no requirement for a specific growth regime, specialized media or cell washing, cell protoplasting, liposome mediation, or electroporation. As *T. kodakarensis* cells separate, rather than remain associated in clusters following cell division, individual clones are readily isolated as separate colonies on gelrite-solidified plates. *T. kodakarensis* cells can be frozen and recover readily facilitating laboratory stock culture collections and strain archiving.

The first *T. kodakarensis* mutants isolated were clones spontaneously resistant to 5-fluoroorotic acid resistant (5-FOA^R). As expected, these strains were also uracil auxotrophs and had mutations in *pyrE* (TK2138) or *pyrF* (TK2276), consistent with the universally conserved role of the *pyrE* and *pyrF* products in pyrimidine metabolism. Incubation of these strains with chromosomal DNA from the parental strain resulted in clones that grew in the absence of uracil and this established the *T. kodakarensis* transformation protocol (Sato et al. 2003). This selection is still used to isolate transformants but may be complicated by the presence of trace amounts of uracil in commercial gelrite preparations. One uracil-independent transformant, designated *T. kodakarensis* KW128 (Table 4.8.1), was generated by transformation with DNA that had a functional copy of *pyrF* inserted within *trpE* (TK0254). The chromosomal *trpE* gene was replaced by the [*trpE::pyrF*] construct. *T. kodakarensis* KW128

Table 4.8.1

Selectable traits, genes, and *T. kodakarensis* strains

Selectable trait[a]	Gene[b]	Transformable strain[c] (Genotype)	Reference
Tryptophan	TK0254	KW128 (Δ*pyrF*; Δ*trpE*::*pyrF*)	Sato et al. (2005)
Uracil	TK2276	KU216 (Δ*pyrF*)[d] KUW1 (Δ*pyrF*, Δ*trpE*)	Sato et al. (2003, 2005)
Agmatine[e]	TK0149	TS559 (Δ*pyrF*; Δ*trpE*::*pyrF*, ΔTK0664[f]; ΔTK0149)	Santangelo et al. (2010)
Arginine	PF0207 + PF0208	All strains[g] with citrulline added	This chapter
Simvastatin[e] Mevinolin[e]	PF1848	All strains	Matsumi et al. (2007) Santangelo et al. (2007)
6-Methylpurine	TK0664	TS517 (Δ*pyrF*; Δ*trpE*::*pyrF*, ΔTK0664)	Santangelo et al. (2010)

[a]Growth in the absence of the listed nutrient, or presence of the inhibitor.
[b]Numerical designation of the *T. kodakarensis* (TK) or *P. furiosus* (PF) gene that confers the selectable trait.
[c]Transformants of this strain can be obtained with this selection.
[d]Deletion or inactivation of *pyrF* confers resistance to 5-fluoro-orotic acid (5-FOA).
[e]Transformants can be selected on nutrient-rich media.
[f] Deletion or inactivation of TK0664 confers resistance to 6-methyl-purine (6-MP).
[g]The wild-type isolate, and all derivatives, naturally require arginine for growth.

cannot synthesize anthranilate synthase and is a non-reverting tryptophan auxotroph (Sato et al. 2005). In many genetic experiments, *T. kodakarensis* KW128 has been transformed with DNA that expresses a functional copy of *trpE* and transformants are selected, with essentially no background of spontaneously growing non-transformants, on a medium that does not contain tryptophan.

To obtain targeted integration of transforming DNA into the *T. kodakarensis* chromosome, a selectable gene must be flanked on both sides by ≥500 bp of *T. kodakarensis* chromosomal DNA. Using standard molecular biology techniques, the flanking DNA molecules are amplified from the target site in the *T. kodakarensis* chromosome, ligated to the selectable gene, and the construct is cloned and replicated within a plasmid in *Escherichia coli*. A plasmid mini-preparation from *E. coli* can then be used to transform *T. kodakarensis* directly; there is no requirement for DNA methylation or plasmid linearization. The flanking regions of chromosomal DNA direct the integration of the selectable gene into the *T. kodakarensis* chromosome. If only a single recombination occurs, the entire plasmid DNA is integrated into the *T. kodakarensis* chromosome but, in practice, two recombination events frequently occur. In this case, the target gene at the site of integration is deleted and replaced by the transforming DNA that confers the selectable trait, e.g., growth in the absence of tryptophan (*Fig. 4.8.1a*). Transformant clones form visible colonies at a frequency of ~1 in 10^7 cells plated (~100 transformants/10^9 cells/μg of transforming DNA) after ~4 days incubation on gelrite-solidified plates at 85°C. Elemental S° in the medium makes the plates initially opaque but,

with colony growth, the S° is solubilized and this clearing facilitates the early detection of colonies. In most experiments, cells from a transformed clone are then used to inoculate an overnight liquid culture from which genomic DNA is isolated and the construction is confirmed by diagnostic PCR, Southern hybridizations, and/or sequencing.

As illustrated in *Fig. 4.8.1*, transformation of *T. kodakarensis* KW128 with selection for tryptophan-independent growth can be used to delete and replace target genes, manipulate expression signals, introduce specific mutations, and add epitope- and purification affinity-tags. These genetic manipulations can be combined, for example, adding a strong promoter to obtain over-expression of a tagged and/or variant version of a *T. kodakarensis* protein or protein complex of interest. They can also be undertaken with other selectable genes and *T. kodakarensis* strains as described below (*Table 4.8.1*).

Genetic Selections for *T. kodakarensis*

Strain construction by transformation of an auxotroph to a prototroph can only be used once, and so cannot be used to construct a strain with several genetic changes. To do so, either genes that confer different selectable traits must be used, or a selection–counterselection procedure developed that can be used repetitively to delete non-essential genes in the same strain. Most of the antibiotics used in bacterial genetics do not inhibit *Archaea*, and so most of the genetic selections developed for *T. kodakarensis* are based on complementation of an auxotrophic requirement. The development of genetics for this hyperthermophile also requires that all growth supplements and inhibitors, and the corresponding enzymes that confer a selectable phenotype, must be stable for several days at 85°C. With these limitation, only one partnership of an antibiotic (simvastatin/mevinolin) and antibiotic-resistance conferring gene [3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase)] has so far been established for *T. kodakarensis* (Matsumi et al. 2007). Each of the selections now available for *T. kodakarensis* is described individually below, and they are summarized with corresponding transformable strains in *Table 4.8.1*. The selective media used to impose these selections are described in publications that document their use (e.g., Sato et al. 2003, 2005; Atomi et al. 2004; Kanai et al. 2007; Yokooji et al. 2009; Santangelo et al. 2010).

Uracil. *T. kodakarensis* mutants spontaneously resistant to 5-fluoro-orotic acid (5-FOA) are uracil auxotrophs and have mutations in *pyrE* or *pyrF* (Sato et al. 2003). Following transformation, growth in defined liquid media lacking uracil can be used to select transformants, but clones must then be selected on plates and screened to confirm the construction and loss of uracil auxotrophy. With gelrite as the solidifying agent there may be some background growth of uracil auxotrophs. If so, additional screening for uracil prototrophy is required to eliminate non-transformants.

Tryptophan. Complementation of tryptophan auxotrophy is a very effective selection for transformants (Sato et al. 2005). The large subunit of anthranilate synthase is encoded by *trpE* and spontaneous growth of strains with *trpE* inactivated [*pyrF*::*trpE*] or deleted (Δ*trpE*) on plates lacking tryptophan is never observed. After transformation with DNA that expresses a functional *trpE*, virtually every *T. kodakarensis* colony that grows on media with all amino acids except tryptophan is a transformant clone. Cross-feeding on plates does not occur. If an unusually low frequency of transformation is encountered, it provides a strong indication that the desired construction is deleterious. In these cases, the few transformants that do grow result from anomalous integrations of *trpE* at non-homologous sites.

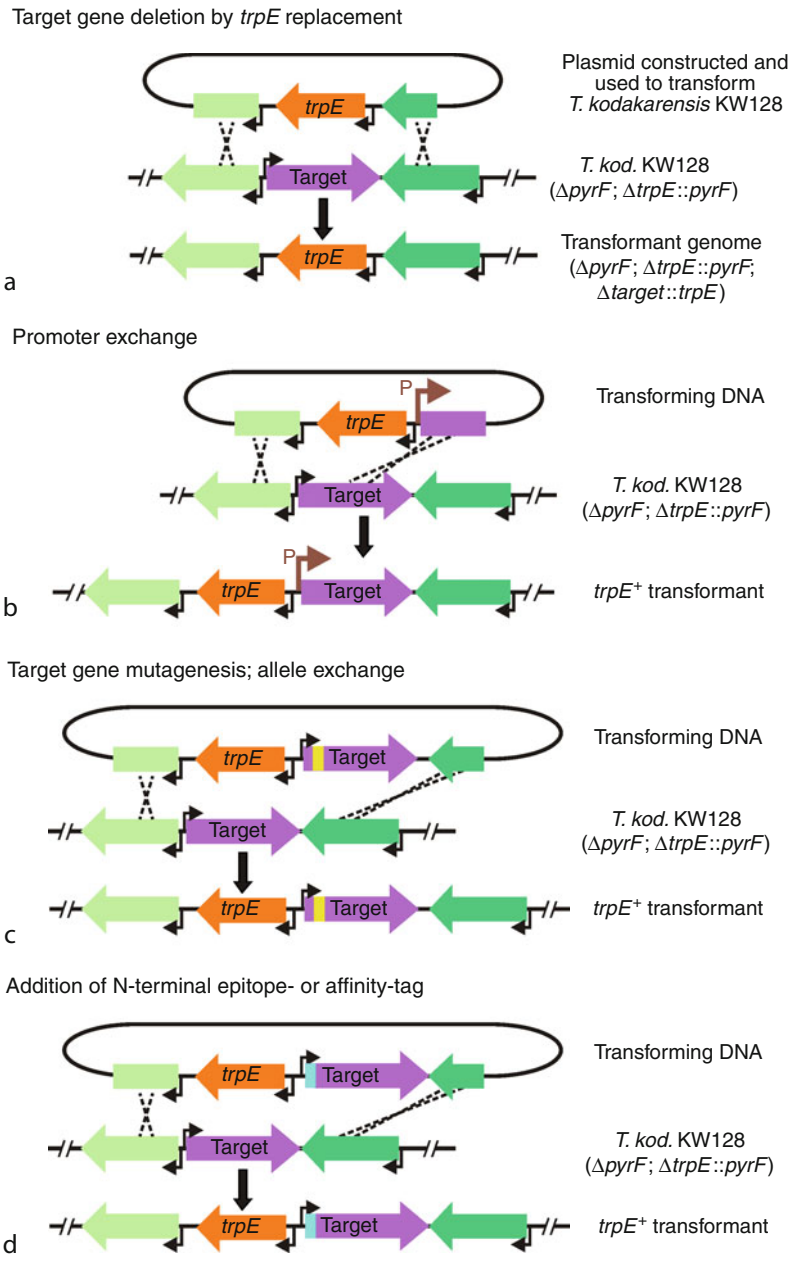

Fig. 4.8.1

Construction of *T. kodakarensis* strains using *trpE* expression to select transformants. Through homologous recombination in the flanking sequences, *trpE* is integrated into the *T. kodakarensis* KW128 chromosome. Transformation can (a) delete, (b) exchange and/or add a regulatory sequence, (c) mutagenize (*yellow bar*), or (d) add an epitope or affinity purification tag (*blue bar*) to the target gene. These procedures are illustrated with *trpE* but can be undertaken and repeated with any gene that confers a selectable trait on a *T. kodakarensis* strain (Table 4.8.1)

Citrulline. *T. kodakarensis* naturally requires arginine for growth (Atomi et al. 2004), whereas closely related *Thermococcales* have complete arginine biosynthetic pathways and grow in the absence of added arginine. In *P. furiosus*, PF0207 and PF0208 encode argininosuccinate synthase and argininosuccinate lyase, respectively, the two enzymes needed to catalyze the synthesis of arginine from citrulline and aspartate. *T. kodakarensis* also does not grow with citrulline added, but does grow when transformed with a constitutively expressed PF0207-PF0208 operon if the medium is supplemented with 5 μg citrulline/ml. *T. kodakarensis* clones spontaneously able to synthesize arginine have never been encountered. Complementation of the natural arginine auxotrophy by transformation with DNA expressing PF0207-PF0208 can be used before or after *trpE* complementation to construct strains with two unlinked chromosomal changes.

Agmatine. Polyamines, spermine, cadaverine, spermidine, and putrescine are essential components of cells in all three domains. In *T. kodakarensis*, a pyruvoyl-dependent arginine decarboxylase (TK0149) converts arginine (obtained from the growth medium) to agmatine which is then used to synthesize putrescine (Fukuda et al. 2008). Deletion of TK0149 results in a strain that can only grow when provided with both arginine and agmatine. The concentration of agmatine in standard nutrient-rich media is too low to support the growth of a *T. kodakarensis* strain with TK0149 inactivated, and therefore transformation with DNA that expresses a functional TK0149 results in transformants that can be selected directly on nutrient-rich plates (Santangelo et al. 2010). Selection on nutrient-rich media results in much faster strain construction. Complementation of a ΔTK0149 mutation by plasmid expression of TK0149 can also be used to maintain the presence of a plasmid in large-volume cultures grown in rich media for enzyme or recombinant protein production.

Mevinolin and Simvastatin. For cholesterol biosynthesis, HMG-CoA reductase is essential and, in common with other HMG-CoA reductases, the *T. kodakarensis* homologue (TK0914) is inhibited by mevinolin and simvastatin. As a result, micromolar concentrations of these statins inhibit the growth of *T. kodakarensis* and spontaneously resistant mutants have increased HMG-CoA reductase levels. Over-expression of HMG-CoA reductase was therefore predicted and confirmed to confer simvastatin- and then mevinolin-resistance on *T. kodakarensis* (Matsumi et al. 2007; Santangelo et al. 2007). To do so, the HMG-CoA reductase encoding gene from *P. furiosus* (PF1848) was cloned and constitutively expressed in *T. kodakarensis*. By using this gene from a related hyperthermophile, recombination of the transforming DNA with the HMG-CoA reductase gene (TK0914) in the *T. kodakarensis* chromosome was much reduced while the inherent heat-resistance of the encoded enzyme was retained. Transformants with the PF1848 expression cassette (= Mev^R) integrated into the *T. kodakarensis* chromosome can be selected in liquid media, or on plates containing up to 20 μM mevinolin or simvastatin per ml. They are then stable and can be propagated in nutrient-rich or minimal media without an added statin. Expression of Mev^R from a plasmid can be used to maintain a plasmid in any *T. kodakarensis* strain grown for any purpose in nutrient-rich media with simvastatin or mevinolin present.

Repeated Selection and Counterselection of Multiple Mutations

Two procedures are available to introduce and then remove a selectable gene. These can therefore be used repetitively to construct *T. kodakarensis* strains with multiple unlinked non-lethal mutations. In both procedures, an auxotrophic *T. kodakarensis* strain is first

transformed with DNA that expresses the complementing wild-type gene flanked by sequences that direct the integration of the complementing gene adjacent to the target gene(s) to be deleted. This generates an intermediate strain in which flanking sequences are duplicated and when a second (counter) selection is applied, clones are isolated in which a spontaneous recombination between the duplicated sequences has deleted the complementing gene and the target gene(s). In the first system (Sato et al. 2005; Fujiwara et al. 2008), the transforming DNA has a functional *pyrF* gene and the intermediate strain is isolated by transformation of a *T. kodakarensis* uracil auxotroph (KU216 or KUW1; *Table 4.8.1*) and selection of clones that grow without added uracil. The counterselection is applied by plating on 5-FOA, and mutants that are spontaneously resistant to 5-FOA have both *pyrF* and the target gene(s) deleted (*Fig. 4.8.2a*). In the second system, the starting strain (TS517; (*Table 4.8.1*) has both *trpE* and TK0664 deleted (Santangelo et al. 2010). This strain is therefore a tryptophan auxotroph, and deletion of the TK0664-encoded hypoxanthine guanine phosphoribosyltransferase results also in resistance to 6-methylpurine (6MP). The DNA used to transform T. kodakarensis TS517 has functional *trpE* and TK0664 genes divergently expressed from constitutive promoters and transformants are selected by growth on plates lacking tryptophan. With TK0664 also expressed, these intermediate strains are sensitive to 6MP. The counterselection is applied by plating on 6MP, and clones isolated as $6MP^R$ have undergone a spontaneous deletion of *trpE*, TK0664, and the target gene(s) (*Fig. 4.8.3a*). By starting with a strain that has both TK0149 and TK0664 deleted (TS559; *Table 4.8.1*), the 6MP counterselection can be combined with agmatine complementation. By using expression of PF0207-PF0208 to complement the natural arginine requirement as the initial selection, the 6MP counterselection can be used to delete genes in any *T. kodakarensis* strain that has TK0664 deleted or inactivated.

When the transforming DNA is a circular plasmid and integration into the *T. kodakarensis* chromosome occurs via a single crossover, the entire plasmid DNA is integrated into the chromosome of the intermediate strain. This can simplify the construction of the DNA used for transformation, but has the disadvantage that the counterselected clones can result from a single crossover at either of two alternative sites, one generates the desired deletion and the second restores the starting chromosome organization (Figs. *4.8.2b* and *4.8.3b*). Screening several counterselected clones will usually identify a strain with the desired deletion. If screening identifies only strains with the starting chromosome, this provides a strong indication that the desired construct is incompatible with laboratory viability.

Shuttle Plasmids that Replicate and Express Genes in Both *T. kodakarensis* and *E. coli*

T. kodakarensis does not naturally have plasmids and although there are chromosomal sequences that suggest virus-like integrations, there are also no viruses known for *T. kodakarensis*. There are sequences in the genome consistent with transposable elements but these have not been further investigated (Fukui et al. 2005). Fortunately, many related thermococcal species have been isolated from high-temperature environments (Lepage et al. 2004; Teske et al. 2009) and screening of natural isolates identified three plasmids in *Thermococcus nautilus* (Soler et al. 2007), a species with growth features very similar to *T. kodakarensis*. Sequencing the smallest of these plasmids (pTN1; 3,619 bp) revealed the presence of two ORFs and sites at which pTN1 could be combined with an *E. coli* plasmid to

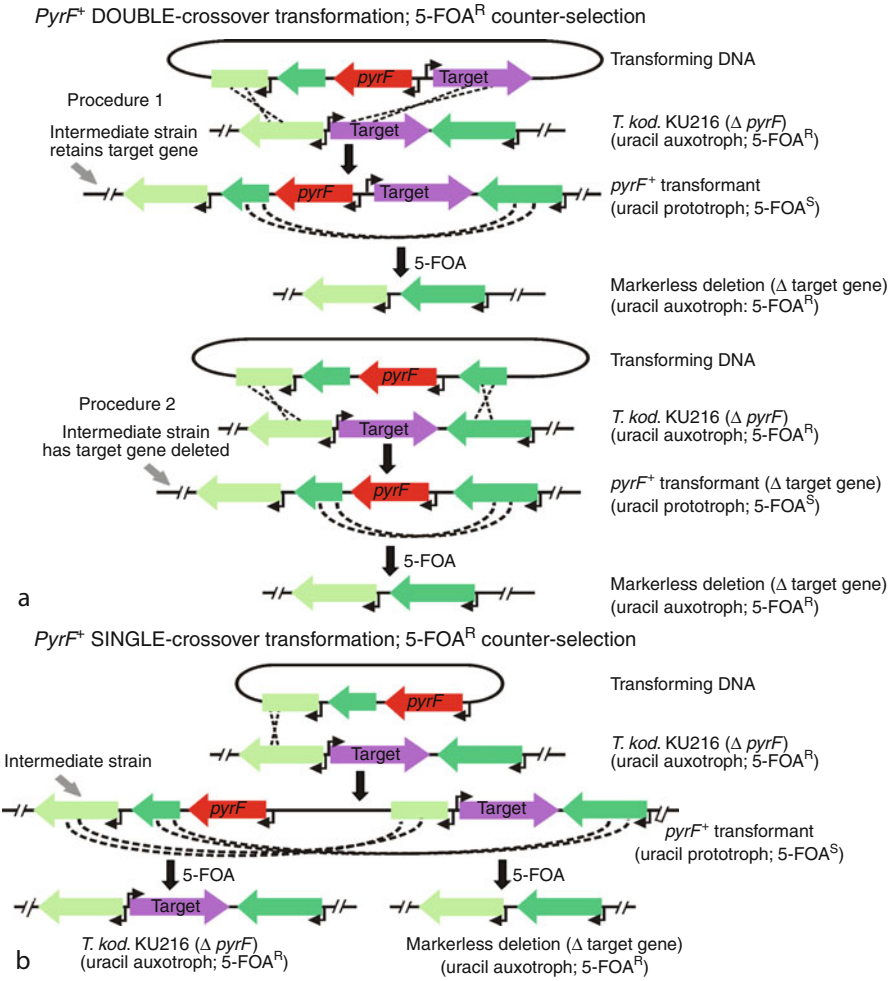

Fig. 4.8.2

Construction of markerless deletions by transformation of *T. kodakarensis* KU216 to uracil-independent growth followed by counterselection of 5-fluoro-orotic acid resistant (5-FOA^R) mutants. (a) Integration of the transforming DNA into the *T. kodakarensis* chromosome via two recombination events, one on either side of *pyrF*. As illustrated, in Procedure 1 the transforming DNA is constructed so that the target gene remains in the intermediate strain and recombination between duplicated flanking sequences then deletes both *pyrF* and the target gene. In Procedure 2, the target gene is deleted with construction of the intermediate strain and the subsequent recombination deletes *pyrF*. In both procedures, the desired strain generated is ΔpyrF and so can be subjected to a second round of selection and counterselection to delete a second and then more target genes. (b) Integration of the transforming DNA by a single crossover. The entire transforming plasmid DNA is integrated into the *T. kodakarensis* chromosome. As illustrated, in spontaneous mutants counterselected as 5-FOA^R, recombination between duplicated flanking sequences can generate either the desired construct or restore the original *T. kodakarensis* KU216 chromosome organization

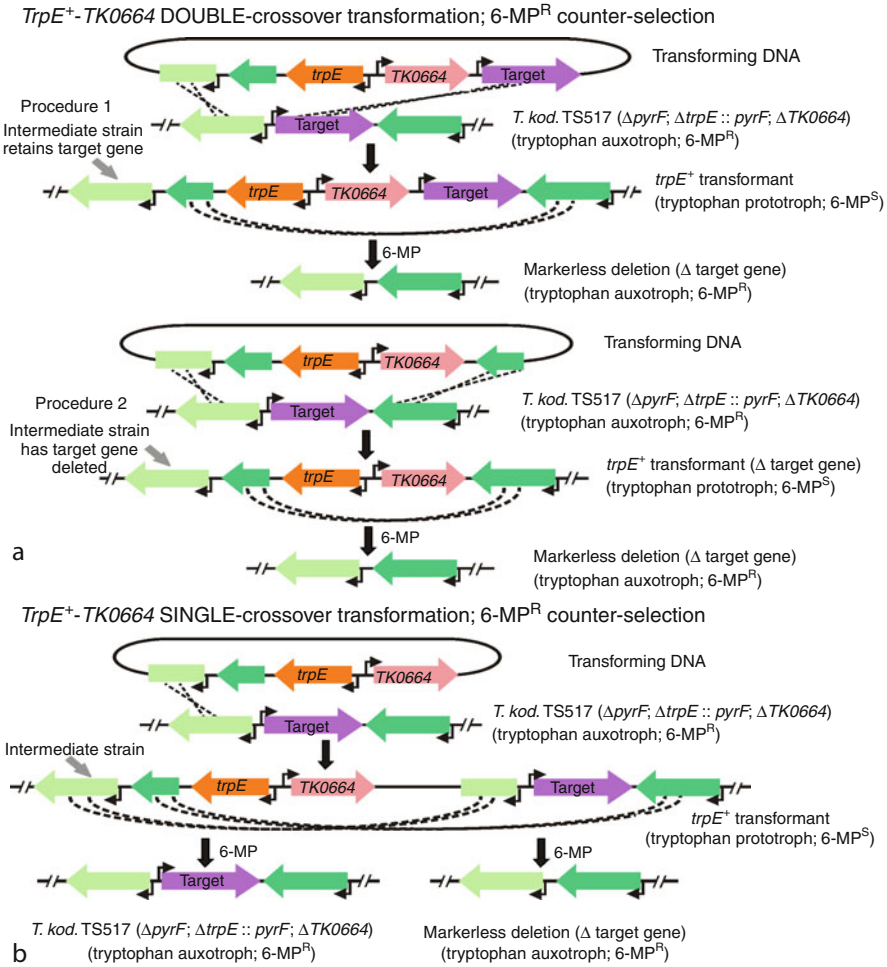

Fig. 4.8.3

Construction of markerless deletions by transformation of *T. kodakarensis* TS517 to tryptophan-independent growth followed by counterselection of 6-methylpurine resistant (6-MP^R) mutants. (a) Integration of the transforming DNA into the *T. kodakarensis* chromosome via two recombination events, one on either side of the *trpE* plus TK0664 expression cassette. As in Fig. 4.8.2a, in Procedure 1 the target gene is present in the intermediate strain, whereas in Procedure 2 the target gene is deleted with construction of the intermediate strain. In Procedure 1, recombination between duplicated flanking sequences deletes the *trpE* plus TK0664 expression cassette and the target gene, and in Procedure 2 deletes the expression cassette. In both procedures, the desired strain generated retains the Δ*trpE::pyrF;* ΔTK0664 mutations and so can be subjected to a second round of selection and counterselection to delete additional genes. (b) Integration of the transforming DNA by a single crossover. The entire transforming plasmid DNA is integrated into the *T. kodakarensis* TS517 chromosome. As illustrated, in spontaneous mutants isolated as 6-MP^R, recombination between duplicated flanking sequences can generate either the desired construct or restore the original *T. kodakarensis* TS517 chromosome organization

construct a shuttle plasmid that would replicate in both *T. kodakarensis* and *E. coli*. As illustrated (Fig. 4.8.4), the entire pTN1 sequence was cloned into the multiple cloning site of pCR2.1-TOPO generating pTK01, and *trpE* and PF1848 (Mev^R) were added to confer selectable traits for transformation into *T. kodakarensis* (Fig. 4.8.4). The resulting shuttle plasmid, pLC70, and derivatives replicate and are stably retained at a copy number of ~3 in *T. kodakarensis*, and any protein or protein variant can be synthesized in the cytoplasm of *T. kodakarensis* by expression from these plasmids (Santangelo et al. 2008b).

Reporter Systems

Research on gene expression ideally combines in vivo and in vitro investigative approaches. For *T. kodakarensis*, RNA hybridizations to genome microarrays can establish the in vivo transcriptome response to a physiological change or a specific mutation (Kanai et al. 2007, 2010), but this is not a technology used routinely to measure individual promoter responses to

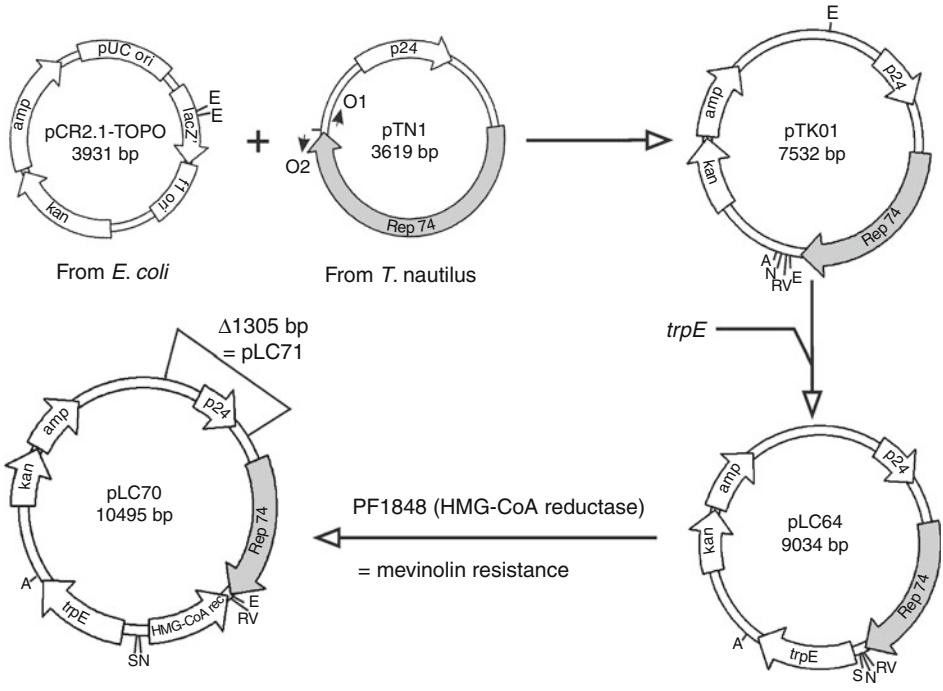

Fig. 4.8.4

Construction of the pLC70 shuttle plasmid. A linearized version of pTN1, generated by PCR using primers designated O1 and O2 that hybridized at the sites identified by the arrowheads, was cloned into pCR2.1-TOPO to produce pTK01. Constitutively expressed *trpE* (TK0254) and PF1848 (Mev^R) were added to provide selectable traits for transformation into *T. kodakarensis*. Transformation of *E. coli* provides resistance to kanamycin (kan) and ampicillin (amp). Restriction sites are identified: EcoRI (E), EcoRV (RV), ApaI (A), NotI (N), and SalI (S). As indicated, deletion of 1,305 bp from pLC70 resulted in a smaller shuttle plasmid, pLC71 (Santangelo et al. 2008b)

imposed changes. This information may be obtained by northern blot analyses or RT-PCR (Fujiwara et al. 2008) but is most easily obtained by reporter gene assays. Two related reporter-gene systems are available for *T. kodakarensis*.

TK1761 encodes a β-glycosidase that hydrolyzes ortho-nitrophenylglucopyranoside (ONP-gluco) and ortho-nitrophenylgalactopyranoside (ONP-galacto) releasing ortho-nitrophenol that can be readily detected and quantified spectrophotometrically in *T. kodakarensis* cell lysates (Ezaki et al. 1999). TK1761 is naturally expressed at a very low level. When expressed at a much higher level from a strong constitutive archaeal promoter, targeted mutagenesis of the promoter resulted in the predicted changes in β-glycosidase levels consistent with TK1761 expression reporting the promoter activity (Santangelo et al. 2008a). TK1761 expression has been used to confirm and quantify the functions of DNA sequences that direct intrinsic transcription termination, ribosome binding of mRNAs, and translation initiation (Santangelo et al. 2008b, 2009). Many *T. kodakarensis* ORFs have GUG or UUG rather than AUG as the translation initiating codon. Replacing the AUG that occurs naturally in TK1761 with GUG had only a marginal effect on TK1761 expression, but with UUG as the translation initiating codon, expression of the reporter was substantially reduced.

Approximately 50% of *T. kodakarensis* genes appear to be located in multi-gene operons and so are transcribed into polycistronic mRNAs (Fukui et al. 2005). Electron microscopy of chromatin spreads revealed that transcription and translation are coupled in *T. kodakarensis* (French et al. 2007), and using the TK1761 reporter system established that premature translation termination stimulated downstream transcription termination (Santangelo et al. 2008a). As in *Bacteria*, this phenomenon of operon polarity is likely exploited naturally in *Archaea* to regulate gene expression. It does, however, raise a concern for strain constructions that delete or replace a gene within an operon. If such a construction disrupts translation of the polycistronic transcript, it will probably result in an anomalous decrease in expression of promoter-distal genes.

The second reporter system also employs a β-glycosidase, the enzyme encoded by TK1827 that hydrolyzes ONP-gluco and ortho-nitrophenylmannopyranoside (ONP-manno) but not ONP-galacto. Expression of TK1827 from its natural promoter is almost undetectable in *T. kodakarensis*, but can be increased substantially by adding a strong promoter and translation signals without any negative effect on growth. TK1761 and TK1827 are not essential genes, and a *T. kodakarensis* strain (TS1079) is available with both genes deleted that provides a clean background for both reporter assays (Santangelo et al. 2010). The TK1761 and TK1827 reporter systems can be used together to assay different regulatory components in the same cell by taking advantage of the specificity of the TK1761 system for ONP-galacto and the TK1827 system for ONP-manno. Both reporter genes can be integrated and expressed from chromosomal sites, or from replicating plasmids. Plasmid expression is apparently increased through gene dosage, and regulation of plasmid expression may not therefore faithfully reflect regulation that would occur naturally at a single chromosomal location.

Addition of Epitope- and Affinity-tags

With the genetic technologies available, any *T. kodakarensis* gene or heterologous can now be expressed at high levels in *T. kodakarensis*. Purification of the encoded protein can be simplified by adding an N- or C-terminal peptide that provides an epitope bound by a commercial antibody [e.g., the nine amino acids that constitute the hemagglutinin epitope (HA-tag)] or

that binds strongly to an affinity matrix [e.g., a hexa-histidine sequence (His_6-tag)] (Fig. 4.8.1d). When the tag is used to isolate the protein directly from a *T. kodakarensis* cell lysate, all proteins that co-assemble with the tagged protein to form a stable complex in vivo are also isolated, and can then be identified by mass spectrometry (Dev et al. 2009). Hints at the functions of proteins may also be obtained by following the expression of a tagged variant. For example, using an anti-HA antibody, western blots will reveal the levels of expression under different growth conditions and at different growth stages, and whether the protein is cytoplasmic, membrane or surface located.

By combining site-specific mutagenesis and gene tagging, proteins can be generated in *T. kodakarensis* with any desired residue change that can also be isolated by tag-affinity (Fig. 4.8.1c, d). If the protein is incorporated into a complex, the complex can similarly be isolated by using the tag and the consequences of the variant protein on the activity of a complex assembled in vivo can be determined. For example, by site-specific mutagenesis and tagging of RpoB, an archaeal RNAP was generated and tag-purified from *T. kodakarensis* that has a conserved peptide loop deleted from the second largest subunit of this 12 subunit enzyme (Hirata et al. 2008a, b). This variant RNAP was found to initiate transcription normally but is defective in transcript elongation (Santangelo and Reeve 2010).

T. kodakarensis as a Hyperthermophilic Archaeal Expression System

Direct purification of proteins from *Archaea* is often limited by their naturally low levels of expression and/or the difficulty of growing sufficient cell mass. Over-expression as recombinant proteins in a surrogate host, most often in a mesophilic bacterium, can also be limited by protein mis-folding and/or the lack of essential metals, co-factors, polypeptide partners, and/or post-translation modifications. Over-expression of archaeal genes in *T. kodakarensis*, especially genes from other hyperthermophilic *Archaea* that are difficult to grow to high cell densities, is now practical and attractive. For functional studies of archaeal membrane proteins, expression in an *Archaeon* may be essential to provide the correct archaeal-lipid environment (Matsuno et al. 2009). Similarly, an archaeal protein may require maturation, for example, metal and/or cofactor incorporation, by machinery present only in an *Archaeon*. Expression of heterologous genes (PF0207, PF0208 and PF1848) has already been used to add genetically useful functions to *T. kodakarensis* and this can certainly also be extended to synthesize novel products and to change metabolic flux for biotechnological applications.

Summary

The genetic technologies available can now be used to precisely delete any non-essential *T. kodakarensis* gene, and to add and modify any cellular function or biochemical pathway. Novel strains can be constructed quickly by standard molecular biology and microbiology procedures using selections imposed on cells growing in nutrient-rich media. With the selection–counterselection procedures established, strains can be constructed with an essentially unlimited number of unlinked deletions. Almost all of the selections and gene manipulation procedures can be combined and focused on one strain to probe pathways and complexes in depth.

There are, however, still some tools needed to complete the *T. kodakarensis* genetic tool box, most notably an equivalent of P_{lac}, a promoter that can be induced and repressed instantly by the simple addition or removal of a gratuitous inducer. The promoter for the gene (TK2164) that encodes fructose-1,6-bisphosphatase is ~15-fold more active in *T. kodakarensis* cells grown under gluconeogenic conditions (with 1% sodium pyruvate) than in cells grown under glycolytic conditions (with 1% maltodextrin), and this can be used to obtain high versus low levels of gene expression (Hirata et al. 2008a) This growth-dependent regulation cannot however be imposed quickly and reversibly to activate or inactivate gene expression as required, for example, to determine the effects of inhibiting the synthesis of an essential gene product in growing cells. It would also be very useful to have expression vectors that add membrane localization and protein secretion signals, an in vivo transposon mutagenesis system, and additional plasmids (Soler et al. 2010) that are compatible with the pLC70 family of shuttle vectors in *T. kodakaraensis* (Santangelo et al. 2008b).

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4.9 Thermophilic Protein Folding Systems

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Introduction

The study of adaptive stress responses was initiated at the molecular level with the observation of dramatic chromosome puffs coupled to the inducible transcription of specific proteins that were observed in Drosophila under heat stress (Ritossa 1962). Since then, stress responses have been observed in virtually all classes of organisms. Although a wide variety of survival strategies are deployed when cells are exposed to environmental challenges, such as heat stress, desiccation, chemical stress, or starvation, usually, the effector proteins are generically referred to as Heat Shock Proteins (HSPs). HSPs are diverse in structure and function, and are usually classified based on their subunit molecular weights. Classes that occur in microorganisms and in the majority of thermophiles include HSP100, HSP90, HSP70, HSP60, and the small HSPs (Trent 1996). Most of these proteins function as molecular chaperones, catalyzing the refolding of denatured proteins and assisting the folding of newly synthesized proteins, or else preventing protein aggregation, and a species will generally possess more than one set of HSPs.

In this review, we focus on the HSPs from thermophiles, which grow at temperatures up to 122°C (Blochl et al. 1997), and include some multi-extremophiles like the thermoacidophiles isolated from solfataric environments at pH 0–1 at moderately high temperatures. *Picrophilus torridus*, which grows optimally at pH 0.8, has an intracellular pH of 4.6 unlike other thermoacidophiles, which maintain neutral pH (ref, Schleper). Therefore, in this case, both extracellular and intracellular proteins must be stabilized against low pH in addition to temperature stress (Futterer et al. 2004). Protein folding is arguably more challenging in thermophiles than it is in mesophiles due to the novel structural adaptations that enhance protein stability at high temperature. While these strategies are to some extent still under active debate, some principles have emerged, including highly charged exterior surfaces, rigid folds maintained by multiple ion-pair networks (Robb and Maeder 1998), tight hydrophobic core packing, and overall more compact and densely packed protein structures achieved that minimize internal voids (Vetriani et al. 1998). High optimal growth temperature is accompanied by compositional changes in amino acid content, with increased content of acidic and basic amino acids (lysine, arginine, glutamate, and aspartate) resulting in increased surface charge and in the formation of buried ion pairs (Vetriani et al. 1998). The formation of ion pairs in the hydrophobic interior milieu of proteins has implications for the energetics of folding at high temperatures. The localization of a charged residue in the hydrophobic interior of a protein uses an additional increment of free energy, the desolvation penalty (Thomas and Elcock 2004). The effect of high temperatures, near boiling, in lowering the dielectric properties of water is to lower the desolvation penalty associated with burying charged residues during folding and thus ion pair networks may be especially suited to stabilizing hyperthermostable proteins. The recombinant expression of thermostable proteins in mesophilic hosts is often accompanied by misfolding and the formation of inclusion bodies, suggesting that thermophile-specific folding pathways might occur. We examine the current literature on heat shock–inducible protein chaperones characterizing the mechanisms that may contribute to the protein stability in thermophiles that determines their survival under extraordinary duress.

Nascent Polypeptide-associated Complex and Trigger Factor

The polypeptides emerging from the ribosome are in random coil conformation and appear in the cytosol in a linear parade from the N- to the C-termini. Folding of these proteins begins

with their recognition by chaperones that bind and restrain the random coil polypeptides until they enter a folding pathway. In the Domain Bacteria, a ubiquitous chaperone named the Trigger Factor carries out this function (ref). In the Archaea, the nascent polypeptide-associated complex (NAC), a homolog of a chaperone that was first isolated etc from bovine brain cytosol and is represented by multiple subunits that were first characterized in yeast (Reimann et al. 1999). Several hypotheses have been advanced for the action of eucaryal NAC complexes and studies are ongoing. It appears likely that NAC proteins prevent inappropriate interaction between newly synthesized polypeptide chains and other cellular factors. NAC functions in archaea may be similar to bacterial trigger factor (TF), since TF homologs are absent from all archaeal genomes. Unlike eucarya, archaea have single NAC homologs per genome and appear to form homodimers, as reported for *Sulfolobus solfataricus* (Spreter et al. 2005). The monomers have two domains, formed by the N- and C-terminal regions of the protein, with the N-terminal domain being homologous to NAC α-subunits in eucarya. A C-terminal ubiquitin-associated (UBA) domain is encoded by all of the archaeal NAC genes (Spreter et al. 2005). Putative ubiquitin homologs occur in several archaeal genomes, but are missing from several others, and consequently, the UBA domain, which is strongly conserved in all archaeal genomes, may have functions unrelated to ubiquitin binding.

Prefoldins

The Prefoldin complex, alternatively termed the Gim Complex (GimC) (Geissler et al. 1998), is universally present in eucarya and archaea, with similar structures, but they are thus far absent in bacteria (Geissler et al. 1998; Vainberg et al. 1998). The prefoldins are "holdase" chaperones whose crystal structure was first resolved from the archaeon, *Methanothermobacter thermoautotrophicum* (Siegert et al. 2000; Stirling et al. 2003). In the archaea, with one exception, prefoldins are hexamers consisting of two α subunits and four β subunits, which act as generalized holding chaperones. The chaperone structure has been likened to a jelly-fish. A globular "body" consisting of complexed hydrophobic domains in a double beta barrel forms an oligomer that supports six canonical, antiparallel coiled coils with their N- and C-domains oriented outwardly from the globular domain. The so-called "tentacles," one per subunit, form a cavity lined with hydrophobic patches to secure nonnative target proteins (Lundin et al. 2004). The archaeal prefoldins bind to a wide range of nonnative proteins in vitro. Although similar in overall structure to archaeal prefoldins, the eucaryal versions consist of six nonidentical subunits (two α-class and four β-class subunits) and in contrast to archaeal prefoldins, they bind specifically and exclusively to the ribosome-nascent forms of actins and tubulins (Leroux et al. 1999). Prefoldin acts as a cochaperonin of group II chaperonins in eukaryotes and archaea by capturing nonnative proteins and delivering them to the group II chaperonin. Here, we review a series of studies on the molecular mechanisms of archaeal PFD function. Particular emphasis is placed on the molecular structures revealed by X-ray crystallography, molecular dynamics simulation, and cryo-EM.

The prefoldin tentacles are capable of flexing outwards to accommodate both small (14kDa, lysozyme) and large (62 kDa, firefly luciferase) proteins in the cavity formed by the "tentacles" to prevent their aggregation (Leroux et al. 1999; Lundin et al. 2004). A recent electron microscopic study showed that the number of prefoldin coiled coils involved in the interaction with the unfolded substrates increases with the size of the denatured protein (Martin-Benito et al. 2007). The prefoldin from the hyperthermophile *Pyrococcus horikoshii* OT3 prefoldin captures

unfolded protein substrates through a hydrophobic groove at the distal region of each tentacle (Ohtaki et al. 2008). This mechanism is different from that of eukaryotic prefoldin, which encapsulates the substrates inside the cavity (Martin-Benito et al. 2007). The coiled coils in the groove are highly flexible and widen the central cavity upon capturing nonnative proteins. The beta subunits initiate substrate binding, whereas the alpha subunit determines the width and length of the central cavity (Ohtaki et al. 2008). The holding-and-release mechanism of the archaeal prefoldins has recently been elucidated (Iizuka et al. 2008; Kida et al. 2008; Ohtaki et al. 2008; Okochi et al. 2005; Shomura et al. 2004; Zako et al. 2005; Zako et al. 2006). The transfer of nonnative substrates to chaperonins has been well characterized using surface plasmon resonance and takes place between the prefoldins and chaperonins from one species of *Pyrococcus*, but not when chaperones from different *Pyrococcus* species are used (Okochi et al. 2005; Zako et al. 2005; Zako et al. 2006). Recent studies on the *Thermococcus KS1* prefoldin indicated that prefoldin complexes (Iizuka et al. 2008) transfer substrate to Class II chaperonins via a direct interaction, as visualized elegantly for the first time by electron microscopy studies of *Pyrococcus horikoshiii* (Ph) chaperones (Martin-Benito et al. 2007). A major difference between archaeal and eukaryal prefoldins is that unlike the eukaryotic prefoldin, which can bind one or both rings of CCT simultaneously, archaeal prefoldins form an asymmetric complex with one cavity of the chaperonin at a time (Martin-Benito et al. 2007).

The hyperthermophilic methanogen, *Methanocaldococcus jannaschii,* encodes genes for the α and β subunits of prefoldin. However, a unique third prefoldin subunit is encoded by the *pfd* gene, and is heat shock regulated, unlike the α and β subunits (Boonyaratanakornkit et al. 2005; Horwitz 1992; Jacob et al. 1993; Muchowski et al. 1999). The α and β subunits of prefoldin assemble readily in vitro into standard hexameric complexes, whereas the γ subunit did not coassemble the other subunits. Instead, polydisperse prefoldin assemblies appearing as long filaments could be seen by electron microscopy with negative staining. The γ subunit is not a suitable candidate for a hypothetical filamentous cytoskeleton structural elements as has been proposed for some thermophilic HSP60 filaments (Trent et al. 1997) because the prefoldin is relatively flexible (Whitehead et al. 2007). The chains performed efficiently as molecular holdase chaperones (Whitehead et al. 2007). This system opens new questions regarding the functional assignments of the heat shock–inducible γ-prefoldin and the sHSPs, since they have overlapping chaperone activities in vitro and they are coregulated by heat stress in vivo.

Small Heat Shock Proteins

The smallest class of holdase chaperones are homologs of the vertebrate a-B crystalline eye lens protein and are encoded by all archaeal genome sequences including *Nanoarchaeum equitans* and practically all bacterial genomes released to date. In the Bacteria, the sHSP homologs are frequently referred to as an abbreviation of Inclusion Body Protein (IBP). This nom-de-plume was earned in *E. coli* where the sHSPs are entrained during formation of insoluble aggregates of recombinant proteins. The sHSPs and α-crystallins have a monomeric molecular weight range of 15–40 kDa, and form polydisperse multimeric complexes in vivo. However, in the archaea, biochemical characterization is limited to thermophilic and hyperthermophilic organisms, with the exception of a recent study on the sHSP of the psychrophilic methanogen, *Methanococcoides burtonii* (Laksanalamai et al. 2009).

The prototype crystal structure of a sHSP was from the laboratory of Sung-Ho Kim at the University of California, Berkeley (Kim et al. 2003). This critical breakthrough involved the

hyperthermophile *Methanococcus jannaschii* sHSP 16.5. The crystal structure revealed a hollow sphere composed of 24 identical subunits. Newly purified recombinant Mj sHSP16.5 was actually heterogeneous (Cao et al. 2008; Kim et al. 2003); however, preheating could activate Mj sHsp, forming a more compact homogeneous species (Cao et al. 2008). Although the amino acid sequence similarity of these sHSP proteins is low and their quaternary structures are dissimilar, the monomeric folds of the dimeric building blocks of the complexes are almost identical. Many sHSPs share a common central building block structure, but the variation in their mechanisms of action may be defined by their individual quaternary structures and specific interactions with other chaperones. The sequences of the N- and C-terminal domains of archaeal sHSPs are especially divergent, and this variability is responsible for the diversity of multisubunit structures observed. The N-terminal domain of the *M. jannaschii* sHSP16.5 is essential for chaperone functions; however, it is disordered in the crystal structure. Low resolution features have been resolved by cryoelectron microscopy (Kim et al. 2003). While most sHSPs from extremophiles are "holdases" that protect denatured proteins from aggregation under very severe conditions, in some cases, they are able to refold denatured proteins (Kim et al. 1998; Laksanalamai et al. 2001; Roy et al. 1999; Usui et al. 2001). Electron microscopy showed that sHSP forms two distinct types of octahedral oligomers of slightly different sizes, indicating structural flexibility in the process of oligomeric assembly. Under conditions of heat stress, the distribution of the structurally different sHSP assemblies changed, and this change was correlated with increased chaperone activity. Temperature-induced conformational regulation of the activity of sHsps may be a general phenomenon in thermophilic archaea (Haslbeck et al. 2008).

The copy number of sHSP encoding genes is variable among archaeal species. The thermophilic and hyperthermophilic archaea contain one, two, or three *shsp* homologs. Hyperthermophilic species growing optimally near 100°C have one *shsp* gene with the exception of *Pyrobaculum aerophilum* which has two homologs (Laksanalamai et al. 2001). *Thermoplasma acidophilum* and all the *Sulfolobus spp* represented by genome sequences each have three *shsp* homologs. However, one of the sHSPs in *T. acidophilum* appears to have domains that are similar to the two ATPase domains of ArsA from *E. coli* (Ruepp et al. 2001). *Sulfolobus solfataricus* and *S. tokodaii* have one 14–15 kDa and two 20–21 kDa sHSPs each. The mesophilic methanogens, *Methanosarcina acetivorans* and *Methanosarcina mazei* GoE1 contain three and four *shsp* homologs, respectively. However, one of the two sHSPs from *M. acetivorans* (NP_619401) does not appear to belong to the α-crystallin type HSPs. The genome sequence of *Halobacterium* NRC-1 has the highest paralogy, encoding five sHSPs that clearly belong to the α-crystallin family. It seems likely that the multiple sHSPs encoded in a single species perform a range of potentially overlapping cellular functions; however, this has not been experimentally assessed.

The sHSPs can maintain solubility of nonnative proteins under physiological conditions indefinitely, for example, in the eye lens, displaying a remarkable capacity for securing the solubility of nonnative target proteins present in greater concentration than the chaperones. The binding capacity of eucaryal α,β crystallins for nonnative proteins is greatly stimulated by serine phosphorylation of the sHSP, and the dynamic reordering of sHSP complexes is required for solubilization of nonnative proteins (Shashidharamurthy et al. 2005). Although recent studies on archaeon *Halobacterium salinarum* revealed the existence/conservation of Ser/Thr/Tyr phosphorylation sites on many chaperones (Aivaliotis et al. 2009), direct experimental proof of sHSP phophorylation is still lacking.

Recently, reconstitution of a protein refolding pathway in vitro was described (Laksanalamai et al. 2009; Laksanalamai et al. 2006; Luo et al. 2009). Denatured taq polymerase

was reactivated cooperatively at 100°C by a mixture of sHSP or HSP60 from *P. furiosus*, in an ATP-dependent folding pathway. The cooperativity between sHSP and CPN was further confirmed by Surface Plasmon Resonance technology (Laksanalamai et al. 2009; Luo et al. 2009). However, sHSP cooperates with CPN by a different mechanism than that of Cpn and prefoldin. In the presence of ATP, CPN offloaded denatured substrate captured by sHSP without direct contact with sHSP (Luo et al. 2009). This experiment shows that proteins that became denatured due to heat shock or other stresses avoid aggregation by binding at saturating levels to sHSP. The sHsp releases folding intermediates such as molten globules that are subsequently bound to Cpn for subsequent maturation in the ATP-dependent cycle. Lower levels of denatured protein, relative to the sHSP concentration, are restrained as bound denatured proteins and released from sHSP to CPN.

Recently, a sHSP from *Methanococcoides burtonii* was found to function at temperatures far above the optimum growth of 24°C (Laksanalamai et al. 2009). This is the first characterized psychrophile sHSP and the study of its interaction with the HSP60 chaperonin from the same strain has revealed a cooperative folding process. Unfolded lysozyme substrate could be bound tightly to immobilize sHSP in the Biacore instrument. Significantly, the substrate could be released by the addition of HSP60 to the microfluidic flow and release was greatly accelerated by ATP (Laksanalamai et al. 2009).

Group I Chaperonins (GroEL)

The chaperonins are multisubunit complexes with two back-to-back rings. Each ring complex encloses a cavity that can alternate between an open or closed conformation and accommodate nonnative protein substrate undergoing folding. The double-ring complex enhances protein folding or refolding reactions by transiently confining the protein substrates in their cavities and then releasing them in highly cooperative conformational changes powered by hydrolysis of ATP (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Chaperonins are divided into two groups based on whether they need cochaperones to fullfill chaperone functions: group I Chaperonins, represented by GroEL, in all bacteria, mitochondria, and chloroplasts, and group II Chaperonins, occurring in eukaryotes and archaea (Hartl and Hayer-Hartl 2002; Sigler and Horwich 1995). The two groups share similar quaternary structures consisting of a double toroid cylinder assembled into two rings stacked back to back. This arrangement generates two internal cavities that open and shut in an alternating cycle. Group I Chaperonins are composed of 14 identical subunits arranged in two heptameric rings and require a cochaperone, GroES, to facilitate protein folding. Group II Chaperonins form double rings with an eight- or ninefold rotary symmetry. Group II chaperonins utilize a built-in lid instead of GroES to perform the folding functions (See discussion in Part II). GroEL and GroES are both required for folding proteins when the spontaneous folding reactions do not proceed rapidly, whether under stress or normal growth conditions. The detailed mechanism of the binding or encapsulation of protein substrate by GroEL and GroES, which is critical to the folding of nonnative protein substrate, is subject to ongoing investigation and details remain unclear. The putative model is that an open ring of GroEL captures nonnative protein substrates through binding to three to four of the seven hydrophobic sites on an open ring of GroEL (Farr et al. 2000). Binding of ATP and GroES to a GroEL ring triggers global, cooperative conformational changes in each subunit within the ring that both sequesters the binding sites and creates an expanded, closed cavity. The internal surface of the closed cavity exposes hydrophilic residues, thus trapping the substrate

polypeptide in a hydrophilic chamber that encourages protein folding (Lin and Rye 2006). The key features of the interaction between nonnative protein substrates and GroEL are isolation (prevention of aggregation), unfolding (passive, possibly active to release kinetically trapped states), and confinement (acceleration of folding inside the GroEL–GroES cavity due to restriction of conformational space) (Brinker et al. 2001). Several recent studies have been performed to understand the interaction between nonnative protein substrates and GroEL (Elad et al. 2007). A cryo EM structure of GroEL–MDH complexes revealed that the distorted nonnative substrate was seen in several distinct states, preferentially binding deep inside the cavity, leaving space for GroES to bind without direct competition. The images of GroEL with bound substrate show distortions in the GroEL ring, so that the apical domains are displaced from their sevenfold symmetric positions. This distortion is transmitted to the opposite ring and may be an important mechanism in negative cooperativity, which maintains the alternation of cycles between the two rings (Elad et al. 2007).

Studies on the folding and assembly of Ribulose bisphosphate carboxylase oxygenase (Rubisco, Rbc) provided the initial breakthrough in understanding the role of GroEL (Goloubinoff et al. 1989). Rbc subunit folding by the GroEL/GroES chaperonin is tightly coupled with assembly mediated by the chaperone RbcX(2). RbcL monomers remain partially unstable and retain high affinity for GroEL until captured by RbcX(2). As revealed by the structure of a RbcL(8)-(RbcX(2))(8) assembly intermediate, RbcX(2) acts as a molecular staple in stabilizing the RbcL subunits as dimers and facilitates RbcL(8) core assembly. Finally, addition of RbcS results in RbcX(2) release and holoenzyme formation. Specific assembly chaperones may be required more generally in the formation of complex oligomeric structures when folding is closely coupled to assembly (Liu et al. 2010). RbcX dimers provide a binding site for a surface-exposed C-terminal peptide of Rubisco large subunit, preventing aggregation during the assembly process.

Archaeal HSP60s/CPNs

The archaeal group II chaperonins form toroidal double rings with an eight- or ninefold symmetry, consisting of homologous subunits (Gutsche et al. 1999). The archaeal chaperonins are composed of up to five sequence-related subunits. *Sulfolobus* species (Archibald et al. 1999; Kagawa et al. 2003), *Haloferax volcanii* (Large et al. 2002), *Methanosarcina mazei* (Klunker et al. 2003), and *Methanococcoides burtonii* (Cavicchioli et al. 2000) contain three chaperonin genes. *Table 4.9.1* lists the number of subunits per genome and subunit composition of chaperonins from characterized members of the archaea. Recently, it was found that there are five chaperonin subunits (Hsp60-1, -2, -3, -4 and -5) in *Methanosarcina acetivorans*. Among them, Hsp60-1, Hsp60-2, and Hsp60-3 have orthologs in *Methanosarcinacea*, but others, Hsp60-4 and Hsp60-5, occur only in *M. acetivorans*. The HSP60-4 and Hsp60-5 paralogs may represent the third class of chaperonin that may be ancestral to two widely distributed group I and II orthologs (Maeder et al. 1999).

The subunit composition of the chaperonin complexes in several archaea changes with growth temperature (Izumi et al. 2001; Yoshida et al. 2001; Kagawa et al. 2003). The chaperonin from the hyperthermophilic archaeon, *Thermococcus* sp. strain KS-1 (*T.* KS-1) is composed of two highly sequence-related subunits, α and β (Yoshida et al. 1997), that form a hetero-oligomer with variable subunit composition in vivo (Yoshida et al. 2001). Expression of α and β subunits is regulated differently, and only the β subunit is thermally inducible

Table 4.9.1

Structural and functional characteristics of archaeal group II chaperonins

Organism	Subunit species	Native or Recombinant	Rotational symmetry	ATPase activity	Arrest activity[a]	Folding activity	Reference
Crenarchaeota							
Sulfolobus shibatae	3	Native	9	Trace	+	NR	(Kagawa et al. 2003; Trent et al. 1991)
Sulfobus solfataricus	3	Native	9	Trace	+	+	(Archibald et al. 1999), (Guagliardi et al. 1994; Guagliardi et al. 1995; Knapp et al. 1994; Marco et al. 1994)
Sulfolobus tokodaii	3	Native	NR	Trace	+	–	(Archibald et al. 1999; Nakamura et al. 1997)
		Recombinant (α, β)[b]	NR	Trace	+	–	
Pyrodictium brockii	NR	Native	8	NR	NR	NR	(Phipps et al. 1991)
Pyrodictium occultum	2	Native	8	+	NR	NR	(Phipps et al. 1991), (Minuth et al. 1998)
		Recombinant (α, β)[f]	8	+	+	NR	
		Recombinant (α+β)[c]	8	+	+	NR	
Euryarchaeota							
Archaeoglobus fulgidus	2	Native	8	NR	NR	NR	(Emmerhoff et al. 1998; Phipps et al. 1991)
Haloferax volcanii	3	Native	NR	+	NR	NR	(Large et al. 2002)
Methanococcus jannaschii	1	Native	NR	+	+	–[d]	(Kowalski et al. 1998)
Methanococcus thermolithotrophicus	1	Recombinant[e]	8	+	+	+	(Furutani et al. 1998)
Methanococcus maripaludis	1	Recombinant[g]	NR	+	+	+	(Kusmierczyk and Martin 2003)
Methanopyrus kandleri	1	Native	8	+	NR	NR	(Andra et al. 1996; Benaroudj et al. 2003)
		Recombinant	8	+	+	NR	

Pyrococcus horikoshii	1	Recombinant	NR	+	+	+	(Okochi et al. 2005)
Thermoplasma acidophilum	2	Native	8	Trace	+	NR	(Bigotti and Clarke 2005; Nitsch et al. 1997; Waldmann et al. 1995)
		Recombinant (α, β)	8	+	NR	NR	
		Recombinant (α+β)	8	+	Trace	Trace	
Thermococcus kodakaraensis	2	Recombinant (α, β)	NR	+	+ (β)[h]	NR	(Izumi et al. 1999; Yan et al. 1997)
Thermococcus sp. strain KS-1	2	Native	NR	+	+	+	(Yoshida et al. 2002b; Yoshida et al. 1997)
		Recombinant (α, β)	8	+	+	+	

[a] "Arrest activity" denotes binding activity to nonnative proteins.
[b] α and β subunits are separately expressed in *E. coli* and purified.
[c] α and β subunits are coexpressed in *E. coli* and purified.
[d] This measurement was carried out at 30°C.
[e] Reconstituted using purified subunit.
[f] Reconstituted with α and γ subunits.
[g] Reconstituted with α, β, and γ subunits.
[h] Purified β subunit prevent thermal inactivation of yeast alcohol dehydrogenase.
NR, not reported.

(Yoshida et al. 2001). The proportion of the β subunit in *T.* KS-1 chaperonin increases with temperature, and the β subunit-rich chaperonin is more thermostable than the α subunit-rich chaperonin (Yoshida et al. 2002a). The hyperthermoacidophilic archaeon, *Sulfolobus shibatae*, contains group II chaperonins composed of up to three different subunits (α, β and γ). Expression of the β and γ subunits is increased by heat shock, and decreased by cold shock (Kagawa et al. 2003). On the other hand, expression of the α subunit gene is undetectable at heat shock temperatures and low at normal growth conditions, but induced by cold shock (Kagawa et al. 2003). The halophilic archaeon *Haloferax volcanii* has three group II chaperonins genes, *cct1*, *cct2*, and *cct3*, which are all expressed but to differing levels (Large et al. 2002). Interestingly, deletion of *cct3* has no effect on the activity of the chaperonin complex, but loss of *cct1* leads to ∼50% reduction in the purified chaperonin ATPase activity (Lund et al. 2003). The precise functional properties and physiological significance of the heterologous subunit composition of archaeal group II chaperonin subunits is still the subject of active investigation.

So far, there are only two available crystal structures of archaeal group II chaperonins (Ditzel et al. 1998; Shomura et al. 2004). The first structure was determined for CPN from *T. acidophilum* (Ditzel et al. 1998). The prototype crystal structure of the group II chaperonin is shown in *Fig. 4.9.1*. It is shown that the subunit architectures are very similar to group I chaperonins, except for differences in the helical protrusion region (Braig 1998; Ditzel et al. 1998; Klumpp and Baumeister 1998). It consists of two eight-membered rings of alternating α and β subunits with a spherical shape; each ring has a central cavity. Crystal structure of the *Thermococcus KS1* CPN is also available (Shomura et al. 2004), showing a similar structure to that of the *T. acidophilum* CPN. Archaeal group II CPN requires no GroES like cochaperonin to carry out folding function. The helical protrusion in group II chaperonins may provide an equivalent functional role to the GroES subunit, by sealing off the central cavity of the chaperonin complex (Llorca et al. 2001; Meyer et al. 2003; Iizuka et al. 2004) (*Fig. 4.9.1*). In both structures, the rings were closed, with the helical protrusion covering the central cavity, but it is highly possible that the protrusion may move under physiological conditions, allowing denatured substrates to enter the cavity. So far, the evidence of open rings came from cryo-electron microscopy studies of *T. acidophilum* and *Sulfolobus shibatae* CPNs (Nitsch et al. 1998; Schoehn et al. 2000a; Schoehn et al. 2000b), and SAXS (small-angle X-ray scattering) (Iizuka et al. 2004; Iizuka et al. 2005). A recent review has more detailed discussion on this (Large et al. 2009). Cryo-EM studies on CPN from *Methanosarcina mazei* found that local conformational changes triggered by ATP hydrolysis lead to an alteration of intersubunit contacts within and across the rings, ultimately causing a rocking motion that closes the ring (Zhang et al. 2010).

Supporting the hypothesis that hyperthermophilic chaperonins might be used as a salvage pathway for proteins, CPN from a hyperthermophilic archaeum, *Thermococcus* KS-1 was found useful for the stabilization of free and immobilized enzymes and applicable to various fields of biotechnology (Kohda et al. 2006). A group II chaperonin from the hyperthermoacidophilic archaeon *Sulfolobus shibatae* and cellulases was engineered to make multienzyme structures (named as rosettazyme). The rosettasomes have increased cellulose-degrading activity compared to their activity in free solution (Mitsuzawa et al. 2009). A self-renaturing enzyme-chaperone chimera consisting of penicillin amidase and a single-subunit Cpn from *Methanocaldococcus jannaschii* functions at high activity in aqueous-organic mixtures (Bergeron et al. 2009). The total turnover number of immobilized chimera for amoxicillin synthesis was almost 3 times higher than that of the immobilized penicillin amidase lacking

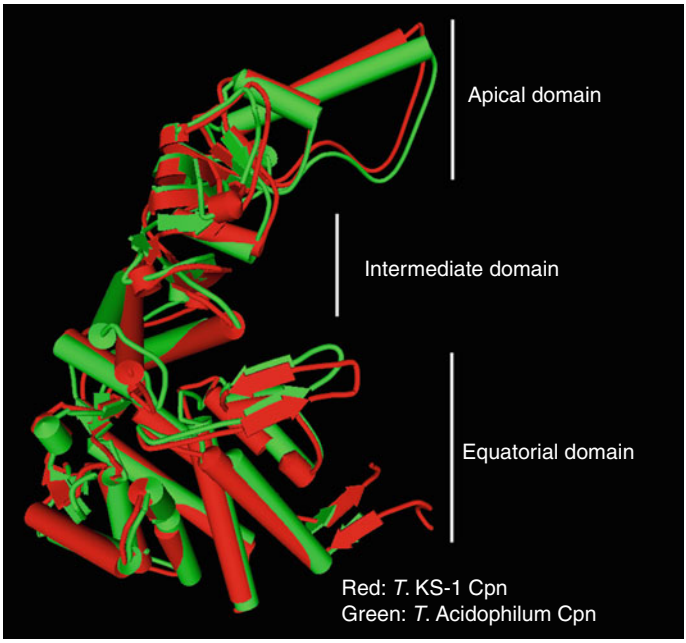

Fig. 4.9.1
The alignment of monomeric structures of *T. acidophilum* Cpn (*green*) and T. Ks1 α-Cpn (*red*)

a chaperone domain (Bergeron et al. 2009). This indicated an enzyme-chaperone chimera as a new approach to enzyme stabilization.

Recently, several works focused on the stabilization mechanism of group II Cpn. The yohda group reported that single mutation K323R of Cpn from *T.* KS-1 resulted in the higher activity at relatively low temperatures (Kanzaki et al. 2010). This mutation induced higher mobility of the built-in lid of the Cpn to make it more active at lower temperature, but they also stated that the cold adaptation of K323R is not sufficient for practical application. Our lab carried out systematic mutagenesis on Pf Cpn and found out that archaeal group II chaperonins adapt to different growth temperatures by changing the number of negatively charged residue in the carboxyl terminal (unpublished material). A model was proposed to demonstrate how the mutations affect double ring formation and ring stability (*Fig. 4.9.2*).

The Proteasome: An ATP-Dependent Protein Degradation Machine

The housekeeping problem of disposing of irretrievably misfolded proteins in the thermophilic Archaea and some Bacteria is assigned to a large hollow complex termed the 20S proteasome. The eukaryotic and archaeal 20S proteasomes share overall size and structure, but differ significantly in complexity – the archaeal proteasome is based on fewer unique proteins. Consequently, the 20S archaeal proteasome serves as a simplified model system, suitable for examining the significance of subunit composition on the functional properties of the multimeric protease. The archaeal proteasome is presumably related to the an ancestral proteasome precursor and may provide insight into how the proteasomes developed into the

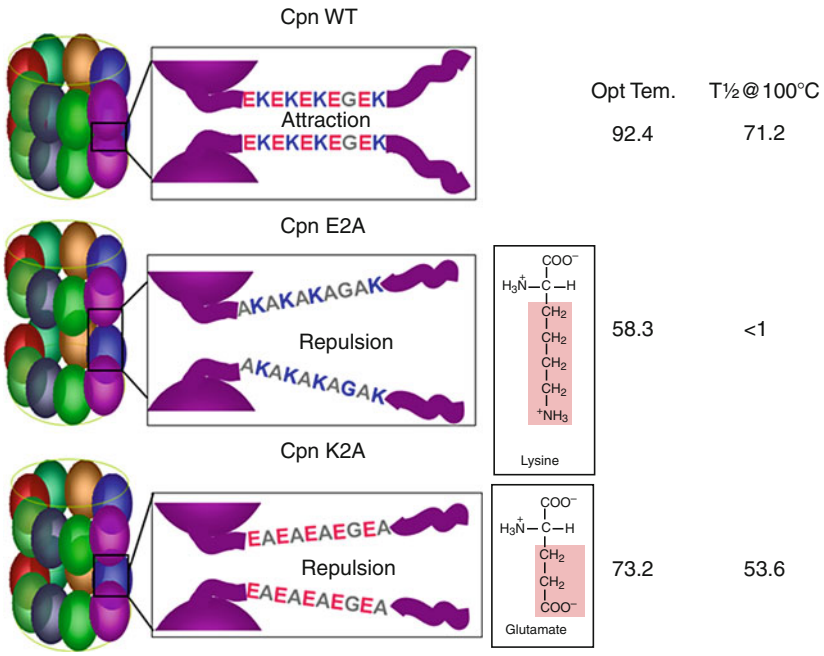

Fig. 4.9.2

A model of inter- and intraring ion pairs stabilizing the double ring structure. The C terminus is not necessary for single/double ring formation, but the ion pairs (E-K) randomly formed among EK-rich motifs stabilize the huge Cpn complex by enhancing the intra- and interring interactions. When the Glu or Lys residues in the EK-rich motif were mutated to Ala, on the one hand, the ion pairs were disrupted; and the left-over residues with the same charge generate repulsion forces. Glu and Lys did not contribute equally to protein stability. Lys residue has longer side chain than Glu residue. The longer side chain generated stronger repulsion forces among the subunits in 0E. The stronger repulsion resulted in two consequences: more dissociation of double rings and higher flexibility of the single ring. On the contrary, the repulsive force is weaker because Glu residue has shorter side chain. Thus the double rings of 0K still stick together and the structure of 0K also remained compact. Lys residues in the C terminus are not as important as Glu residues. These findings provide a potential way for the rational design of cold-adapted or hot-adapted Cpns

complex proteases described in modern day eukaryotes (Barber and Ferry 2001) A very few instances of proteasomes have been reported in Bacteria, presumably as a result of lateral gene transfer from the Archaea sharing niches (Tamura et al. 1995).

20S Proteasome Structure

Eukaryotic genomes encode up to 23 unique proteasome subunit proteins; the *T. acidophilum* proteasome is comprised of only two different subunits (one α and one β) with molecular weights of 20 and 35 kDa, respectively (Puhler et al. 1992; Zwickl et al. 1992).This simple basis for 20S proteasome structure is typical among all archaea, whose genomes encode between 2 and 4 different subunit proteins. (Groll and Clausen 2003; Maupin-Furlow et al. 2004).

The usefulness of thermophiles as model systems is illustrated by the report of the 20S proteasome from the thermophilic archaeon *Thermoplasma acidophilum*, the first archaeal proteasome structure resolved. This structure was subsequently found to be closely related to all archaeal proteasomes examined so far (Lowe et al. 1995). Like its eukaryotic counterpart, the 20S archaeal proteasome is composed of four stacked heptametric rings that form a barrel-like structure with a hollow channel extending down the center (Rechsteiner et al. 1993). Protein degradation in the cytosol by unrestrained proteasomes is avoided by sequestering the active sites within the interior channel of the 20S proteasome. The overall length of the cylindrical enzyme is 148 Å with maximum and minimum diameters of 113 Å and 75 Å, respectively, whereas the eukaryotic 20S proteasome core is inaccessible through the ends, except by major rearrangement of the α subunit N-termini that creates an opening of 10 Å (Lowe et al. 1995; Zwickl et al. 1992).

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4.10 Physiology, Metabolism, and Enzymology of Thermoacidophiles

Reconstruction of the Central Carbon Metabolic Network of Thermoacidophilic Archaea

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_4.10,

Introduction

Adaptation and specialization to harsh environments represent hallmarks of members of the Archaea and this was originally, besides the presence of unique metabolic pathways (i.e., methanogenesis), regarded as a typical archaeal feature. However, meanwhile a wide distribution of mostly uncultured members in ordinary habitats such as ocean and lake waters or soil has been proven and Archaea are known to play major roles in the global ecosystems (DeLong 1998; DeLong and Pace 2001; Francis et al. 2005; Leininger et al. 2006).

Some extremophiles survive and thrive at temperatures over 100°C or down to 0°C, in extremely alkaline (around pH 11) acidic waters (pH < 1), extremely saline environments (>30% (w/v) salts), or combinations thereof. Typical environments from which these Archaea have been isolated include rift vents in the deep sea (e.g., black smokers), geysers, hot acidic springs and sulfuric waters, or salt lakes. Life under such extreme conditions requires effective adaptation strategies. Considerable high effort has been made to unravel the underlying molecular mechanisms and principles, which are also of great interest for biotechnological applications (e.g., heat- and acid-stable proteins; Serour and Antranikian 2002; Hess et al. 2008). Although some Archaea have been studied in detail, information about the biochemical and physiological features as well as regulatory traits of archaeal metabolism is still limited in comparison to Bacteria and Eucarya.

The thermoacidophilic lifestyle, e.g., life in hot acidic springs like solfatares, is characterized by simultaneously coping with high temperatures, wide temperature fluctuations (>60 up to >100°C), low pH values (<4), and low availability of organic material. One striking finding is that all known thermoacidophilic Archaea either prefer an extremely low pH (<2) with optimal growth temperatures around 60°C (e.g., *Picrophilus* and *Thermoplasma* species) or possess an optimal growth temperature above 80°C (hyperthermophiles), but a pH optimum not lower than 3 (for references see *Table 4.10.1*). Thus, so far, there is no extremely acidophilic hyperthermophile (optimal growth temperature >80°C and pH < 2) described. The same is true for thermoacidophilic Bacteria, which exhibit optimal growth at temperatures between 45–60°C and at pH values around 4 (e.g., *Alicyclobacillus acidocaldarius* ssp. *acidocaldarius* (pH 3–4, 60°C; DSM446; Darland and Brock 1971; Wisotzkey et al. 1992); *Alicyclobacillus acidiphilus* (pH 4, 45°C; DSM14558; Matsubara et al. 2002)). Therefore, it is tempting to speculate that both extreme environmental conditions simultaneously – acidophily and hyperthermophily – are generally too challenging to cope with for organisms. Although, some *Sulfolobus* species (e.g., *S. solfataricus* and *S. tokodaii*; *Table 4.10.1*) and *Acidianus infernus* (85–90°C, pH 2; Segerer et al. 1986) are quite close to this border (>80°C, pH < 2).

Another interesting finding represents the fact that all thermoacidophilic Archaea described so far have an aerobic or at least microaerophilic (e.g., *Caldivirga maquilingensis*) metabolism (*Table 4.10.1*), whereas most hyperthermophilic Archaea are anaerobes that gain energy, for example, by the oxidation of various organic compounds and often use elemental sulfur as final electron acceptor.

The adaptation strategies required for life in hot acid have been investigated in more detail in the recent years and has led to several assumptions of how these organisms cope with challenges like DNA damage and membrane, protein, or metabolite instabilities (e.g., Ahmed et al. 2004; Fütterer et al. 2004; Angelov et al. 2005; Ciaramella et al. 2005; Angelov and Liebl 2006; Fröls et al. 2007; Auernik et al. 2008a). For example, it has been shown that thermoacidophiles like *Picrophilus* sp., which live in extremely acidic environments (pH < 1), possess a special membrane that is highly impermeable for protons at low pH (<4). This allows for

Table 4.10.1

Physiological and metabolic characteristics of different thermoacidophilic cren- and euryarchaeal strains with available complete genome sequence information

Organism	Strain	Isolated from	Physiology		Lifestyle	Carbon and energy sources	Electron acceptors	References
			Growth temperature °C (optimal)	pH (optimal)				Organisms and genomes
Crenarchaeota								
Sulfolobales								
Sulfolobus solfataricus	P2 DSM1617	Terrestrial, hot solfataric spring (Pisciarelli, Italy)	60–92 (80)	2–4 (3)	Obligate aerobic, chemoheterotrophic	Various sugars (e.g., arabinose, glucose, galactose, sucrose), amino acids or peptides	O_2	Zillig et al. (1980) She et al. (2001a) Grogan (1989)
S. acidocaldarius	98–3 DSM639	Terrestrial, hot solfataric spring (Locomotive Spring, Yellowstone National Park, USA)	55–85 (75–80)	1–5.9 (2–3)	Obligate aerobic, chemoheterotrophic	Complex organic substrates (e.g., yeast extract, tryptone, casamino acids), some sugar polymers (e.g., dextrin) enhance growth	O_2	Brock et al. (1972) Chen et al. (2005) Grogan (1989)
S. tokodaii	Strain7 JCM10545	Beppu Hot Springs (Kyushu Island, Japan)	70–85 (80)	2–5 (2.5–3)	Obligate aerobic, chemoheterotrophic	Sugars, e.g., glucose, complex organic substrates (yeast, casamino acids)	O_2	Suzuki et al. (2002) Kawarabayasi et al. (2001)

Table 4.10.1 (Continued)

Organism	Strain	Isolated from	Physiology		Lifestyle	Carbon and energy sources	Electron acceptors	References
			Growth temperature °C (optimal)	pH (optimal)				Organisms and genomes
S. islandicus	L.S.2.15 Lassen #1	Lassen volcanic National Park (California, USA)	65–88 (78)	2–5 (2.7)	Obligate aerobic, chemoheterotrophic	Sugars, e.g., glucose, galactose, arabinose, dextrin, complex organic substrates, e.g., trypton	O_2	Whitaker et al. (2003) Reno et al. (2009)
Metallosphaera sedula	ATCC51363 DSM5348	Thermal pond (Pisciarelli Solfatara, Italy)	50–80 (75)	1–4.5 (2)	Aerobic	Complex organic substrates or H_2/CO_2, respectively	O_2	Huber et al. (1989) Auernik et al. (2008b)
Thermoproteales								
Caldivirga maquilingensis	IC-167 DSM13496 JCM10307	Acidic hot spring (Philippines)	60–92 (85)	2.3–6.4 (3.7–4.2)	Anaerobic-microaerobic, chemoheterotrophic	Glycogen, gelatin, beef extract, peptone, tryptone and yeast extract	Sulfur/S^0, thiosulfat/$S_2O_3^{2-}$, sulfate/SO_4^{2-}	Itoh et al. (1999) JGI (2007)

Euryarchaeota Thermoplasmatales								
Picrophilus torridus	DSM9790	Volcanic solfataric field (Hokkaido, Japan)	45–65 (60)	around 0–3.5 (0.7)	Obligate aerobic, chemoheterotrophic	Yeast extract, peptone, preferably polymeric sugar (e.g., starch, sucrose), glucose, lactose (only in presence of yeast extract)	O_2	Schleper et al. (1995b) Fütterer et al. (2004)
Thermoplasma acidophilum	DSM1728	Self-heating coal refuse piles (USA)	45–63 (59)	0.8–4 (2)	Aerobic or anaerobic, chemoheterotrophic	Yeast extract, peptone preferably polymeric sugars (e.g., starch, pectin), glucose (only in presence of yeast extract)	O_2/S^0	Darland et al. (1970) Ruepp et al. (2000) Smith et al. (1975)
Thermoplasma volcanium	GSS1 JCM9571	Volcanic, solfataric fields (Solfatara, Italy)	33–67 (59)	0.8–4 (2)	Aerobic or anaerobic, chemoheterotrophic	Yeast extract, sugar polymers, glucose	O_2/S^0	Segerer et al. (1988) Kawashima et al. (2000)

keeping an internal pH of 4.6 in an extremely acidic surrounding (Van de Vossenberg et al. 1998; Fütterer et al. 2004). However, at higher pH values (>4) the membrane becomes proton permeable and the organism is no more able to maintain membrane integrity, which displays the unique adaptation of this organism to its extreme environment (Van de Vossenberg et al. 1998). For other thermoacidophiles like *S. acidocaldarius* with a pH optimum for growth not lower than 2, an internal pH value close to neutral (pH 6.5) has been demonstrated (Luebben and Schaefer 1989). Most thermoacidophiles are able to utilize polysaccharides such as dextrins or starch, and thus, another remarkable adaptive trait is their possession of extremely heat- and acid-stable extracellular hydrolytic enzymes (e.g., the extremely acid-stable glucoamylase from *P. torrdius*; Serour and Antranikian 2002).

Thermoacidophilic Archaea are distributed among the two archaeal kingdoms of Crenarchaeota and Euryarchaeota. From the approximately 30 described thermoacidophilic archaeal strains (only 4 of which belong to the Euryarchaeota), 14 genomes (3 euryarchaeal, 11 crenarchaeal) have been sequenced and are available (http://archaea.ucsc.edu/) allowing for network reconstruction and comparative analysis. In this chapter, the reconstructed central carbon metabolic (CCM) network based on biochemical and genomic information of different thermoacidophilic crenarchaeal members of the Sulfolobales (*Sulfolobus solfataricus* (P2), *Sulfolobus acidocaldarius* (DSM639), *Sulfolobus tokodaii* (strain 7), *Sulfolobus islandicus* (L.S.2.15), *Metallosphaera sedula* (ATCC51363)); the Thermoproteales (*Caldivirga maquilingensis* (IC-167)) as well as euryarchaeal members of the Thermoplasmatales (*Picrophilus torridus* (DSM9790), *Thermoplasma acidophilum* (DSM1728), and *Thermoplasma volcanium* (GSS1)) is presented.

Thermoacidophilic Archaea

Crenarchaeal Thermoacidophiles

Within the Crenarchaeota, acidophilic thermophiles belong to the orders of the Sulfolobales and Thermoproteales. Members of these groups have been isolated from terrestrial volcanic and thermal active areas, such as hot acidic solfataric or mud springs, marine hydrothermal vents, and are found all around the globe.

The members of the order **Sulfolobales** are characterized by a diverse metabolism ranging from an aerobic or facultatively anaerobic and a chemolithoautotrophic or chemoorganoheterotrophic lifestyle. The order consists of the six genera *Sulfolobus* (10 species), *Acidianus* (seven species), *Metallosphaera* (three species), *Desulfurolobus*, *Stygioglobus*, and *Sulfurisphaera* (one species each). Here, focus will be on those species and strains whose complete genome sequence information is available, i.e., the four *Sulfolobus* species *S. solfataricus* (P2), *S. islandicus* (L.S.2.15), *S. acidocaldarius* (98–3), *and S. tokodaii* (strain 7), as well as *M. sedula* (ATCC51363) (*Table 4.10.1*).

Most *Sulfolobus* species grow heterotrophically on various organic substrates, e.g., different sugars, amino acids, tryptone, and yeast extract, and only a few have the ability to fix carbon dioxide (CO_2), like the facultative heterotrophic *M. sedula*, which uses a new, recently described CO_2 fixation pathway (Huber et al. 1989; Berg et al. 2007).

Some *Sulfolobus* strains (i.e., *S. solfataricus* and *S. acidocaldarius*) became model systems for studying archaeal metabolism, information processing pathways, and adaptation strategies to

extreme environments, mainly, because they are easy to maintain in the laboratory, the whole genome sequences are available (She et al. 2001a, Chen et al. 2005), and genetic systems (gene deletion, expression system) became available recently (Worthington et al. 2003; Albers et al. 2006; Albers and Driessen 2008; Wagner et al. 2009; Deng et al. 2009).

The model organism *S. solfataricus* (strain P2, DSM1617; Zillig et al. 1980) was isolated from a solfataric field (Pisciarelli) near Naples, Italy, and optimally grows at 80°C (60–92°C) and pH 2–4. The organism is a strict aerobe, growing heterotrophically on various carbon and energy sources, such as many different sugars, like arabinose, hexoses (e.g., glucose, galactose, mannose), disaccharides (e.g., maltose, sucrose), polysaccharides (e.g., starch, dextrin), tryptone, amino acids, and peptides (*Table 4.10.1*; Grogan 1989). The genome of the *S. solfataricus* strain P2 is about 3 Mbp and contains approximately 3,033 predicted open reading frames (ORFs) (She et al. 2001a). The genome shows a high level of plasticity with about 200 diverse insertion sequence elements (Martusewitsch et al. 2000; Redder et al. 2001; She et al. 2001b; Bruegger et al. 2002, 2004).

The *S. acidocaldarius* strain 98–3 (DSM 639) was isolated from Locomotive Spring in Yellowstone National Park by T. Brock et al. (1972) as the first hyperthermophilic archaeon. The organism thrives at a pH 2–3 and at 75–80°C. In contrast to *S. solfataricus*, *S. acidocaldarius* is not able to utilize sugars like arabinose, glucose, galactose, mannose, or disaccharides (e.g., cellubiose or maltose), but the organism is able to grow on polysaccharides such as dextrin, starch, or maltotriose in the presence of peptides or tryptone (*Table 4.10.1*). For the genome (2.2 Mbp) about 2,329 putative ORFs are predicted (Chen et al. 2005).

The *Sulfolobus tokodaii* strain JCM10545 (formerly *Sulfolobus* sp. strain 7, isolated from Beppu hotsprings in Kyushu (Japan) in 1983; Suzuki et al. 2002; *Table 4.10.1*) optimally grows at 80°C, pH 2.5–3, and under aerobic conditions. *S. tokodaii* is an obligate aerobe, grows heterotrophically on some sugars (e.g., glucose), complex organic substrates (yeast, casamino acids), and metabolizes sulfur. The whole genome sequence has been determined (Kawarabayasi et al. 2001).

Sulfolobus islandicus (strain REN1H1) was firstly isolated by Zillig and coworkers in 1994 from the Reykjanes solfataric field in Iceland (Zillig et al. 1994). Recently, a total of seven different *Sulfolobus islandicus* strains from three different locations (hot acidic springs) on the Kamchatka Peninsula (Russia), Lassen volcanic National Park (USA), and Yellowstone National Park (USA) were isolated and their genomes have been analyzed (Reno et al. 2009; *Table 4.10.1*). The different locations show a great diversity and show their own genome and genome components, meaning that each *S. islandicus* strain is evolving in its own unique way driven by the geographic isolation (Whitaker et al. 2003; Reno et al. 2009). However, the pathways of the CCM network of these isolated *S. islandicus* strains do not show major differences. Implicating that the overall pathways of the central carbon metabolism are conserved within the six different strains (unpublished data).

For the CCM network reconstruction the genome of the *S. islandicus* strain L.S.2.15 Lassen #1 (2.74 Mbp, 3,000 defined ORFs; Reno et al. 2009) has been chosen. The strain was isolated from the location "Sulfur Works" in Lassen volcanic National Park (USA) and grows optimally at 78°C and a pH of 2.7 on different carbon sources (Zillig et al. 1994).

Metallosphaera sedula (strain ATCC 51363/DSM5348; *Table 4.10.1*) was isolated from a thermal pond in Pisciarelli Solfatara (Italy) and represents a separate genus of Sulfolobales (Huber et al. 1989). *M. sedula* is an aerobic, facultative heterotrophic, metal-mobilizing Crenarchaeon which grows chemoheterotrophically on complex organic substrates such as

casamino acids, peptone, or tryptone, and for autotrophic growth the organism couples, for example, hydrogen, sulfur, or iron oxidation with CO_2 assimilation. Fixation of CO_2 under autotrophic growth conditions is performed via the modified 3-hydroxypropionate/4-hydroxybutyrate cycle that has been described recently (Huegler et al. 2003a, b; Huegler and Fuchs 2005; Berg et al. 2007).

The genome of the *M. sedula* strain DSM 5348 has been deciphered to be 2.19 Mbp and contains about 2,342 predicted ORFs (Auernik et al. 2008b). *Metallosphaera sedula* is very resistant to toxic metals and is able to mobilize metals from metal sulfides (production of soluble sulfates). Therefore, the organism is of special interest for biomining, e.g., to recover metals from sulfidic ores (Rawlings and Johnson 2007). Besides *M. sedula*, also *Sulfolobus metallicus* and *Acidianus* species (Sulfolobales) are bioleaching extreme thermoacidophiles.

Members of the order **Thermoproteales** (comprising five genera) are generally known to be rod-shaped crenarchaeotes that are widely distributed in terrestrial hot springs and submarine hydrothermal systems.

The order harbors only the genus *Caldivirga* that shows a thermoacidophilic lifestyle and so far, only one species (*Caldivirga maquilingensis*) has been described (Itoh et al. 1999; *Table 4.10.1*). The *C. maquilingensis* strain IC-167 has been isolated from a hot acidic spring in the Philippines (Itoh et al. 1999) and its genome sequence has been released in 2007 by the DOE Joint Genome Institute (http://www.jgi.doe.gov/). *Caldivirga maquilingensis* grows optimally at 85°C and, compared to the other thermoacidophiles, at a less extreme pH around 4 (3.7–4.2). The organism is able to grow anaerobically as well as microaerophilically, and is a chemoheterotrophe that is able to utilize complex organic substrates such as glycogen, peptone, tryptone, or yeast extract as carbon and energy sources.

Euryarchaeal Thermoacidophiles

Within the Euryarchaeota, acidophilic thermophiles belong to the order **Thermoplasmatales**. This order consists of the genus *Picrophilus* with two described species (*P. torridus* and *P. oshimae*), *Thermoplasma* (*T. acidophilum* and *T. volcanium*) and the recently described genus *Thermogymnomonas* (*T. acidicola*; Itoh et al. 2007). The most extreme acidophiles (hyperacidophiles) belong to the genus *Picrophilus*. *P. torridus* and *P. oshimae* optimally grow at pH 0.7 or even below and at 60°C (47–65°C; Schleper et al. 1995a,b; Schleper et al. 1996; *Table 4.10.1*). They are strict aerobic heterotrophes, utilizing different sugar polymers as carbon and energy source. With a size of 1.55 Mbp the genome of *P. torridus* (strain DSM 9790) represents the smallest genome of a non-parasitic free-living organism showing a very high coding density (1,583 predicted genes), which is discussed as an adaption to the extreme constraints of the environment (Fütterer et al. 2004).

The two *Thermoplasma* species, *T. acidophilum* and *T. volcanium*, thrive at temperatures around 60°C and pH 2 in similar thermal and acidic environments like members of the Sulfolobales, e.g., solfataric fields (Darland et al. 1970; Segerer et al. 1988; *Table 4.10.1*). Their heterotrophic lifestyle is adapted to aerobic as well as anaerobic (sulfur-respiration) growth conditions. Another characteristic for species of this genus is that they do not have a rigid cell wall, but only possess a plasma membrane. The complete genome sequences of both

the *T. acidophilum* strain DSM 1728 and the *T. volcanium* strain GSS1 have been deciphered (Ruepp et al. 2000; Kawashima et al. 2000).

Reconstructed CCM Network of Thermoacidophilic Archaea

The availability of genome sequence information and modern high throughput technologies revolutionizes life sciences and allows scientists to address and ask new, previously impossible, exciting questions. However, one of the basis for a decent understanding and a broad use of the available genome sequence information is the derived functional prediction and annotation. Major draw-backs in functional assignments are (i) the great number of hypothetical proteins in many available genomes (approximately 40%, Gerlt and Babbitt 2000), (ii) the great number of errors "wrong/misleading annotations" that are often taken over from previous annotations, and (III) therewith linked missing updates.

In most cases the predicted protein functions are assigned during genome annotation, based on the amino acid sequence similarity of the predicted proteins/enzymes to previously annotated proteins/enzymes, which are in only few cases functionally characterized proteins. In addition, increasing biochemical and structural information about single enzymes as well as enzyme families, super-, and suprafamilies reveals that the assignment of function from the sequence information alone is often misleading. As nicely reviewed by Gerlt and Babbitt (2000) even if two proteins are homologous and thus derived from a common ancestor, they do not necessarily catalyze the same reaction. This scenario is also reflected by the fact that for 38% of characterized enzyme activities in ENZYME database no protein sequence is known yet (Karp 2004). Therefore, the great number of unknown ORFs identified in microbial genome sequencing projects and their functional assignment are major challenges to bioinformaticians and biochemists in post-genomic biology. In Archaea this is especially challenging, since Archaea harbor many new, unusual pathways and enzymes that show no similarity to their bacterial and eucaryal counterparts. The KEGG pathway maps in Archaea resemble a cloze with many pathway holes.

Sulfolobus solfataricus is the established thermoacidophilic model organism, and compared to the other thermoacidophilic Archaea its CCM has been well studied (e.g., Lamble et al. 2003; 2005; Kim and Lee 2005, 2006; Snijders et al. 2006; Ettema et al. 2008; Zaparty et al. 2010). In the *Sulfolobus* Systems Biology (SulfoSYS, http://www.sulfosys.com, Albers et al. 2009) project the CCM network of *S. solfataricus* (P2; Zillig et al. 1980) has been reconstructed in detail, based on available biochemical and genome sequence information, including genomic context analyses (http://string.embl.de/) and comparative genomics (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?page=home; http://wwwarchbac.u-psud.fr/genomics/GenomicsToolBox.html). Blast search analyses (BLASTP 2.2.22+, Altschul et al. 1997; NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi) with previously characterized archaeal, bacterial, or eucaryal CCM network candidates (http://www.brenda-enzymes.org/) were performed for network reconstruction. This work has been complemented by extensive biochemical and enzymatic studies of candidates (Albers et al. 2009; Zaparty et al. 2010; unpublished data).

This established *S. solfataricus* blue print model has now been used for the CCM network reconstruction of other thermoacidophilic Archaea with available genome sequence information, including: (i) the branched Entner–Doudoroff (ED) pathway, (ii) the

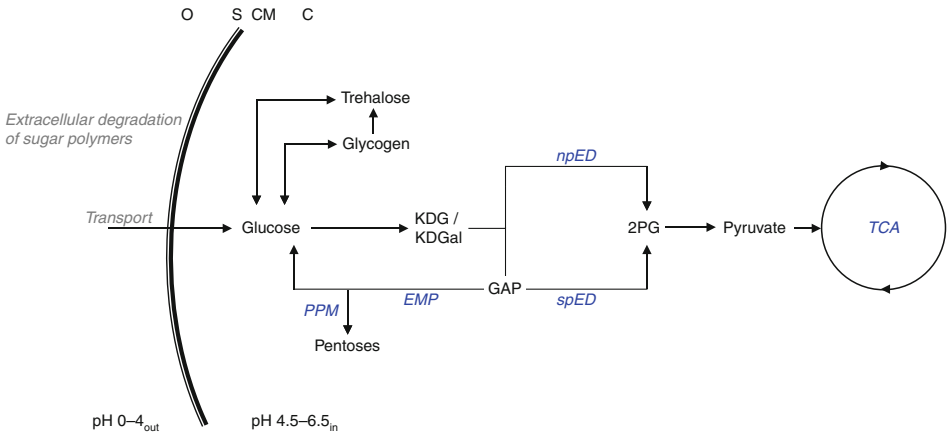

Fig. 4.10.1

Schematic overview of the central carbon metabolic (CCM) network of thermoacidophilic Archaea. C, cytoplasm; CM, cytoplasmic membrane; EMP, Embden-Meyerhof-Parnas; KDG/KDGal, 2-keto-3-desoxy-gluconate/galactonate; npED, non-phosphorylative Entner-Doudoroff branch; O, outside; PPM, pentose phosphate metabolism; S, S-layer; spED, semi-phosphorylative entner-Doudoroff branch; TCA, tricarboxylic acid cycle. The indicated cell envelope (S-layer) is missing in the cell-wall-less *Thermoplasma* species

Embden–Meyerhof–Parnas (EMP) pathway, (iii) the oxidative tricarboxylic acid (TCA) cycle, (iv) the pentose phosphate metabolism (reversed ribulose monophosphate (RuMP) pathway), as well as (v) glycogen and trehalose metabolism (*Fig. 4.10.1*).

Glycolysis and Gluconeogenesis

Archaea utilize modifications of the classical ED and EMP pathway, known from Eucarya and Bacteria. Whereas the upper part of the EMP pathway is unique, the lower part is shared by many pathways, like the ED pathway, and is generally regarded as funnel for degradation of various metabolites. Initial studies on sugar metabolism in Archaea suggested that only ED modifications are used for sugar degradation, due to missing phosphofructokinase activity (Danson and Hugh 1992). However, by now, different phosphofructokinases were identified in Archaea and also the EMP pathway has been established as route for sugar degradation in the third domain of life. Archaeal phosphofructokinases (PFKs) are, compared to their bacterial and eucaryal counterparts, either characterized by unusual co-substrate specificity (pyrophosphate (PP_i), ADP) and/or are members of new enzyme families (e.g., ADP-PFK of the ribokinase superfamily). Worth mentioning is that all archaeal sugar kinases characterized so far harbor no allosteric properties and therefore highlight an alternative mode of pathway regulation in Archaea (for review see Siebers and Schoenheit 2005).

The current understanding suggests the presence of EMP modifications in (facultatively) anaerobic and ED modifications in aerobic Archaea, resembling the distribution of both pathways in Bacteria. The only exception known so far is the anaerobic hyperthermohile

Thermoproteus tenax, which utilizes both pathways in parallel (for review see Zaparty et al. 2008b). Interestingly, the modifications seem to be predominantly manifested on protein and not on intermediate level: Many of the players (enzymes) involved in both pathways show no similarity to their bacterial and eucaryal counterparts; however, the catalyzed enzymatic reactions as well as the intermediates are similar, underlining the importance of basic thermodynamic constraints governingmetabolism.

Interestingly, the invention of new enzyme activities/reactions (e.g., the non-phosphorylating glyceraldehyde-3-phosphate (GAP) dehydrogenase (GAPN, Brunner et al. 2001), GAP oxidoreductase (GAPOR, Mukund and Adams 1995)) has been reported for (hyper)thermophiles, with only few exceptions (Ettema et al. 2008). Both enzymes catalyze the unidirectional, non-phosphorylating oxidation of GAP to 3-phosphoglycerate in the presence of pyridine nucleotides (GAPN) and ferredoxin (GAPOR). Therefore, they substitute for the classical GAPDH-phosphoglycerate kinase (PGK) enzyme couple, for which a gluconeogenic function is proposed (for review see Siebers and Schönheit 2005). The utilization of non-phosphorylating enzymes in (hyper)thermophiles is accompanied by the loss of ATP gained in the PGK reaction and has been discussed in respect to metabolic thermoadaptation due to the thermal instability of 1,3 diphosphoglycerate (Ahmed et al. 2004; Ettema et al. 2008). In addition, GAPN is the only allosteric, glycolytic enzyme reported so far, and GAP conversion has been shown to be the first, important control point in Archaea (for review see van der Oost and Siebers 2007; Zaparty et al. 2008a).

In the thermoacidophilic Archaea discussed here, the modified ED pathway is the general route for sugar degradation, whereas the EMP pathway seems to be only used for gluconeogenesis (*Table 4.10.2*, *Fig. 4.10.2*). The first modification of the *ED pathway*, the so-called *semi-phosphorylative pathway*, has been reported in Bacteria *(Clostridium aceticum*, Andreesen and Gottschalk 1969) and was afterwards demonstrated in halophilic Archaea (Tomlinson et al. 1974). Later on, a second modification, the *non-phosphorylative ED pathway*, has been identified in (hyper)thermohilic Archaea (De Rosa et al. 1984; Budgen and Danson 1986) as well as the fungal genus *Aspergillus* (Elzainy et al. 1973). However, recent studies revealed that all Archaea, which use ED modifications for sugar degradation, except *Halobacterium* sp. NRC1, seem to utilize both modifications in parallel (Ahmed et al. 2005; Jung and Lee 2005; Kehrer et al. 2007). The so-called branched ED pathway, characterized by a non-phosphorylative (npED) and a semi-phosphorylative (spED) branch, seems to be therefore the common route for sugar degradation in ED utilizing Archaea (Ahmed et al. 2005) (*Fig. 4.10.2*).

In this *branched ED pathway* the initial phosphorylation, characteristic in the classical ED pathway, is omitted and glucose is directly oxidized to gluconate via glucose dehydrogenase and converted via gluconate dehydratase (GAD) to one of the key intermediates of the pathway: 2-keto-3-desoxygluconate (KDG). In the spED branch, KDG is phosphorylated by KDG kinase. The formed 2-keto-3-desoxy-6-phosphogluconate (KDPG) is subsequently cleaved by the KD(P)G aldolase, which is active in both branches (*Fig. 4.10.2*), forming glyceraldehyde 3-phosphate (GAP) and pyruvate. GAP is further converted to pyruvate via GAPN, phosphoglycerate mutase, enolase, and pyruvate kinase in the lower, common shunt of the EMP pathway. In the npED branch KDG is directly cleaved by the bifunctional KD(P)G aldolase forming pyruvate and glyceraldehyde (GA). GA is oxidized by GA dehydrogenase or GA oxidoreductase to glycerate, which is subsequently phosphorylated by glycerate kinase (*Fig. 4.10.2*). The formed 2-phosphoglycerate is channeled into the EMP pathway and converted to a second molecule of pyruvate by enolase and pyruvate kinase. There is no net energy (ATP) yield for both ED branches, considering that GAPN is utilized in the spED branch.

Table 4.10.2

Identified candidate genes encoding enzymes involved in the branched ED and the EMP pathway of thermoacidophilic Archaea with available genome sequence information

# *Fig. 4.10.2*	Enzyme	EC #	Gene ID best hit and e-value									References
EMP and ED pathway			SSO	LS215 (e-value)	Saci (e-value)	ST (e-value)	M_{sed} (e-value)	C_{maq} (e-value)	PTO (e-value)	Ta (e-value)	TVN (e-value)	
1A	Glucose dehydrogenase (GDH)	1.1.1.47	3003	2525 (0.0)	1079 (1e-155)	1704 (6e-81)	1301 (5e-81)	1714 (9e-122)	0639 (2e-109)	0897 (1e-49)	1019 (3e-47)	Lamble et al. (2003); Angelov et al. (2005)
1B	Gluconolactonase (GL)	3.1.1.17	2705	2771 (7e-156)	1674 (2e-53)	2555 (9e-113)	1292 (4e-47)	0241 (1e-51)	0907 (5e-46)	0648 (2e-53)	0929 (2e-54)	
2	Gluconate dehydratase (GAD)	4.2.1.39	3198	2325 (0.0)	0885 (5e-180)	2366 (0.0)	1303 (5e-173)	0074 (8e-120)	0485 (3e-91)	0085 m (1e-87)	0168 (2e-85)	Ahmed et al. (2005); Kim and Lee (2005); Zaparty et al. (2010)
3	KD(P)G aldolase (KD(P)GA)	4.1.2.-	3197	2326 (2e-163)	0225 (3e-79)	2479 (4e-85)	1296 (1,296)	0935 (2e-25)	1026 (4e-50)	0619 (5e-56)	0669 (7e-48)	Ahmed et al. (2005); Lamble et al. (2003, 2005); Reher et al. (2010)
4	Aldehyde fd: oxidoreductase (AOR), β-subunit	1.2.7.5	2636	2837 (3e-154)	2269 (3e-118)	1783 (5e-78)	0299 (4e-70)		1119 (3e-47)			Kardinahl et al. (1999)
	AOR, γ-subunit	1.2.7.5	2637	2836 (2e-90)	2270 (2e-80)	1782 (8e-68)	0298 (4e-68)		1120 (5e-42)			Kardinahl et al. (1999)
	AOR, α-subunit	1.2.7.5	2639	2835 (0.0)	2271 (0.0)	1781 (0.0)	0297 (0.0)		1121 (3e-85)			Kardinahl et al. (1999)
4	Glyceraldehyde dehydrogenase (GADH)	1.2.1.3							0332 (0.0)	0809	1021 (0.0)	Jung and Lee (2006); Reher and Schoenheit (2006)

5	Glycerate kinase (GK)	2.7.1.-	0666	1579 (0.0)	0113 (3e-100)	2037 (3e-103)	2013 (3e-103)	0816 (4e-54)	1442 (7e-41)	0453m (4e-30)	0783 (1e-34)	Reher et al. (2006b); Noh et al. (2006), unpublished data
6	Enolase (ENO)	4.2.1.11	0913	1395 (0.0)	2375 (1e-152)	1212 (8e-156)	1668 (8e-155)	0197 (1e-57)	1234 (3e-60)	0882 (3e-59)	0981 (4e-55)	Unpublished data
7	Pyruvate kinase (PK)	2.7.1.40	0981	1329 (0.0)	1648 (1e-126)	1617 (2e-130)	1412 (8e-135)	0580 (6e-66)	0336 (2e-55)	0896 (4e-62)	1020 (1e-59)	Unpublished data
8	KDG/Gal Kinase (KDGK)	2.7.1.45	3195	2327 (3e-169)	0226 (6e-100)	2478 (7e-103)	1297 (1e-90)	0369 (2e-75)	0011 (4e-55)	0122	0199 (5e-114)	Ahmed et al. (2005); Lamble et al. (2005); Kim and Lee (2006); Jung and Lee (2005)
9	GAP dehydrogenase, phosphorylating, (GAPDH)	1.2.1.12/ 1.2.1.13	0528	1689 (0.0)	1356 (5e-136)	1356 (5e-143)	1652 (1e-137)	1790 (0.0)	0742 (2e-80)	1103 (1e-79)	0458 (1e-84)	Russo et al. (1995); Jones et al. (1995)
10	Phosphoglycerate kinase (PGK)	2.7.2.3	0527	1688 (0.0)	1355 (1e-172)	1357 (4e-168)	1653 (1e-163)	1789 (0.0)	1514 (9e-72)	1075 (1e-70)	0530 (5e-73)	Unpublished data
11	GAP dehydrogenase, non-phosphorylating, (GAPN)	1.2.1.9	3194	2328 (0.0)	0227 (0.0)	2477 (0.0)	1298 (0.0)	0979 (0.0)	0225 (3e-47)	0439 (4e-46)	1054 (8e-46)	Ettema et al. (2008)
	GAP oxidoreductase (GAPOR)	1.2.7.6						0263 (0.0)*				
12	Phosphoglycerate mutase (PGAM)	5.4.2.1	0417	1815 (0.0)	0837 (1e-168)	0377 (6e-178)	2238 (5e-160)	0340 (5e-49)	1271 (1e-95)	0413 (9e-91)	1158 (4e-90)	Potters et al. (2003)
13	PEP synthetase (PEPS)	2.7.9.2	0883	1423 (0.0)	1417 (0.0)	1235 (0.0)	1694 (0.0)	0499 (5e-24)	1519 (0.0)	0886 (7e-33)	0976 (7e-33)	

Table 4.10.2 (Continued)

# *Fig. 4.10.2*	Enzyme	EC #	Gene ID best hit and e-value									References
14	Pyruvate phosphate dikinase (PPDK) domain	2.7.9.1	2820	2644 (3e-170)		0698 (4e-125)	0431 (8e-75)					
15	Triosephosphate isomerase (TIM)	5.3.1.1	2592	2885 (2e-119)	0117 (1e-88)	2030 (6e-91)	1451 (1e-88)	0703 (7e-39)	0348 (8e-32)	0313 (1e-33)	1287 (2e-33)	Unpublished data
16	Fructose-1,6-bisphosphate aldolase (FBPA), class Ia	4.1.2.13	3226	2301 (2e-146)	**	2350 (3e-122)	0237 (1e-102)	1259 (7e-27)				
17	Fructose-1,6-bisphosphate aldolase/ phosphatase (FBPA/ase)	3.1.3.11	0286	1954 (0.0)	0671 (0.0)	0318 (0.0)	2259 (0.0)	1151 (5e-128)	0807 (4e-123)	1428 (2e-131)	1445 (4e-131)	Say and Fuchs (2010) Unpublished data
19	Phosphoglucose/-mannose isomerase (PGI)	5.3.1.9/ 5.3.1.8	2281	0092 (1e-152)	0151 (5e-57)	2245 (1e-58)	1856 (6e-55)	1967 (1e-21)	1226 (5e-21)	1419 (5e-21)	1438 (3e-21)	Hansen et al. (2003)
20	Hexokinase (HK)	2.7.1.1	3218	2305 (1e-149)	0424 (6e-78)	2354 (3e-85)	1390 (6e-75)	0831 (1e-26)	0011 (2e-20)	0122 (7e-19)	0199 (2e-21)	Nishimasu et al. (2006)

Characterized candidates are underlined and the respective references are given.

*Blast search with the *P. aerophilum* GAPOR (PAE1029; Reher et al. 2007).

**The ORF encoding the FBPA class Ia in *S. acidocaldarius* is not defined in the published genome (DSM 639, She et al. 2001a). The ORF has been identified by homology searches against the complete *S. acidocaldarius* genome sequence (gi|70605853; position 358904–359720).

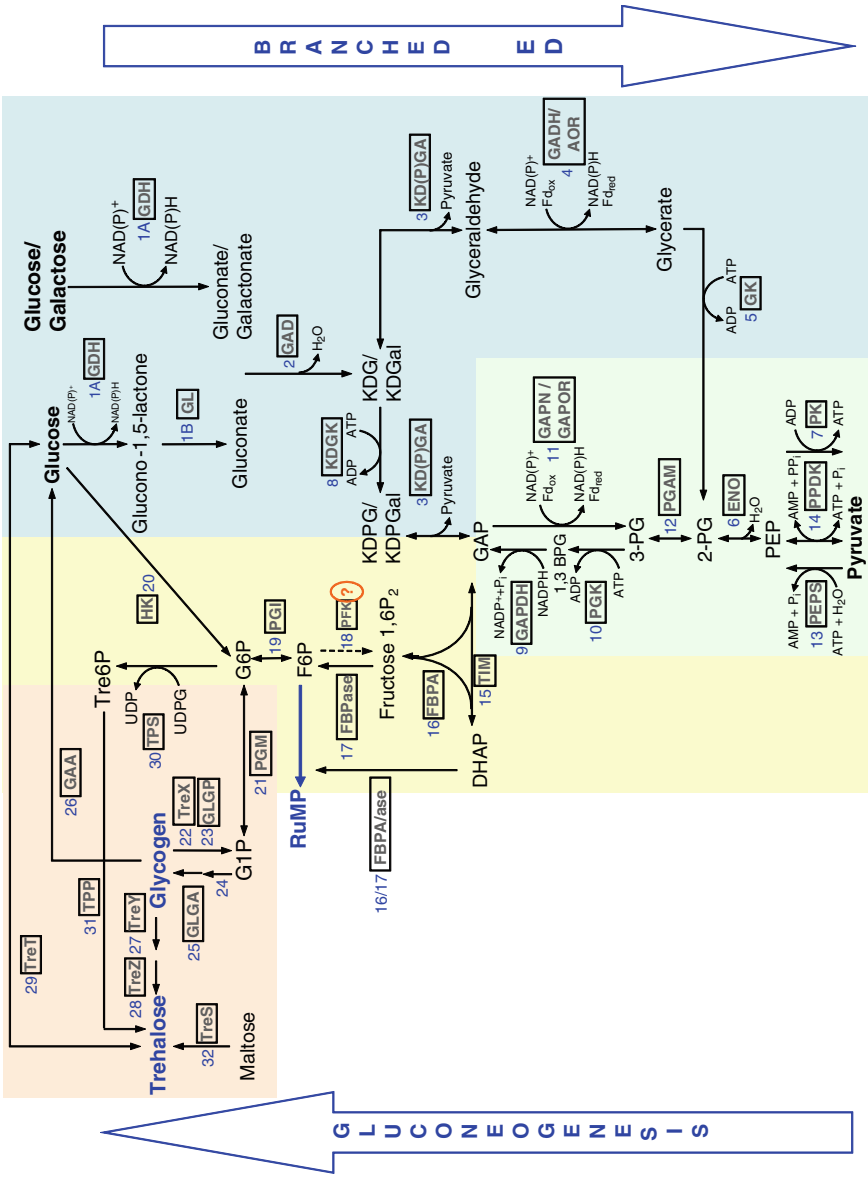

Fig. 4.10.2 (Continued)

Interestingly, the archaeal enzymes GAD and KD(P)G aldolase show no similarity to the famous key enzymes of the bacterial ED pathway gluconate dehydratase (EDD) and KDPG aldolase (EDA) (Lamble et al. 2003; Kim and Lee 2005; Ahmed et al. 2005).

In *S. solfataricus* the modified branched ED pathway is well established and has been shown to be promiscuous for glucose and galactose degradation (Lamble et al. 2003, 2005). Key enzymes of the pathway are the bifunctional KD(P)G aldolase as well as the KDG kinase (KDGK) and glycerate kinase (GK), the latter two catalyze the phosphorylation steps in the spED and npED branch, respectively. Using the *S. solfataricus* blue print model for pathway reconstruction in all crenarchaeal thermoacidophiles, necessary candidates for a functional branched ED pathway were identified, confirming the central role of this pathway in sugar degradation. Additional available biochemical information for euryarchaeal thermoacidophiles helps to complete the reconstruction: In *T. acidophilum* a novel KDG kinase has been characterized (Ta0122; Jung and Lee 2005), which is not related to the enzyme of *S. solfataricus* and *T. tenax* (ribokinase superfamily, pfkB family carbohydrate kinase, PF00294; Kehrer et al. 2007, unpublished), but is a member of the BadF/BadG/BcrA/BcrD ATPase family (PF01869). Homologs of this KDG kinase were identified in all members of the Thermoplasmatales (Euryarchaeota, Jung and Lee 2005). Furthermore, a novel KDG-specific aldolase from *P. torridus* has been characterized recently (Reher et al. 2010) and the authors

Fig. 4.10.2

Overview reconstructed glycolytic, branched ED, and gluconeogenic EMP pathway, as well as glycogen and trehalose metabolism in thermoacidophilic Archaea. Boxed in blue: reactions of the branched ED pathway; boxed in yellow: reactions of the anabolic EMP pathway; boxed in green: shared reactions of the ED and EMP pathway; orange box: reactions of glycogen and trehalose metabolism. For details on missing candidates identified in some thermoacidophiles see *Tables 4.10.2* and *4.10.5* and discussion in the text. *Intermediates*: 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; DHAP, dihydroxy acetonephosphate; fructose 1,6P_2, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; $Fd_{ox/red}$, ferredoxin oxidized/reduced form; GAP, glyceraldehyde 3-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; KD(P)G/Gal, 2-Keto-3-deoxy 6-(phospho)gluconate/-galactonate; PEP, phosphoenolpyruvate; Tre6P, trehalose 6-phosphate. *Abbreviations enzymes (reaction number)*: (1A) GDH, glucose dehydrogenase; (1B) GL, gluconolactonase; (2) GAD, gluconate dehydratase; (3) KD(P)GA, bifunctional KD(P)G aldolase; (4) GADH/AOR, glyceraldehyde dehydrogenase/aldehyde oxidoreductase; (5) GK, glycerate kinase; (6) ENO, enolase; (7) PK, pyruvate kinase; (8) KDGK, KDG kinase; (9) GAPDH, glyceraldehyde-3-phosphate dehydrogenase; (10) PGK, phosphoglycerate kinase; (11) GAPN/GAPOR, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase/GAP oxidoreductase; (12) PGAM, phosphoglycerate mutase; (13) PPDK, phosphate, pyruvate dikinase; (14) PEPS, phosphoenolpyruvate synthetase; (15) TIM, triosephosphate isomerase; (16) FBPA, fructose-1,6-bisphosphate aldolase; (17) FBPase, fructose-1,6-bisphosphatase; (18) PFK, phosphofructokinase; (19) PGI, glucose-6-phosphate isomerase; (20) HK, hexokinase; (21) PGM, phosphoglucomutase/phosphomannomutase, bifunctional; (22) TreX, glycogen-debranching protein; (23) GLGP, glycogen phosphorylase; (24) ADP/UDP-glucose pyrophosphorylase; (25) GLGA, glycogen synthase; (26) GAA, glucan-1,4-α-glucosidase; (27) TreY, maltooligosyltrehalose synthase; (28) TreZ, trehalose hydrolase; (29) TreT, trehalose glycosyl-transferring synthase; (30) TPS, trehalose-6-phosphate synthase (OtsA); (31) TPP, trehalose-6-phosphate phosphatase (OtsB); (32) TreS, trehalose synthase

suggest a strictly non-phosphorylative ED pathway for this organism. The encoding gene (PTO1026) shares 38% (111/286) amino acid similarity to the characterized, bifunctional KD(P)G-aldolase from *S. solfataricus* (SSO3197; Ahmed et al. 2005).

The ferredoxin-dependent aldehyde oxidoreductase (AOR) from *S. acidocaldarius* has been characterized previously (Kardinahl et al. 1999) and the encoding genes were identified by the determined N-terminal sequence of the three subunits (Saci_2269, _2270, _2271). The enzyme shows a significant preference for glyceraldehyde, and homologs were identified in all thermoacidophiles except *Caldivirga marquilingensis, T. acidophilum*, and *T. volcanium*. In addition, the $NADP^+$ specific glyceraldehyde dehydrogenases of *T. acidophilum* and *P. torridus* have been characterized in detail (Ta0809, PTO0332, Jung and Lee 2006; Reher and Schoenheit 2006a). Therefore suggesting that *P. torridus* harbors both AOR and GADH, whereas in *Thermoplasma* sp. only GADH homologs were identified.

Glyceraldehyde dehydrogenases, like GAPN, are members of the aldehyde dehydrogenase (ALDH) superfamily. In the *S. solfataricus* genome five paralogs of the ALDH superfamily were identified (SSO3194, SSO1218, SSO1629, SSO1842, SSO3117, Ettema et al. 2008) and so far only GAPN (SSO3194) and 2,5-dioxopentanoate dehydrogenase (SSO3117) have been characterized (Ettema et al. 2008; Brouns et al. 2006). Due to the high similarity within the ALDH superfamily and the broad substrate specificity reported for some enzymes (e.g. Brouns et al. 2006; unpublished), functional annotation by sequence alone is difficult and the predictions, especially in respect to substrate specificity, should be handled with care, until biochemical information is available. As seen in *Table 4.10.2*, GAPN homologs seem to be present in all crenarchaeal thermoacidophiles, which fits quite well with the proposed function of GAPN and GAPOR in metabolic thermoadaptation (Ahmed et al. 2004; Ettema et al. 2008). However, for a functional confirmation of GAPN in Thermoplasmatales, biochemical studies have to be awaited. Only in the genome of the microaerobic *C. maquilingensis* a GAPOR homolog could be identified. The ORF shows high similarity to the recently characterized enzyme from the hyperthermophilic crenarchaeote *Pyrobaculum aerophilum* (57% amino acid identity, e-value 0.0; PAE1029, Reher et al. 2007).

The upper branch of the *EMP pathway* is supposed to be irreversible, with an exclusive role in gluconeogenesis, in thermoacidophiles (*Fig. 4.10.2*). However, the only enzyme missing for a functional glycolytic EMP pathway in several thermoacidophiles is the phosphofructokinase (PFK, reaction 18, *Fig. 4.10.2*). Although a PFK encoding gene was annotated in the *P. torridus* genome (PTO1237, Fütterer et al. 2004, *Table 4.10.2*), so far no PFK activity has been reported neither in crude extracts nor for isolated enzymes of thermoacidophilic Archaea. Interestingly, hexokinase activity was demonstrated for a member of the ROK (repressors, open reading frames of unknown function, and sugar kinases) family in *S. tokodai* (ST2354, Nishimasu et al. 2006) as well as in *S. solfataricus* crude extracts, but without identification of the responsible enzyme (De Rosa et al. 1984), suggesting that the initial activation of glucose for degradation takes place. An alternative function of hexokinase, beside glycolysis, might be providing the precursors glucose 6-phosphate and by the phosphoglucose/-mannose isomerase (PGI) reaction fructose 6-phosphate for pentose formation via the reversed ribulose monophosphate (RuMP) pathway (see below, *Fig. 4.10.4*). Hexokinase homologs were identified in all analyzed thermoacidophilic genomes (*Table 4.10.2*). However, various sugar kinases with different (co-)substrate specificity are members of the diverse ribokinase superfamily (e.g., ADP-glucokinases, ADP-PFKs, ATP-PFKs, and ATP-dependent KDG kinases) and the ROK family (e.g., ATP-gluco/hexokinases) and several paralogs were identified in available thermoacidophilic genomes (data not shown), making predictions difficult.

Therefore, for a final evaluation of a possible glycolytic function of the EMP pathway in thermoacidophiles ongoing experimental information has to be awaited.

The antagonistic counterpart of PFK, archaeal type FBPase Class V, required for gluconeogenesis has been characterized from *Thermococcus kodakaraensis* (Rashid et al. 2002) and *S. solfataricus* (unpublished data) and is present in all analyzed thermoacidophilic genomes. Strikingly, in the genomes of the Thermoplasmatales (*P. torridus*, *T. acidophilum*, *T. volcanium*) no candidates for the archaeal type class I FBP aldolase and, as shown for all Archaea, no classical class I and class II enzymes could be identified, suggesting the presence of a novel, so far unknown, aldolase in these organisms. In a recent study it was shown that the class V phosphatases of *Cenarchaeum symbiosum, Ignococcus hospitalis, M. sedula* and, *Thermoproteus neutrophilus* is bifunctional and exhibits both fructose-1,6-bisphosphate aldolase as well as phosphatase (FBPA/ase) activity (Say and Fuchs 2010). This bifunctional character has also been demonstrated for the *S. solfataricus* enzyme encoded by SSO0286 (unpublished data, Siebers et al.). However, in the genome of *S. solfataricus* an additional candidate with homology to FBPA class Ia could be identified (SSO3226, *Table 4.10.2*) and its function still has to be clarified.

Like in Bacteria and Eucarya, another important control point of the pathway is established at the level of phosphoenolpyruvate (PEP) – pyruvate conversion by the antagonistic enzyme couple PEP synthetase (PEPS) and pyruvate kinase (PK) (for review see Siebers and Schoenheit 2005; Tjaden et al. 2006; Zaparty et al. 2008a). PEPS and PK candidates were identified in all analyzed genomes (*Table 4.10.2*). In contrast to the unidirectional, anabolic PEPS and glycolytic PK, pyruvate, phosphate dikinase (PPDK) catalyzes the reversible reaction and the previously characterized enzyme of *T. tenax* has been shown to favor the catabolic direction (Tjaden et al. 2006). Putative PPDK candidates are found in all crenarchaeal species with the exception of *S. acidocaldarius* and *C. maquilingensis* (*Table 4.10.2*). However, these candidates only encode the PEP/pyruvate binding domain of PPDK (pfam01326).

Tricarboxylic Acid (TCA) Cycle, Including the Glyoxylate Shunt and C_3/C_4 Conversions

In all thermoacidophiles, organic compounds are completely oxidized to CO_2 via the oxidative TCA cycle under aerobic growth conditions, generating additional reduced electron carriers (NAD(P)H, ferredoxin$_{red}$, $FADH_2$) and energy. The pyruvate derived from the branched ED pathway and other intermediary glycolytic routes is converted to acetyl-CoA via oxidative decarboxylation by pyruvate-ferredoxin oxidoreductase (POR; reaction 33 *Fig. 4.10.3*) in Archaea. In all nine thermoacidophilic genomes, homologs of 2-oxoacid-ferredoxin oxidoreductases could be identified (*Table 4.10.3*). However, a reliable functional assignment of these candidates, based on sequence analysis alone, is not possible unequivocally and requires biochemical analyses in the case of candidates coding for pyruvate synthase (POR) or α-oxoglutatrate ferredoxin oxidoreductase (OOR) (*Table 4.10.3*; reactions 33 and 37, *Fig. 4.10.2*).

In addition, genes coding for the 2-oxoacid dehydrogenase complex known from Bacteria and Eucarya could be identified in Archaea (Sisignano et al. 2009; Heath et al. 2007). However, so far only a function in the degradation of branched amino acids has reported in *Haloferax volcanii* and *T. acidophilum* (Heath et al. 2007; Sisignano et al. 2009). The enzyme of

Table 4.10.3

Identified candidate genes encoding enzymes involved in the TCA cycle, C_3/C_4 conversions, and the glyoxylate cycle of the thermoacidophilic Archaea with available genome sequence information

# *Fig. 4.10.3*	Enzyme	EC #	Gene ID best hit and e-value									References
Tricarboxylic acid cycle			SSO	LS215 (e-value)	Saci (e-value)	ST (e-value)	M_{sed} (e-value)	C_{maq} (e-value)	PTO (e-value)	Ta (e-value)	TVN (e-value)	
33/37	Pyruvate/α-OG fd: oxidoreductase, (POR/OOR) α-Subunit	1.2.7.–	2815	2658 (8e-179)	0209 (0.0) 2306 (0.0)	2300 (0.0) 2435 (0.0)	0525 (0.0)	1071 (0.0)	1000 (7e-148)	0773 (1e-155)	0846 (6e-149)	
	β-Subunit	1.2.7.–	2816	2657 (6e-174)	2307 (6e-128)	2298 (8e-132)	0524 (1e-138)	0785 (1e-113)	0999 (2e-91)	0772 m (3e-97)	0847 (5e-96)	
33/37	Pyruvate/α-OG fd: oxidoreductase, (POR/OOR)	1.2.7.–	2756	2728 (1e-163)	2253 (4e-144)	1787 (8e-118)	0309 (3e-122)	1713 (3e-85)	1361 (2e-09)	0629 (1e-62)	0836 (3e-66)	
	β-Subunit											
	α-Subunit	1.2.7.–	2757	2727 (0.0)	2252 (0.0)	1532 (4e-102)	0308 (1e-167)	1712 (2e-94)	1360 (6e-17)	0628 m (1e-87)	0835 (6e-88)	
	γ-Subunit	1.2.7.–	2758	2725 (9e-97)	2250 (5e-68)	1531 (3e-37)	0306 (2e-57)	1863 (8e-28) 1710 (3e-25)		0626 m (1e-31)	0833 (1e-29)	
33/37	Pyruvate/α-OG fd: oxidoreductase, (POR/OOR) β-Subunit	1.2.7.–	1206	0395 (8e-163)	2253 (2e-79)	1533 (6e-147)	0510 (3e-128)	1713 (3e-83)	1361 (2e-08)	0629 (2e-58)	1334 (5e-05) 0836 (2e-58)	

Table 4.10.3 (Continued)

# *Fig. 4.10.3*	Enzyme	EC #	Gene ID best hit and e-value									References
	α-Subunit	1.2.7.–	1207	0396 (0.0)	2252 (2e-94)	1532 (0.0)	0509 (7e-166)	1712 (3e-103)	1360 (8e-13)	0628 m (1e-84)	0835 (1e-83)	
	γ-Subunit	1.2.7.–	1208	0398 (2e-94)	2250 (4e-34)	1531 (4e-84)	0507 (5e-65)	1710 (1e-29)		0626 m (4e-34)	0833 (9e-32)	
	δ1-Subunit	1.2.7.–	7412	0397 (5e-42) 2726 (6e-20)	2251 (3e-17)		0508 (1e-35)	1711 (1e-23)		0627 (3e-14)	0834 (1e-13)	
	δ2-Subunit	1.2.7.–	11071	2726 (5e-47)	2251 (5e-39)		0307 (1e-27)	1711 (3e-21)		0627 (5e-12)	0834 (7e-12)	
34	Citrate synthase (CS)	2.3.3.1	2589	2888 (0.0)	0243 (1e-122)	1805 (6e-153)	0281 (5e-155)	0306 (2e-89)	0889 (1e-118)	0819 (2e-122)	0239 (1e-120)	Danson et al. (1985); Smith et al. (1987)
35	Aconitase (ACN)	4.2.1.3	1095	1228 (0.0)	1214 (0.0)	0833 (0.0)	1467 (0.0)	0626 (0.0)	0935 (0.0)	0112 (0.0)	0189 (0.0)	Uhrigshardt et al. (2001)
36	Isocitrate dehydrogenase (IDH)	1.1.1.41	2182	2965 (0.0)	2375 (0.0)	2166 (0.0)	0703 (0.0)	1738 (2e-162)	0168 (5e-142)	0117 (4e-126)	0195 (5e-126)	Camacho et al. (1995)
38	Succinyl-CoA synthetase α-Subunit	6.2.1.5	2482	0303 (3e-146)	1266 (8e-103)	0962 (3e-106)	1581 (3e-103)	1221 (1e-89)	1002 (4e-72)	1331 (4e-70)	0273 (8e-70)	Danson et al. (1985)
	β-Subunit	6.2.1.5	2483	0304 (0.0)	1265 (5e-137)	0963 (4e-111)	1582 (2e-126)	1220 (2e-65)	1003 (2e-57)	1332 m (2e-60)	0274 (1e-60)	

39	Succinate dehydrogenase (SDH-A) flavoprotein subunit	1.3.99.1	2356	0186 (0.0)	0982 (0.0)	0497 (0.0)	0677 (0.0)	1869 (4e-133)	0995 (0.0)	1001 (4e-130)	0747 (2e-135)	Janssen et al. (1997)
	SDH-B, iron-sulfur protein	1.3.99.1	2357	0187 (2e-179)	0981 (4e-146)	0498 (3e-151)	0676 (2e-141)	1355 (8e-45)	0996 (5e-42)	1002 (5e-42)	0746 (1e-39)	
	SDH-C, cytochrome-b subunit	1.3.99.1	2358	0188 (2e-164)	0980 (4e-130)	0499 (4e-131)	0675 (1e-130)		0997 (1e-113)			
	SDH-D, flavoprotein subunit	1.3.99.1	2359	0189 (1e-57)	0979 (5e-32)	0500 (3e-27)	0674 (1e-27)		0998 (2e-24)			
40	Fumarate hydratase (FumR), class II	4.2.1.2	1077	1243 (0.0)	0122 (0.0)	2023 (0.0)	1462 (0.0)	0931 (0.0)	0427 (5e-68)	0258 (8e-79)	1336 (1e-71)	Puchegger et al. (1990)
41	Malate dehydrogenase (MDH)	1.1.1.37	2585	2892 (0.0)	0246 (5e-118)	1811 (2e-128)	0455 (1e-127)	1675 (6e-27)	0994 (5e-23)	0952 (2e-26)	1097 (2e-27)	Goerisch et al. (1985)
C_3/C_4 conversions												
42	PEP carboxylase (PEPC)	4.1.1.31	2256	0069 (0.0)	0059 (0.0)	2101 (0.0)	0756 (0.0)	1916 (2e-160)	0964 (8e-158)			Ettema et al. (2004)
43	PEP carboxykinase (PEPCK)	4.1.1.32	2537	0433 (0.0)	1100 (0.0)	1058 (0.0)	1452 (0.0)	0716 (0.0)		0123 (5e-73)	0200 (2e-70)	
44	Malic enzyme (MAE)	1.1.1.38	2869	2611 (0.0)	2154 (0.0)	0114 (1e-167)	1054 (3e-163)	0373 (9e-157)	0957 (3e-125)	0456m (2e-133)	0768 (1e-131)	Bartolucci et al. (1987)
45	Pyruvate carboxylase (PYC)	6.4.1.1	2466	0285 (0.0)	0260 (0.0)	0593 (0.0)	0147 (0.0)	1296 (2e-07)	0899			

Table 4.10.3 (Continued)

# *Fig. 4.10.3*	Enzyme	EC #	Gene ID best hit and e-value									References
Glyoxylate shunt												
46	Isocitrate lyase (ICL)	4.1.3.1	1333	1004 (0.0)	2191 (0.0)	1808 (8e-11)	0279 (2e-11)	0783 (4e-10)	0170 (3e-08)	1227 (1e-12)	0368 (4e-15)	Uhrigshardt et al. (2002)
47	Malate synthase (MS)	2.3.3.9	1334	1003 (0.0)	2190 (0.0)		1042 (0.0)					
48	Acetyl-CoA synthetase (ACD), ADP-forming	6.2.1.13						1093 (7e-89)* 1092 (6e-53)*		1153 (2e-112)*	1233 (2e-113)*	

Characterized candidates are underlined and the respective references are given.
*Blast searches with the *P. furiosus* AcdAI (PF1540) and AcdBI (PF1787) (Musfeldt et al. 1999).

T. acidophilum shows additional minor activity with pyruvate, but the physiological function needs to be clarified (Heath et al. 2007).

Acetyl-CoA is further oxidized forming two molecules carbon dioxide (CO_2) via the "conventional" oxidative tricarboxylic acid (TCA) cycle (Fig. 4.10.3; reactions 34–41). In anaerobic fermentative Archaea, such as *Pyrococcus furiosus*, ADP-forming acetyl-CoA synthetase (ACD; reaction 48 Fig. 4.10.3; α- and β-subunit, PF1540, PF1787; Musfeldt et al. 1999) is responsible for acetyl-CoA conversion to acetate, which is coupled to energy (ATP) conservation. Homologs of both ACD subunits could only be identified in the genome of the facultative microaerophilic *C. maquilingensis* (Table 4.10.3).

In the two facultative anaerobic *Thermoplasma* species, significant candidates for AcdAI were identified (Table 4.10.3), however not for AcdBI (Ta1332m, e-value 0.016 and TVN0274, e-value 0.010).

All required candidate genes for a complete oxidative TCA cycle could be identified in the genomes of the five Sulfolobales and *P. torridus* (Table 4.10.3). Only in the genomes of the facultative microaerophilic *C. maquilingensis* and the two facultative anaerobes *T. acidophilum* and *T. volcanium* the γ- and δ-subunit of the succinate dehydrogenase is lacking (*sdhC* and *sdhD*; reaction 39, Fig. 4.10.3).

Besides its key role in catabolism, the TCA cycle also possesses important functions in biosynthesis by providing several important precursors, e.g., for amino acid biosynthesis. Therefore, anaplerotic reactions, catalyzed by phosphoenolpyruvate (PEP) carboxylase (PEPC; reaction 42, Fig. 4.10.3) and/or pyruvate carboxylase (PYC; reaction 45, Fig. 4.10.3), both forming oxalacetate from PEP, are required in order to maintain the metabolic flux. On the other hand, in case of intermediate accumulation of malate and/or oxalacetate, the flux can be regulated by the action of malic enzyme (MAE, reaction 44, Fig. 4.10.3) and/or PEP carboxykinase (PEPCK, reaction 43, Fig. 4.10.3), forming pyruvate (MAE) or PEP (PEPCK) from malate by oxidative decarboxlation.

These C_3/C_4 converting reactions catalyzed by PEPC, PEPCK, PYC, and/or MAE connect the TCA cycle to pyruvate and PEP in all investigated members of the Sulfolobales as well as in *C. maquilingensis*. In the genomes of the Thermoplasmatales a good candidate for PYC is missing. Furthermore, the two *Thermoplasma* species lack a PEPC and *P. torridus* a PEPCK homolog.

The genomes of all Sulfolobales, with the only exception of *S. tokodaii*, harbor genes coding for isocitrate lyase and malate synthase (Fig. 4.10.3; reactions 46 and 47 Fig. 4.10.3) and, therefore, seem to possess a functional glyoxylate cycle, which is normally employed during growth on acetate. *C. maquilingensis* and the three Thermoplasmatales only possess a gene with similarity to isocitrate lyase, which probably also serves to replenish the TCA cycle (Snijders et al. 2006).

As mentioned above, *M. sedula* is a facultative heterotrophe and is able to fix CO_2 via the recently discovered fifth pathway for CO_2 fixation, the modified 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg et al. 2007). This pathway has also been described for the autotrophic *Sulfolobus* sp. strain VE6 (Huegler et al. 2003a; Berg et al. 2007). Acetyl-CoA is converted to succinyl-CoA (via malonyl-CoA, 3-hydroxypropionate, propionyl-CoA, and methylmalonyl-CoA). Within the cycle two carboxylation steps take place: Acetyl-CoA and propionyl-CoA are carboxylated by the two enzymes acetyl-CoA and propionyl-CoA carboxylase, respectively (Menendez et al. 1999). The formed succinyl-CoA serves as a precursor for anabolic purposes and as precursor for the regeneration of acetyl-CoA (Berg et al. 2007).

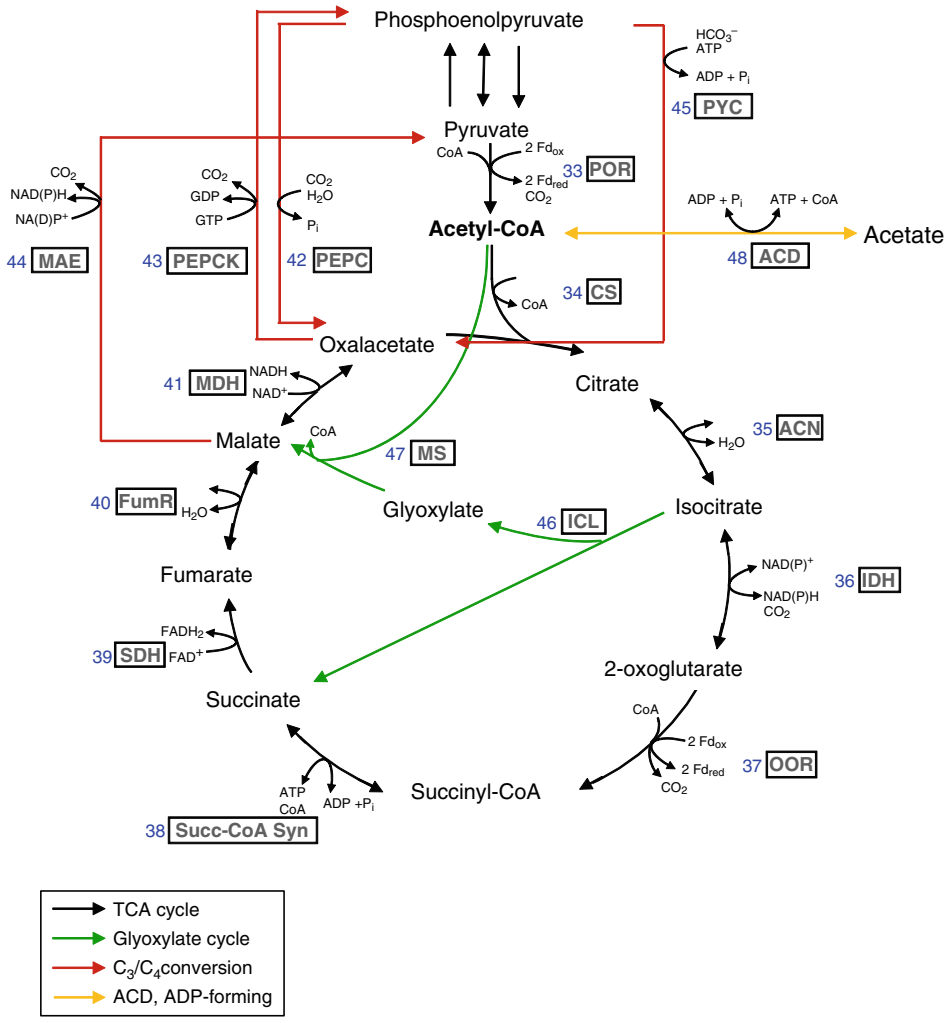

Fig. 4.10.3

The reconstructed tricarboxylic acid cycle including the glyoxylate shunt and C_3/C_4 conversions. Reactions of the TCA cycle are marked by black, the glyoxylate cycle by green, the different enzymes involved in C_3/C_4 conversions by red, and the ACD reaction by orange arrows. For details on missing candidates identified in some thermoacidophiles see *Table 4.10.3* and discussion in the text. *Intermediates*: CoA, coenzyme A; $Fd_{ox/red}$, ferredoxin oxidized/reduced form. *Abbreviations enzymes (reaction number)*: (33) POR, pyruvate synthase; (34) CS, citrate synthase; (35) ACN, aconitase; (36) IDH, isocitrate dehydrogenase; (37) OOR, α-oxoglutarate ferredoxin: oxidoreductase; (38) Succ-CoA Syn, succinyl-CoA synthetase; (39) SDH, Succinate dehydrogenase; (40) FumR, fumarate reductase; (41) MDH, malate dehydrogenase; (42) PEPC, phosphoenolpyruvate (PEP) carboxylase; (43) PEPCK, PEP carboxykinase; (44) MAE, malic enzyme; (45) PYC, pyruvate carboxylase; (46) ICL, isocitrate lyase; (47) MS, malate synthase; (48) ACD, acetyl-CoA synthetase, ADP-forming

Pentose Phosphate Metabolism

In Bacteria and Eucarya, generally a "classical" oxidative pentose phosphate pathway (PPP) is present that is responsible for the generation of reducing power (NADPH) and synthesis of precursors for nucleotide and histidine biosynthesis (ribose 5-phosphate) as well as for aromatic amino acids (erythrose 4-phosphate). In the oxidative phase of PPP (OPPP) 2 molecules of NADPH are generated, and during the non-oxidative phase (NOPPP) biosynthetic building blocks are formed. This conventional oxidative PPP common in Bacteria and Eucarya seems to be generally absent in Archaea (Verhees et al. 2003). No orthologs of the classical oxidative PPP genes encoding glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, and 6-phosphogluconate dehydrogenase/decarboxylase are found in archaeal genomes with one exception: an ortholog of 6-phosphogluconate DH (COG1023) has been found in *Halobacterium* sp. NRC-1 (Soderberg 2005). Instead, in Archaea it appears that pentoses for anabolic purposes are provided by the non-oxidative pentose phosphate (NOPP) pathway and/or the reversed ribulose monophosphate (RuMP) pathway (Soderberg 2005; Orita et al. 2006). The RuMP pathway was originally described as a pathway for the fixation of formaldehyde in methylotrophic Bacteria, but is now recognized as a widespread prokaryotic pathway.

The two key enzymes of the reversed RuMP pathway, the 3-hexulose-6-phosphate isomerase (PHI; reaction 50, *Fig. 4.10.4*) and the 3-hexulose-6-phosphate synthase (HPS; reaction 51, *Fig. 4.10.4*), transform fructose 6-phosphate (F6P) to ribulose 5- phosphate (Ru5P) and formaldehyde (*Fig. 4.10.4*), as recently shown for the HPS-PHI fusion protein of the hyperthermophilic Euryarchaeota *Thermococcus kodakaraensis* (TK0475; Orita et al. 2006) and *Pyrococcus horikoshii* (PHO1938; Orita et al. 2005).

All investigated thermoacidophiles, except the three Thermoplasmatales, harbor two genes coding for single HPS and PHI, respectively (*Table 4.10.4*), thus, indicating that the synthesis

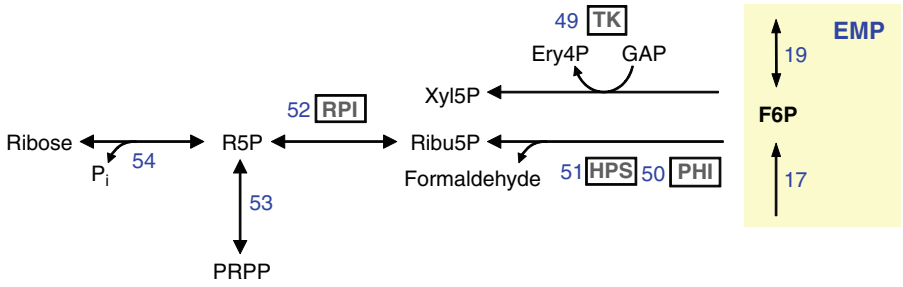

Fig. 4.10.4

Reconstructed pentose phosphate pathway in thermoacidophilic Archaea. For details on missing candidates identified in some thermoacidophiles see *Table 4.10.4* and discussion in the text. *Intermediates*: Ery4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; R5P, ribose 5-phosphate; Ribu5P, ribulose 5-phosphate; PRPP, phosphoribosyl pyrophosphate ; Xyl5P, xylulose 5-phosphate. *Abbreviations enzymes (reaction number)*: (49) TK, transketolase; (50) PHI, 3-hexulose-6-phosphate isomerase; (51) HPS, 3-hexulose-6-phosphate synthase; (52) RPI, ribose-5-phosphate isomerase; (53) ribose phosphate pyrophosphokinase; (54) ribokinase

Table 4.10.4

Identified candidate genes encoding enzymes involved in the pentose phosphate metabolism of the thermoacidophilic Archaea with available genome sequence information

# *Fig. 4.10.4*	Enzyme	EC #	Gene ID best hit and e-value								
Pentose phosphate metabolism			SSO	LS215 (e-value)	Saci (e-value)	ST (e-value)	Msed (e-value)	Cmaq (e-value)	PTO (e-value)	Ta (e-value)	TVN (e-value)
49	Transketolase (TK) N-terminal subunit	2.2.1.1	0299	1940 (8e-179)	0181 (6e-146)	2269 (2e-143)	1878 (6e-136)	0809 (4e-102)	0690 (2e-66)	0617 (8e-73)	0667 (5e-71)
	Transketolase (TK) C-terminal subunit	2.2.1.1	0297	1941 (1e-162)	0180 (2e-123)	2268 (3e-125)	1879 (5e-123)	0809 (2e-54)	0691 (1e-47)	0618 (5e-46)	0668 (2e-51)
50	3-Hexulose-6-phosphate isomerase (PHI)	5.3.1.27	0151	2152 (4e-116)	0747 (2e-87)	0198 (4e-88)	0236 (2e-87)	0308 (5e-69)			
51	3-Hexulose-6-phosphate synthase (HPS)	4.1.2.43	0202	2094 (4e-117)	0802 (2e-71)	0238 (7e-76)	0227 (4e-72)	1871 (3e-28)			
52	Ribose-5-phosphate isomerase (RPI)	5.3.1.6	0978	1331 (2e-112)	1301 (2e-63)	1302 (8e-66)	2287 (3e-61)	0742 (1e-25)	0565 (7e-15)	0878 (4e-15)	0717 (6e-18)
53	Ribose phosphate pyrophosphokinase	2.7.6.1	1045	1273 (1e-154)	1282 (7e-94)	0946 (7e-101)	1790 (8e-101)	0488 (2e-49)	0906 (9e-30)	0119m (1e-34)	0197 (2e-31)
54	Ribokinase	2.7.1.15	0004	2276 (1e-154)	0272 (6e-101)	2328 (3e-104)	0185 (6e-92)	0147 (9e-28)			
	Purine nucleoside phosphorylase	2.4.2.1	1519	2770 (2.6)	1247 (0.023)	2449 (7e-131)	1578 (0.014)	0392 (3e-06)			
			2706	2770 (3e-126)	1247 (1e-89)	0975 (2e-95)	1578 (8e-94)	1546 (1e-56)	0123 (3e-31)		
	2-Deoxyribose-5-phosphate aldolase (DERA)	4.1.2.4								0684 (1e-36)*	0175 (2e-35)*

*Blast search with the *T.kodaraensis* DERA (TK2104; Rashid et al. 2004).

Table 4.10.5

Identified candidate genes encoding enzymes involved in glycogen and trehalose metabolism of thermoacidophilic Archaea with available genome sequence information

# *Fig. 4.10.2*	Enzyme	EC #	Gene ID best hit and e-value									References
Glycogen metabolism			SSO	LS215 (e-value)	Saci (e-value)	ST (e-value)	M_{sed} (e-value)	C_{maq} (e-value)	PTO (e-value)	Ta (e-value)	TVN (e-value)	
21	Phosphomanno-/phosphoglucomutase (PMM/PGM)	5.4.2.2	0207	2090 (0.0)	0806 (0.0)	0242 (0.0)	0223 (0.0)	1688 (2e-75)	1166 (9e-81)	0568 (4e-86)	0620 (3e-88)	Solow et al. (1998); Ray et al. (2005)
22	Glycogen-debranching protein (TreX)	3.2.1.33	2094	0444 (0.0)	1439 (0.0)	0928 (0.0)	0912 (4e-33)					Park et al. (2007); Woo et al. (2008)
23	Glycogen phosphorylase (GLGP)	2.4.1.1	2538	0434 (0.0)	0294 (4e-69)	0893 (0.0)	1844 (3e-05)	1332 (2e-100)				
		2.4.1.1	2775	2708 (2e-176)	1907 (3e-103)	2386 (3e-114)	1844 (1e-100)	1471 (2e-13)	1239 (2e-16)	0340 (1e-13)	0880 (1e-41)	
24	NDP-glucose pyrophosphorylase	2.7.7.-	0813	1018 (9e-89)	0422 (1e-23)	2352 (3e-23)	1811 (2e-88)	0056 (4e-12)	0307 (2e-15)	1486 (2e-07)	0529 (1e-18)	
			1782	1052 (3e-159)	1703 (6e-127)	1971 (8e-178)	1845 (3e-68)	1475 (8e-49)	0307 (8e-106)	1486 (5e-32)	0899 (8e-70)	
			0831	1052 (5e-163)	1703 (4e-123)	1971 (3e-174)	1845 (1e-71)	1475 (5e-48)	0307 (2e-104)	1486 (2e-33)	0899 (2e-72)	

Table 4.10.5 (Continued)

# *Fig. 4.10.2*	Enzyme	EC #	Gene ID best hit and e-value									References
			0745	1492 (0.0)	0619 (7e-129)	0452 (8e-129)	0068 (9e-122)	0561 (1e-22)	0307 (6e-30) / 0702 (4e-24)	1486 (4e-32)	0081 (4e-34) / 0899 (9e-26)	
25	Glycogen synthase (GLGA)	2.4.1.11	0987	1324 (0.0)	1201 (0.0)	0818 (0.0)	1417 (0.0)	1455 (8e-05)	1239 (2e-06)	0340 (4e-05)	0430 (6e-05)	Cardona et al. (2001)
26	Glucan-1,4-α-glucosidase (GAA)	3.2.1.3	0990	1321 (0.0)	1198 (0.0)	0815 (0.0)	1420 (0.0)	0090 (6e-49)	1492 (1e-104)	0342 (2e-110)	0428 (4e-110)	Serour and Antranikian (2002)
			2473	0291 (0.0)	1198 (4e-79)	2017 (0.0)	1420 (1e-78)	0090 (2e-43)	1492 (4e-104)	0342 (3e-111)	0428 (1e-108)	
			2742	2741 (0.0)	1816 (3e-177)	1124 (0.0)	1322 (0.0)	0090 (4e-15)	0598 (3e-63)	0286 m (2e-61)	1315 (1e-62)	
			2754	2730 (0.0)	1250 (0.0)	0973 (0.0)	1322 (5e-135)	0090 (2e-18)	0598 (6e-86)	0286 m (2e-86)	1315 (4e-87)	
Trehalose metabolism												
27	Maltooligosyltrehalose synthase (TreY/ MTSase)	5.4.99.1	2095	0443 (0.0)	1436 (0.0)	0929 (0.0)						Maruta et al. (1996)
28	Trehalose trehalohydrolase (TreZ/ MTHase)	3.2.1.141	2093	0445 (0.0)	1440 (0.0)	0927 (1e-138)						Maruta et al. (1996)

29	Trehalose glycosyl-transferring synthase (TreT)	2.4.1.B2	2609	2870 (0.0)	1827 (0.0)	0404 (1e-171)	0129 (4e-175)	0444 (6e-08)				
30	Trehalose-6-phosphate synthase (OtsA/TPS)	2.4.1.15							1209 (2e-70)*	1210 (5e-68)*	1254 (7e-67)*	
31	Trehalose-6-phosphate phosphatase (OtsB/TPP)	3.1.3.12							1210 (9e-06)	1209 m (8e-10)**	1255 (1e-13)**	
32	Trehalose synthase (TreS)	5.4.99.16							0069 0071	0138 (5e-19)	0217 (6e-17)	Chen et al. (2006)

*Blast search with the *T.kodaraensis* DERA (TK2104; Rashid et al. 2004).

**Blast searches with the *E. coli* TPS (OtsA) and TPP (OtsB) (Kaasen et al. 1994; Giæver et al. 1988).

of the required precursor molecule R5P is accomplished by the reversed RuMP pathway in these organisms. For Thermoplasmatales it is tempting to speculate if an alternative, novel route is involved.

The precursor for the synthesis of aromatic amino acids, erythrose 4-phosphate (E4P), is gained from F6P and GAP via the transketolase reaction (reaction 49, *Fig. 4.10.4*). Homologs are identified in all nine described thermoacidophiles (*Table 4.10.4*). In most Archaea the two ORFs encoding the N- and the C-terminal half of the transketolase (*tktA*, *tktB*) seem to form an operon. In all crenarchaeal genomes, *tktA*, *tktB* (*tktAB* in *C. maquilingensis*) cluster with genes involved in the synthesis of the aromatic amino acids, e.g., coding for chorismate synthase (*aroG*), 3-dehydroquinate synthase (*aroB*), or shikimate 5-dehydrogenase (*aroE*) supporting the predicted function.

Another metabolic link between the pentose phosphate metabolism and the central pathways of glycolysis and gluconeogenesis is established in some Archaea by deoxyribose-phosphate aldolase (DERA, archaeal type class I FBP aldolase (DhnA family); EC 4.1.2.4; *Table 4.10.4*), which was recently characterized from *T. kodakaraensis* (Rashid et al. 2004). DERA catalyzes the reversible conversion of 2-deoxy-D-ribose 5-phosphate to GAP and acetaldehyde and is involved in interconversion between nucleosides and central carbon metabolism. Homolgs could only be identified in the genomes of the two *Thermoplasma* species and are absent in the crenarchaeal genomes and *P. torridus*. Both homologs share significant similarity to the characterized DERA of *T. kodakaraensis* (Rashid et al. 2004; Ta0684, 1e-36 and TVN0175, e-value 2e-35).

Glycogen and Trehalose Metabolism

In many Archaea, the branched glucose polymer *glycogen* (α1,4- and α1,6- linked glucosyl moieties) has been shown to serve as long-term carbon and energy storage (Koenig et al. 1982; Mizanur et al. 2008).

NTP-glucose-1-phosphate uridylyltransferase (NDP-glucose pyrophosphorylase; reaction 24, *Fig. 4.10.2*) catalyzes the synthesis of NDP-glucose from glucose 1-phosphate (G1P) and NTP. G1P is formed from glucose 6-phosphate (G6P) by the phosphoglucomutase (PGM, reaction 21, *Fig. 4.10.2*) reaction (*Table 4.10.5*). NDP-glucose pyrophosphorylase and PGM homologs are encoded in all nine genomes analyzed (*Table 4.10.5*). The *S. solfataricus* ORFs SSO1782, SSO0831, and SSO0813 show similarity (3e-23, 4e-23 and 5e-20) to the recently characterized sugar nucleotidyltransferase from *P. furiosus*, which shows an unusually broad substrate tolerance (PF1356; Mizanur et al. 2004). Glycogen synthase (GLGA; reaction 25, *Fig. 4.10.2*) is a UDP-glucose-glycogen glycosyltransferase that adds glucose from activated glucose to the growing polymeric chain $[(1,4\text{-}\alpha\text{-D-glucosyl})_{n+1}]$. Respective GLGA homologs with high similarity to the *S. acidocaldarius* enzyme (Cardona et al. 2001) could be identified in the five Sulfolobales genomes and in *C. maquilingensis* (*Table 4.10.5*). However, in the genomes of the three Thermoplasmatales respective candidate genes could not be identified unequivocally.

The glycogen-debranching enzyme, which catalyzes the hydrolysis of 1,6- α bonds (TreX; reaction 22, *Fig. 4.10.2*), and glycogen phosphorylase (GLGP; reaction 23, *Fig. 4.10.2*), which catalyzes the P_i-dependent degradation of glycogen forming glucose-1-phosphate, are responsible for the degradation of the storage compound. The GLGP of the hyperthermophilic Crenarchaeote *T. tenax* (Siebers et al. 2004) and the Euryarchaeote *Pyrococcus furiosus*

(Mizanur et al. 2008) have recently been characterized. In crenarchaeal genomes two GLGP candidates could be identified. The *S. solfataricus* candidate SSO2538 shares 50 % identity to the characterized enzyme from *T. tenax*. TreX homologs are present in the five Sulfolobales genomes, but not in *C. maquilingensis* (Table 4.10.5), whereas all these six organisms possess a gene coding for GLGP (Table 4.10.5). In Thermoplasmatales no obvious candidates for TreX and GLGP were identified. In addition, for *P. torridus* and *T. acidophilum* it was difficult to differentiate between GLGA and GLGP candidates.

Beside the phosphorolytic degradation of glycogen, catalyzed by GLGP, hydrolytic mobilization of glucose from the storage compound via glucoamylase (glucan-1,4- α glucosidase, GAA; reaction 26, Fig. 4.10.2) also seems to take place. GA catalyzes the hydrolysis of α-1,4 glycosidic bonds and, therefore, releases glucose, which will enter the branched ED pathway. GA homologs were identified in all analyzed genomes (Table 4.10.5).

Clustering of the *treX* gene, with the genes coding for enzymes required for the synthesis of the compatible solute trehalose (*treZ-treX-treY* operon; Maruta et al. 1996; see below) in all Sulfolobales (except *M. sedula*), points to close functional relation between glycogen and trehalose metabolism in these organisms. The TreX of *S. solfataricus* (SSO2094; Table 4.10.5) has been shown to possess bifunctional glycogen-debranching activity, catalyzing the hydrolysis of α-1,6-glycosidic bonds, and in addition exhibits α-1,4-transferase activity (α-1,4-glucanotransferase; EC 2.4.1.25; Park et al. 2007; Woo et al. 2008). Therefore, glycogen-debranching activity of the enzyme provides maltodextrins from glycogen that are subsequently converted to trehalose by TreY and TreZ (SSO2095, SSO2093, Table 4.10.5; Maruta et al. 1996; see below) or glucose.

Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a naturally occurring alpha α-1,1-linked glucose disaccharide that is present in a great variety of organisms (Bacteria, Archaea, fungi, plants, invertebrates, mammals). Besides an initially supposed function as carbon and energy source (Elbein 1974), trehalose has been shown to be involved in stress response, e.g., to anhydrobiosys, temperature (heat, cold), or salt stress, in Bacteria and Eucarya (Giæver et al. 1988; Kaasen et al. 1994; De Virgilio et al. 1994). In contrast to glycogen, trehalose represents an osmotically active compound that can be accumulated up to extremely high intracellular concentrations (e.g., up to 0.4 M in *E. coli* in response to high osmolarity (Strom and Kaasen 1993); up to 1 g/g protein in *S. cerevisiae* due to heat stress (Hottiger et al. 1987; De Virgilio et al. 1994)) without negative effects on metabolism (compatible solute; Crowe et al. 1984, Crowe and Crowe 1992; Arguelles 2000). Due to its physical and chemical properties, which include high hydrophilicity, high thermo- and pH-stability, and the absence of internal hydrogen bonds, trehalose finds broad industrial application, e.g., in food or pharmaceutical industry. It has also become a widely valued preservative (Arguelles 2000).

Trehalose has also been identified in several members of the Archaea e.g., *S. solfataricus*, *T. acidophilum*, *M. sedula* or *A. ambivalens* (Nicolaus et al. 1988; Martins et al. 1997). However, the physiological function of the disaccharide has not yet been solved. From biochemical and genomic analysis a total of five different pathways for the biosynthesis of the disaccharide have been described within the archaeal domain. The most common and best studied pathway, the *TPS/TPP or OtsA/OtsB pathway*, utilized by several Bacteria and Eucarya, involves the enzymes trehalose-6-phosphate synthase (TPS; OtsA in *E. coli*) and trehalos-6-phosphate phosphatase (TPP; OtsB in *E. coli*) (Giæver et al. 1988; Kaasen et al. 1994). So far this pathway has only been characterized in the hyperthermophilic crenarchaeon *Thermoproteus tenax* (Siebers et al. 2004; Zaparty 2007).

In the genomes of the three euryarchaeal Thermoplasmatales (*P. torridus*, *T. acidophilum*, and *T. volcanium*), genes with similarity to *E.coli* TPS (OtsA) and *E.coli* TPP (OtsB) were identified (reactions 30 and 31 Fig. 4.10.2; Table 4.10.5).

In contrast, the crenarchaeal thermoacidophiles produce trehalose from maltooligosaccharides via the so-called *TreY-TreZ-pathway* that has been characterized in *S. solfataricus* (Maruta et al. 1996; Kobayashi et al. 1996; Di Lernia et al. 1998; Gueguen et al. 2001; reactions 27 and 28, Fig. 4.10.2). The maltooligosyltrehalose (MOT) synthase (TreY/MTSase) catalyzes the conversion of the terminal α-1,4-linkage of the glucose polymer (e.g., glycogen, starch) into an α-1,1-linkage. In a second step the MOT hydrolase (TreZ/MTHase) releases the terminal α-1,1-linked disaccharide, i.e., trehalose (Maruta et al. 1996). Both enzymes share high similarity to members of the α-amylase enzyme family. TreY and TreZ homologs are encoded in the genomes of Sulfolobales (*S. islandicus*, *S. acidocaldarius*, and *S. tokodaii*, with the only exception of *M. sedula*) (Table 4.10.5). The genome of *P. torridus* harbors two genes annotated as 1,4 alpha-glucan branching enzyme (PTO0067 and PTO0068; EC 2.4.1.18) that show low similarity to SSO2093 (TreZ, 5e-28) and SSO2095 (TreY, 0.001), respectively. Here, a functional analysis of the enzymes is required, in order to determine a role either in glycogen or trehalose metabolism.

A third pathway for trehalose synthesis is catalyzed by *trehalose synthase* (TreS), which converts the α-1,4-linkage of the disaccharide maltose into an α-1,1-linkage forming trehalose. This pathway was firstly described for thermophilic members of the Bacteria, e.g., *Thermus aquaticus* (Tsusaki et al. 1997). Two TreS encoding genes were recently also discovered in *P. torridus* (PTO0069 and PTO0071; Fütterer et al. 2004; reaction 32, Fig. 4.10.2) and PTO0069 has been characterized in detail (Chen et al. 2006). Homologs of the *P. torridus* TreS were identified in the genomes of the other two Thermoplasmatales (Table 4.10.5).

Recently, a fourth pathway has been described for the marine and hyperthermophilic Archaeon *Thermococcus litoralis*, which is characterized by a *glycosyl-transferring trehalose synthase (TreT)* (reactions 29, Fig. 4.10.2). The enzyme catalyzes the reversible formation of trehalose from UDP/ADP-glucose and glucose (Qu et al. 2004). However, due to the organization of the *treT* gene in an operon comprising genes coding for a trehalose/maltose ABC transporter and since the organism does not utilize trehalose as compatible solute, a preferred function of TreT in trehalose degradation has been proposed for *T. litoralis*. Surprisingly, the TreT of *T. tenax*, which also has been characterized, turned out to be an *unidirectional* enzyme that only catalyzes the formation of trehalose (Kouril et al. 2008) (reaction 29, Fig. 4.10.2). This pathway is discussed as a fifth pathway for trehalose biosynthesis.

TreT homologs could be identified in the genomes of all Sulfolobales and *C. maquilingensis*, whereas the pathway seems to be absent in the three Thermoplasmatales (Table 4.10.5). However, in the case of TreT, biochemical analyses have to be awaited, since on the one hand it is not possible to distinguish between the reversible and the unidirectional TreT pathway at sequence level, and on the other hand the TreT also shares high similarity with TreS as well as trehalose phosphorylase (TreP, see below). The genome organization of the *treT* gene in Sulfolobales and *C. maquilingensis* supports an unidirectional functional assignment in these organisms (Fig. 4.10.2, Kouril et al. 2008).

The most common pathway for the degradation of trehalose, described in Bacteria and Eucarya, is hydrolysis by trehalases. In some species, e.g., *Euglena gracilis* (Maréchal and Belocopitow 1972), trehalose phosphorylase (TreP) catalyzes the reversible phosphohydrolysis of trehalose to glucose 1-phosphate and glucose and a mjar function in degradation is

proposed. In *E. coli* (Rimmele and Boos 1994) and *Bacillus subtilis* (Helfert et al. 1995) phosphotrehalase (TreC) converts trehalose 6-phosphate to glucose 6-phosphate and glucose. Interestingly, with the exception of the reversible TreT pathway from *T. litoralis* (Qu et al. 2004), virtually nothing is known about trehalose degrading pathways in Archaea.

In summary, the reconstruction of metabolic networks on the basis of the genome sequence information and available biochemical information sheds some light into the complexity of archaeal metabolism. Network reconstruction allows for the identification of pathway gaps and, thus, points to the presence of new, alternative enzymes and pathways. However, as discussed previously, it is often difficult to predict function from sequence alone and biochemical analyses are required. The network reconstruction in thermoacidophilic Archaea revealed some obvious differences between Crenarchaeota and Euryarchaeota. In the Thermoplasmatales candidates for several essential enzymes, such as FBP aldolase in glucoenogenesis, PHI and PHS of the reversed ribulose monophosphate (RuMP) pathway, as well as GLGP and TreX in glycogen metabolism, seem to be absent. However, since at least gluconeogenesis as well as the formation of pentose phosphate is mandatory, these findings raise questions about the presence of alternative, new enzymes or even pathways in Thermoplasmatales.

This work underlines once again the great potential of the combination of modern high-throughput approaches, here genomics, with classical biochemistry and highlights the amazing diversity of archaeal metabolism, a treasure chest for biotechnology.

Cross-References

4.2 Carbohydrate-Active Enzymes from Hyperthermophiles: Biochemistry and Application

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Part 5

Extremophiles: Piezophiles

5.1 Distribution of Piezophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_5.1,

Numerous cold deep-sea adapted microorganisms (piezophilic, formerly referred to as "barophilic" bacteria) have been isolated using deep-sea research submersibles and/or several sediment/animal sampling systems. Many of the isolates from cold sea bottom are novel psychrophilic bacteria, and we have identified several new piezophilic species, that is, *Photobacterium profundum*, *Shewanella violacea*, *Moritella japonica*, *Moritella yayanosii*, *Psychromonas kaikoi*, and *Colwellia piezophila*. These piezophiles involve five genera in gamma-Proteobacteria subgroup and produce significant amounts of unsaturated fatty acids in their cell membrane fractions to maintain the membrane fluidity in cold and high-pressure environments. Piezophilic microorganisms have been identified in deep-sea bottoms of many of the world's oceans. Therefore, these microbes are well distributed on our planet. This chapter focuses on the distribution and taxonomy of the piezophilic microorganisms and their growth habitats.

Piezophiles are Adapted to the Deep-Sea Environment

It has been suggested that life may have originated in the deep sea some 3.5–4 billion years ago, where it was protected from the damaging effects of ultraviolet light. The deep-sea is a particularly high-pressure environment and hydrostatic pressure could have been a very important stimulus for early forms of life. Scientists have proposed that life might have originated in deep-sea hydrothermal vents (Stetter 1993) and thus it appears possible that high-pressure-adapted mechanisms of gene expression could represent a feature present in early forms of life (Kato and Horikoshi 1996). It has recently been reported that the primary chemical reactions involved in the polymerization of organic materials (i.e., amino acids) could have occurred in such high-pressure and high-temperature environments (Imai et al. 1999). Thus, the study of deep-sea microorganisms may not only enhance our understanding of specific adaptations to abyssal and hadal ocean realms but may also provide valuable insights into the origin and evolution of life on our planet.

In 1949, Zobell and Johnson began investigating the effects of hydrostatic pressure on microbial activities (Zobell and Johnson 1949). They first used the term "barophilic" to define organisms whose optimal growth occurred at pressures higher than 0.1 MPa or a requirement for increased pressure for growth. The term "piezophilic" has been proposed to replace "barophilic," as the prefixes "baro" and "piezo," derived from Greek, mean "weight" and "pressure," respectively (Yayanos 1995). Thus, the word "piezophilic" is more suitable than "barophilic" to describe bacteria that grow better at high-pressure than at atmospheric pressure. Therefore, we have opted to use the term "piezophilic" bacteria, meaning high-pressure-loving bacteria. The definitions of "piezophilic" are shown in *Fig. 5.1.1*.

The Deepest Bottom, Mariana Trench Challenger Deep

The Mariana Trench, Challenger Deep (11°22′N. 142°25′E), is the deepest ocean bottom in the world. In 1996, the sediment samples have been obtained from this extreme environment by the unmanned submersible KAIKO (Kyo et al. 1995) in three dives, numbers 21, 22, and 23, at the depth of 11,000 m (*Fig. 5.1.2*, Kato et al. 1997). It seems likely that these were the first sediment samples recovered from the world's deepest point without any microbiological contamination. In 1960, Jacques Piccard and D. Walsh dived to the world's deepest point

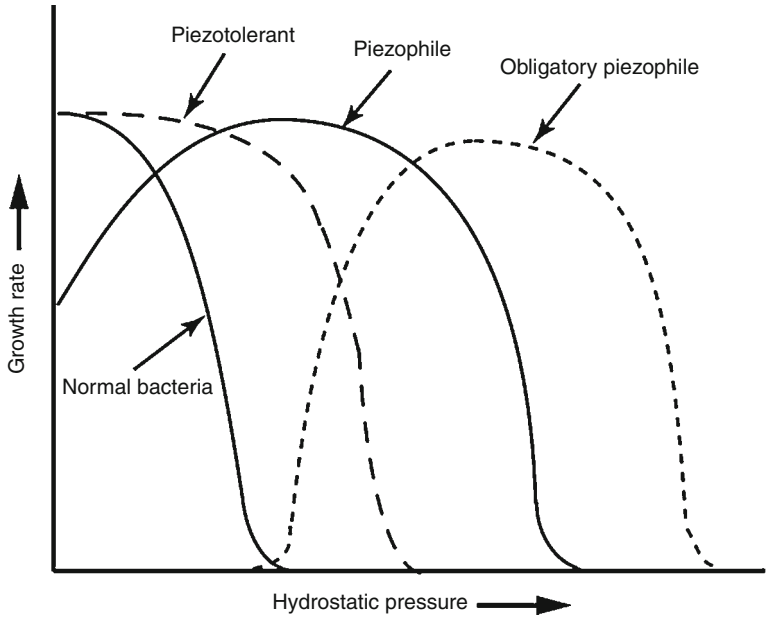

Fig. 5.1.1
Definition of piezophilic growth properties

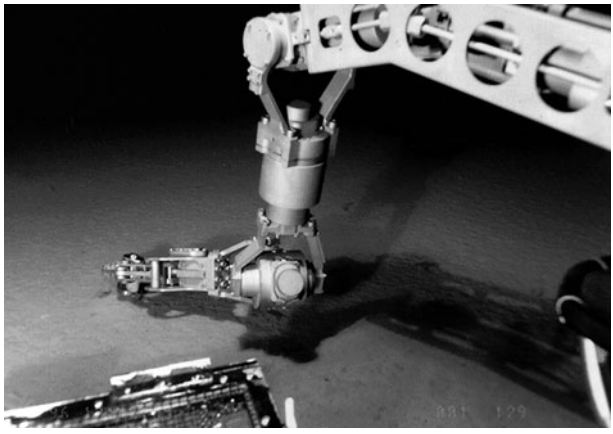

Fig. 5.1.2
Sediment sampling at the Mariana Trench Challenger Deep by means of the unmanned submersible, "KAIKO" system, Dive 10K#21 in 1996

aboard the manned submersible *TRIESTE*. They reported seeing several deep-sea animals and fishes there (Piccard and Dietz 1961). Unfortunately, that dive was not for scientific purposes and no film pictures were made or samples collected, so their story could not be confirmed scientifically.

The Mariana Trench is characterized by cold, dark, remoteness and the highest pressure environment in the ocean bottom. Relatively little is known about the natural bacterial communities in the sediment there because of the need to use uncommon instruments to keep deep-sea organisms at high-pressure and to develop new methods to maintain and cultivate these organisms in the laboratory. There are two isolates, *Pseudomonas bathycetes* and an obligatory barophilic bacterium strain MT41 that have been isolated from the Mariana Trench (Morita 1976; Yayanos et al. 1981). Apparently, *P. bathycetes* is not an organism adapted to the deep-sea environment, as its generation time is 33-days under such conditions (2°C, 100 MPa) (Schwarz and Colwell 1975). This bacterium grows at atmospheric pressure and at temperatures as high as 25°C (Pope et al. 1975). Bacterium strain MT41 was isolated from a deep-sea amphipod (*Hirondellea gigas*) obtained from the Mariana Trench using an insulated trap (Yayanos et al. 1981). This bacterium could grow only under pressure conditions greater than 50 MPa, and is closely related to the genus *Colwellia* (Yayanos et al. 1981; DeLong et al. 1997). The obligatory piezophiles, *M. yayanosii* DB21MT-5 and *Shewanella benthica* DB21MT-2 were isolated from the sediment of the Mariana Trench (Kato et al. 1998; Nogi and Kato 1999). In order to understand the piezophily and natural bacterial communities in the sediment of the Mariana Trench, several future studies have been progressing.

Distribution and Diversity of the Piezophiles

Most *Shewanella* spp. are isolated from ocean environments and some are psychrophilic or psychrotrophic bacteria. The piezophilic *Shewanella* species *S. benthica* and *S. violacea* are also categorized as psychrophilic at atmospheric pressure (Nogi et al. 1998b). *S. gelidimarina* and *S. frigidimarina* isolated from Antarctic ice (Bowmam et al. 1997) and *S. hanedai* isolated from the Arctic Ocean (Jensen et al. 1980) are cold-adapted psychrotrophic bacteria that grow well at low temperature. A phylogenetic tree of these *Shewanella* species within the gamma-Proteobacteria subgroup constructed based on 16S rRNA gene sequences is shown in *Fig. 5.1.3*.

In this tree, two major branches are recognizable in the genus *Shewanella*, indicated by *Shewanella* group 1 and *Shewanella* group 2. Deep-sea *Shewanella* forming the *Shewanella* piezophilic branch are categorized as members of group 1. Interestingly, most *Shewanella* species shown to be psychrophilic or psychrotrophic also belong to group 1. The other species in group 1, *S. pealeana* and *S. woodyi*, isolated from ocean squid and detritus, respectively, grow optimally at 25°C (Leonardo et al. 1999; Makemson et al. 1997) and thus these strains might also be included in the group of cold-adapted bacteria. Most *Shewanella* species in group 2 are not cold-adapted bacteria. They grow well under mesophilic conditions at 25–35°C. *S. frigidimarina*, which can grow optimally below 25°C, is the only exception in this category, although this species belongs to group 2 (Kato and Nogi 2001).

The growth of some of these *Shewanella* species under high-pressure conditions indicates that the members of *Shewanella* group 1 show piezophilic (*S. benthica* and *S. violacea*) or piezotolerant (*S. gelidimarina* and *S. hanedai*) growth properties, although the members of *Shewanella* group 2 generally show piezosensitive growth, that is, no growth at a pressure of 50 MPa (Kato and Nogi 2001). Only a limited number of experiments have been performed examining the growth of these bacteria under high-pressure conditions, but generally members of *Shewanella* group 1 are characterized as cold adapted and pressure tolerant, whereas the

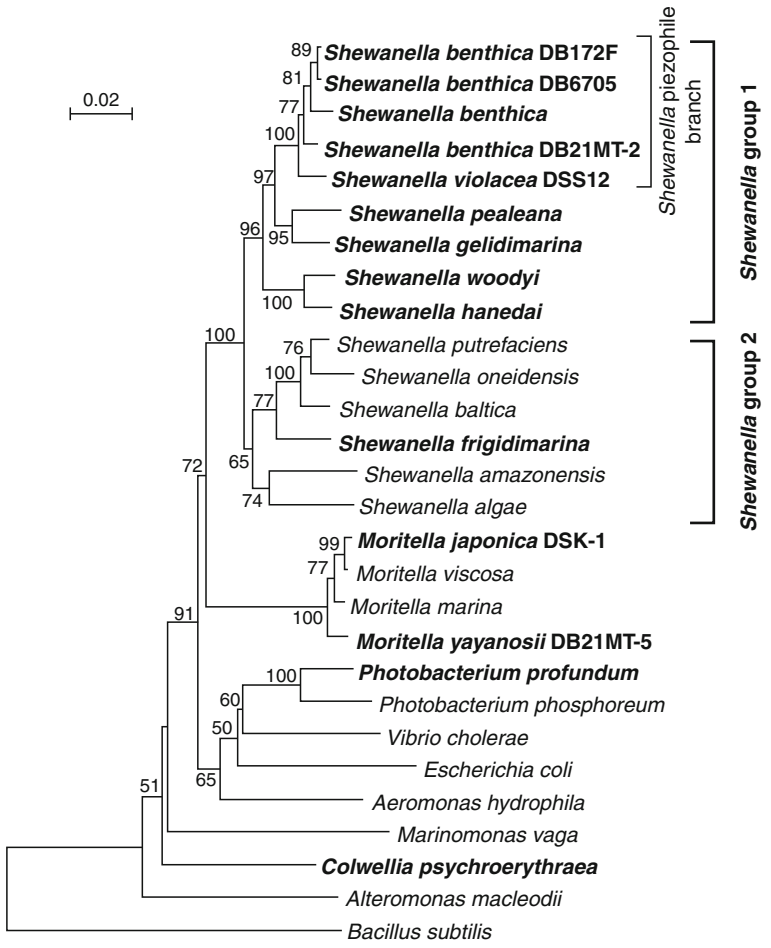

Fig. 5.1.3

Phylogenetic tree showing the relationships of the *Shewanella* species within the gamma-Proteobacteria subgroup constructed based on 16S rRNA gene sequences with the neighbor-joining method. The scale represents the average number of nucleotide substitutions per site. Bootstrap values (%) were calculated from 1,000 trees. Psychrophilic and/or piezophilic bacteria are shown in *bold*

members of *Shewanella* group 2 are mostly mesophilic and pressure sensitive. Some *Shewanella* species are known to produce polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA). It is clear that the members of *Shewanella* group 1 produce substantial amounts of EPA (11–16% of total fatty acids), whereas members of *Shewanella* group 2 produce no EPA or only limited amounts. In terms of other fatty acids, the membrane lipid profiles of members of the genus *Shewanella* are basically similar. This observation also supports the view described above (Kato and Nogi 2001).

On the basis of the properties of *Shewanella* species, we would like to propose that two major branches of the genus *Shewanella* should be recognized taxonomically, *Shewanella*

group 1 and group 2 (Fig. 5.1.3). The two subgenus branches of *Shewanella* would be as follows: *Shewanella* group 1 is characterized as a group of high-pressure, cold-adapted species that produce substantial amounts of EPA and *Shewanella* group 2 is characterized as a group of mostly mesophilic and pressure-sensitive species.

The deep-sea bottom and other cold-temperature environments are probably similar in terms of microbial diversity. Members of *Shewanella* group 1 live in such environments, and most of them show piezophilic or piezotolerant growth properties. In this regard, it is interesting to consider the influence of the ocean circulation as deep ocean water is derived from polar ice (in the Arctic and/or Antarctic regions) that sinks to the deep-sea bottom (Schmitz 1995), probably along with microbes. It was reported that *Psychrobacter pacificensis* isolated from seawater of the Japan Trench at a depth of 5,000–6,000 m was taxonomically similar to the Antarctic isolates *P. immobilis*, *P. gracincola*, and *P. fridigicola* (Maruyama et al. 2000). The occurrence of *Psychrobacter* in cold seawater deep in the Japan Trench and at the surface of the Antarctic sea suggests that bacterial habitation of the deep-sea and their evolution have been influenced by the global deep ocean circulation linked to the sinking of cooled seawater in polar regions. Thus, it is possible that the ocean circulation may be one of the major factors influencing microbial diversity on our planet.

Taxonomy of the Piezophiles

Bacteria living in the deep-sea have several unusual features, which allow them to thrive in their extreme environments. We have isolated and characterized several piezophilic and piezotolerant bacteria from cold deep-sea sediments at depths ranging from 2,500 to 11,000 m using sterilized sediment samplers by means of the submersibles *SHINKAI 6500* and *KAIKO,* systems (Kato 1999; Kato et al. 1995, 2004). Most isolated strains are not only piezophilic but also psychrophilic and cannot be cultured at temperatures higher than 20°C.

The isolated deep-sea piezophilic bacterial strains have been characterized in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants (Kato et al. 1998; Margesin and Nogi 2004; Yayanos et al. 1979). Thus far, all piezophilic bacterial isolates fall into the gamma subgroup of the Proteobacteria according to phylogenetic classifications based on 5S and 16S ribosomal RNA gene sequence information (DeLong et al. 1997; Kato 1999; Margesin and Nogi 2004). The only deep-sea piezophilic bacterial species of these genera were named to be *S. benthica* (Deming et al. 1984; MacDonell and Colwell 1985) in the genus *Shewanella*, and *Colwellia hadaliensis* (Deming et al. 1988) in the genus *Colwellia*, prior to the reports by the JAMSTEC group. We have identified several novel piezophilic species within these genera based on the results of chromosomal DNA–DNA hybridization studies and several other taxonomic properties. Both previously described and novel species of bacteria have been identified among the piezophilic bacterial isolates. Based upon these studies we have indicated that cultivated psychrophilic and piezophilic deep-sea bacteria could be affiliated with one of the five genera within the gamma-Proteobacteria subgroup: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and *Psychromonas*. Figure 5.1.4 shows the phylogenetic relations between the taxonomically identified piezophilic species (shown in bold) and other bacteria within the gamma-Proteobacteria subgroup. The taxonomic features of the piezophilic genera were determined as described below.

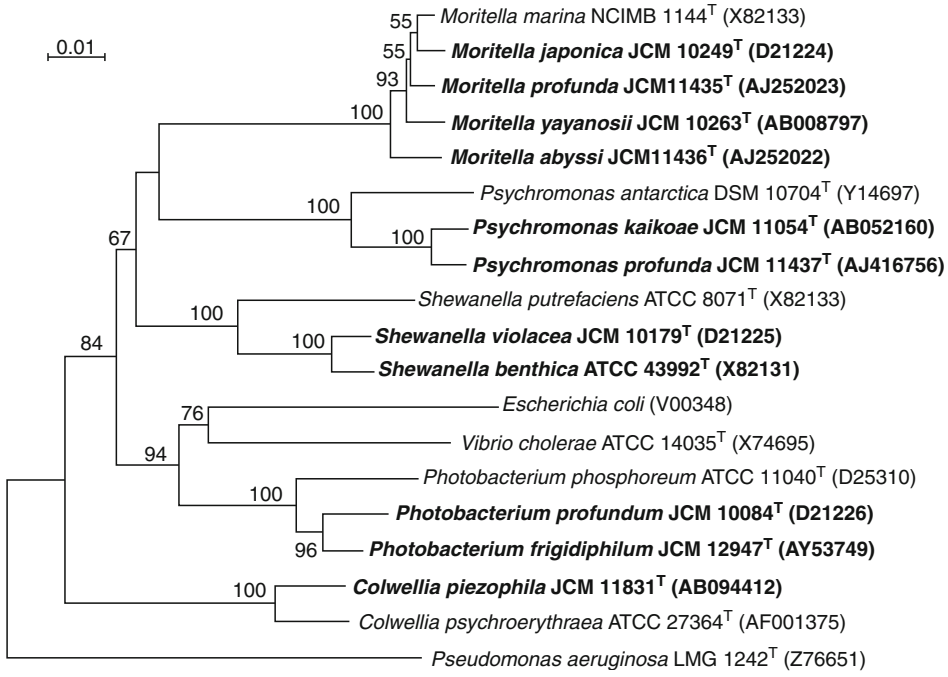

Fig. 5.1.4

Phylogenetic tree showing the relationships between isolated deep-sea piezophilic bacteria (in *bold*) within the gamma-Proteobacteria subgroup determined by comparing 16S rRNA gene sequences using the neighbor-joining method (references for species description are indicated in the text). The scale represents the average number of nucleotide substitutions per site. Bootstrap values (%) are shown for frequencies above the threshold of 50%

The Genus *Shewanella*

Members of the genus *Shewanella* are not unique to marine environments of Gram-negative, aerobic and facultatively anaerobic gamma-Proteobacteria (MacDonell and Colwell 1985). The type strain of this genus is *S. putrefaciens*, which is a bacterium formerly known as *Pseudomonas putrefaciens* (MacDonell and Colwell 1985; Owen et al. 1978). Recently, however, several novel marine *Shewanella* species have been isolated and described. These isolates are not piezophilic species and thus prior to the present report *S. benthica* and *S. violacea* were the only known members of the genus *Shewanella* showing piezophilic growth properties (Nogi et al. 1998b). *Shewanella* piezophilic strains, PT-99, DB5501, DB6101, DB6705, and DB6906, DB172F, DB172R, and DB21MT-2 were all identified as members of the same species, *S. benthica* (Nogi et al. 1998b; Kato and Nogi 2001). The psychrophilic and piezophilic *Shewanella* strains, including *S. violacea* and *S. benthica*, produce eicosapentaenoic acid (EPA) and thus the production of such long-chain polyunsaturated fatty acid (PUFA) is a property shared by many deep-sea bacteria to maintain the cell-membrane fluidity under conditions of extreme cold and high hydrostatic pressure environments (Fang et al. 2003).

S. violacea strain DSS12 has been studied extensively, particularly with respect to its molecular mechanisms of adaptation to high-pressure (Kato et al. 2000; Nakasone et al. 1998, 2002). This strain is moderately piezophilic, with a fairly constant doubling time at pressures between 0.1 and 70 MPa, whereas the doubling times of most piezophilic *S. benthica* strains change substantially with increasing pressure. As there are few differences in the growth characteristics of strain DSS12 under different pressure conditions, this strain is a very convenient deep-sea bacterium for use in studies on the mechanisms of adaptation to high-pressure environments. Therefore, the genome analysis on strain DSS12 has been performed as a model deep-sea piezophilic bacterium (Aono et al. 2010).

The Genus *Photobacterium*

The genus *Photobacterium* was one of the earliest known bacterial taxa and was first proposed by Beijerinck in 1889 (Beijerinck 1889). Phylogenetic analysis based on 16S rRNA gene sequences has shown that the genus *Photobacterium* falls within the gamma-Proteobacteria and, in particular, is closely related to the genus *Vibrio* (Nogi et al. 1998c). *Photobacterium profundum*, a novel species, was identified through studies of the moderately piezophilic strains DSJ4 and SS9 (Nogi et al. 1998c). *P. profundum* strain SS9 has been extensively studied with regard to the molecular mechanisms of pressure regulation (Bartlett 1999) and subsequently genome sequencing and expression analysis (Vezzi et al. 2005). Recently, *P. frigidiphilum* was reported to be slightly piezophily: its optimal pressure for growth is 10 MPa (Seo et al. 2005). Thus, *P. profundum* and *P. frigidiphilum* are the only species within the genus *Photobacterium* known to display piezophily and the only two known to produce the long-chain polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA). No other known species of *Photobacterium* produces EPA (Nogi et al. 1998c).

The Genus *Colwellia*

Species of the genus *Colwellia* are defined as facultative anaerobic and psychrophilic bacteria (Deming et al. 1988), which belong to the gamma-Proteobacteria. In the genus *Colwellia*, the only deep-sea piezophilic species reported was *C. hadaliensis* strain BNL-1 (Deming et al. 1988), although no public culture collections maintain this species and/or its 16S rRNA gene sequence information. Bowman et al. (1998) reported that *Colwellia* species produce the long-chain PUFA, docosahexaenoic acid (DHA). We have recently isolated the obligately piezophilic strain Y223G^T from the sediment at the bottom of the deep-sea fissure of the Japan Trench, which was identified as *C. piezophila* (Nogi et al. 2004). Regarding fatty acids, this strain did not produce EPA or DHA in the membrane layer, whereas high levels of unsaturated fatty acids (16:1 fatty acids) were produced. This observation suggested that the possession of long-chain PUFA should not be a requirement for classification as a piezophilic bacterium; however, the production of unsaturated fatty acids could be a common property of piezophiles.

The Genus *Moritella*

The type strain of the genus *Moritella* is *M. marina*, previously known as *Vibrio marinus* (Colwell and Morita 1964), which is one of the most common psychrophilic organisms isolated

from marine environments. However, *V. marinus* has been reclassified as *M. marina* gen. nov. comb. nov. (Urakawa et al. 1998). *M. marina* is closely related to the genus *Shewanella* on the basis of 16S rRNA gene sequence data and is not a piezophilic bacterium. Strain DSK1, a moderately piezophilic bacterium isolated from the Japan Trench, was identified as *M. japonica* (Nogi et al. 1998a). This was the first piezophilic species identified in the genus *Moritella*. Production of the long-chain PUFA, DHA, is a characteristic property of the genus *Moritella*. The extremely piezophilic bacterial strain DB21MT-5 isolated from the world's deepest sea bottom, the Mariana Trench Challenger Deep at a depth of 10,898 m, was also identified as a *Moritella* species and designated *M. yayanosii* (Nogi and Kato 1999). The optimal pressure for the growth of *M. yayanosii* strain DB21MT-5 is 80 MPa; this strain is unable to grow at pressures of less than 50 MPa but grows well at pressures as high as 100 MPa (Kato et al. 1998). The fatty acid composition of piezophilic strains changes as a function of pressure and, in general, greater amounts of PUFAs are synthesized at higher growth pressures. Approximately 70% of the membrane lipids in *M. yayanosii* are unsaturated fatty acids, which is a finding consistent with its adaptation to very high pressures (Nogi and Kato 1999; Fang et al. 2000). Two other species of the genus *Moritella*, *M. abyssi* and *M. profunda,* were isolated from a depth of 2,815 m off the West African coast (Xu et al. 2003a); they are moderately piezophilic and the growth properties are similar to *M. japonica*.

The Genus *Psychromonas*

The genus *Psychromonas* described psychrophilic bacterium, which also belongs to the gamma-Proteobacteria, is closely related to the genera *Shewanella* and *Moritella* on the basis of 16S rRNA gene sequence data. The type species of the genus *Psychromonas*, *P. antarctica,* was isolated as an aerotolerant anaerobic bacterium from a high-salinity pond on the McMurdo ice-shelf in Antarctica (Mountfort et al. 1998). This strain did not display piezophilic properties. *P. kaikoae*, isolated from sediment collected from the deepest cold-seep environment with chemosynthesis-based animal communities within the Japan Trench at a depth of 7,434 m, is a novel obligatory piezophilic bacterium (Nogi et al. 2002). The optimal temperature and pressure for growth of *P. kaikoae* are 10°C and 50 MPa, respectively, and both PUFAs, EPA and DHA, are produced in the membrane layer. *P. antarctica* does not produce either EPA or DHA in its membrane layer. DeLong and coworkers stated that strain CNPT-3 belonged to an unidentified genus of piezophiles (DeLong et al. 1997) and this strain proved to be closely related to *P. kaikoae*. Thus, the genus *Psychromonas* is the fifth genus reported to contain piezophilic species within the gamma-Proteobacteria. In addition, *P. profunda* is a moderately piezophilic bacterium isolated from deep Atlantic sediments at a depth of 2,770 m (Xu et al. 2003b). This strain is similar to the piezosensitive strain *P. marina*, which also produces small amounts of DHA. Only *P. kaikoae* produces both EPA and DHA in the genus *Psychromonas*.

Fatty Acid Composition of the Piezophiles

The piezophilic and psychrophilic *Shewanella* and *Photobacterium* strains produce EPA (Nogi et al. 1998b, c), *Moritella* strains produce DHA (Nogi et al. 1998a; Nogi and Kato 1999), and *P. kaikoae* produces both EPA and DHA (Nogi et al. 2002) but *C. piezophila* does not produce

such PUFAs (Nogi et al. 2004). The fatty acid composition of piezophilic strains changes as a function of pressure, and in general greater amounts of PUFAs are synthesized under higher-pressure conditions for their growth (DeLong and Yayanos 1985, 1986). Psychrophilic and piezophilic bacteria were believed to produce one of the long-chain PUFAs, either EPA or DHA, but this does not appear to be obligatory. For example, Allen et al. (1999) reported that monounsaturated fatty acids, but not PUFAs, are required for the growth of the piezophilic bacterium *P. profundum* SS9 based on the analysis of pressure-sensitive mutants. In their mutant experiment, 18:1 fatty acids proved to be necessary for growth under low-temperature and/or high-pressure conditions. In the case of *C. piezophila*, this specie had no 18:1 fatty acids but produced a large amount of 16:1 fatty acids in its cell membrane layer. All piezophilic and psychrophilic bacteria analyzed so far have 16:1 fatty acids (*Table 5.1.1*); thus, this fatty acid would appear to be one of the important components required for high-pressure growth.

Table 5.1.1

Whole-cell fatty acid composition (%) of the piezophilic isolates (type strains)

Fatty acid	Sh	Ph	Co	Mo	Ps
12:0	2	2	1		1
14:0	13	3	3	15	6
15:0		1	3	1	1
16:0	14	9	31	13	15
17:0					
18:0		1			
iso-13:0	5	2			
iso-14:0		4			
iso-15:0	11	2			
iso-16:0		15			
14:1		3	9	6	10
15:1			2		
16:1	31	31	50	53	55
17:1					
18:1	2	9		1	2
【EPA】20:5	16	13			2
【DHA】22:6				11	2
3OH-12:0	1	5	1		2
3OH-iso-13:0	5				
3OH-14:0					4
Unsaturated (%)	49	56	61	71	71
Saturated (%)	51	44	39	29	29
Ratio (U/S)	0.96	1.27	1.56	2.45	2.45

Sh Shewanella benthica ATCC 43992^T, *Ph Photobacterium profundum* JCM 10084^T, *Co Colwellia piezophila* Y223G^T, *Mo Molitella yayanosii* JCM 10263^T, *Ps Psychromonas kaikoae* JCM 11054^T.

The fatty acid compositions of those piezophilic strains are distinct depending on their genus and commonly high amounts of unsaturated fatty acids (49–71%) are involved in their membrane layer as shown in *Table 5.1.1*.

Conclusion

Cultured deep-sea piezophilic and psychrophilic bacteria are affiliated with one of the five genera within the gamma-Proteobacteria subgroup: *Shewanella, Photobacterium, Colwellia, Moritella,* and *Psychromonas*. These piezophiles are characterized to contain unsaturated fatty acids in their cell membrane layers but PUFAs, like EPA and DHA, are not obligatory necessary for the high-pressure growth. The diversity of the piezophilic bacteria are closely related with the global deep-sea ocean circulation. These observations indicate that the piezophilic bacteria could be adapted to any of deep-sea cold and high-pressure environments.

Acknowledgments

I am very grateful to Prof. Koki Horikoshi for continued support of my extremophile studies. I also thank my colleagues, Drs. Maria Smorawinska, Lina Li, Takako Sato, Yuichi Nogi and Kaoru Nakasone, for excellent collaboration. Finally, I appreciate the crews of the research ship and members of the submersible operation division at JAMSTEC for their efforts in collecting samples from the deep-sea environment.

Cross-References

5.2 High Pressure and Prokaryotes
5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low-Temperature Piezophiles
5.6 Cultivation Methods for Piezophiles
6.2 Taxonomy

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5.2 High Pressure and Prokaryotes

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Abstract: Many species of prokaryotes isolated from the deep ocean environment have been adapted to the high-pressure conditions, and grown well at such pressure conditions. One of the isolates from deep-sea, psychrophilic, moderately piezophilic bacterium *Shewanella violacea* strain DSS12 grew optimally at 30 MPa and 8°C but also grew well at atmospheric pressure (0.1 MPa) to 70 MPa conditions (*Fig. 5.2.1*, Kato et al. 1995; Nogi et al. 1998). We have examined this strain to elucidate the molecular basis for gene regulation at different pressure conditions because this strain is useful as a model prokaryote for comparing the various features of bacterial physiology under pressure conditions. Proteins, from such deep-sea adapted piezophiles, could be active under high-pressure conditions in general. Actually atmospheric pressure adapted proteins can be inactive under higher-pressure conditions. In the case of respiratory proteins, cell divisional protein FtsZ, RNA polymerase subunit, dihydrofolate reductase (DHFR), and isopropylmalate dehydrogenase (IPMDH), piezophilic proteins were unique for adaptation to high-pressure environment and some of them were much more stable and active under higher-pressure conditions. In addition, we sequenced the genome of this piezophilic bacterium and we expect that many biotechnologically useful proteins can be identified from the genome information.

Pressure-Regulated Promoters and Pressure-Controlling Systems

An operon identified as a pressure-regulated operon whose promoter was activated by growth under high pressure, was cloned and characterized from *S. violacea* DSS12: this operon, which has five transcription initiation sites, is controlled at the transcriptional level by elevated pressure (Nakasone et al. 1998, 1999). Moreover, transcriptional analysis showed that expression of the genes in the pressure-regulated operon is positively controlled at the transcriptional level by elevated pressure and further suggested that most transcripts from the operon at atmospheric pressure coincided with expression. The consensus sequence for the RNA polymerase sigma factor, sigma 54, was found upstream from this operon, and the *S. violacea* sigma 54 was shown to bind to this region (Nakasone et al. 1999). The sigma 54-containing

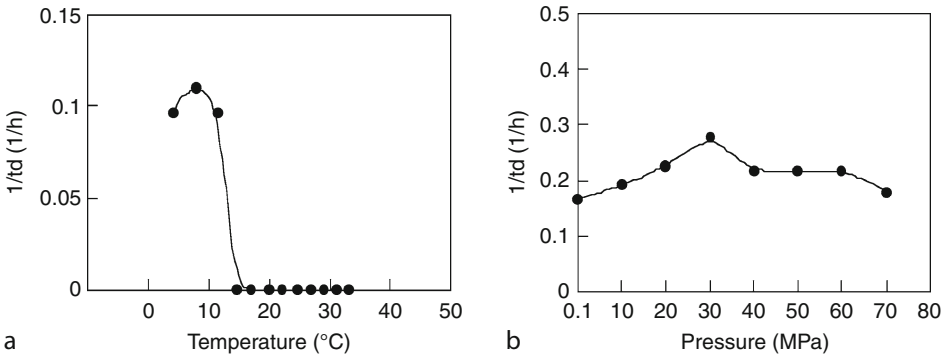

Fig. 5.2.1

Effects of temperature (a) and pressure (b) on growth of *Shewanella violacea* strain DSS12. "td" means doubling time (h)

RNA polymerase has been shown to be responsible for the transcription of several genes, e.g., nitrogen metabolic genes such as the *glnA* operon (Merrick and Edwards 1995). Glutamine synthetase is one of the enzymes involved in nitrogen metabolism. Gene expression of the *glnA* operon is also controlled by elevated pressure conditions at the transcriptional level in *S. violacea*, particularly by factor sigma 54 (Ikegami et al. 2000b). These results suggest that sigma 54 might play an important role in pressure-regulated transcription in piezophilic bacteria, although the expression of sigma 54 itself is not regulated by pressure conditions (Ikegami et al. 2000a; Nakasone et al. 2002).

Our approach toward understanding the molecular basis for gene expression under defined conditions is by detailed characterization of the components of the transcriptional machinery and the accessory factors involved. This regulation is mediated by one of the sigma factors, sigma 54, and a two-component regulatory system composed of the bacterial signal-transducing protein NtrB and the bacterial enhancer-binding protein NtrC in piezophilic *S. violacea*. In other bacteria, transcription from the sigma 54-dependent promoter, such as in the case of *glnAp2*, is regulated by enhancer-binding protein NtrC (Ninfa et al. 1987). The NtrC-binding sites are essential for the regulation of transcription by the sigma 54-containing RNA polymerase. Two NtrC-binding consensus sites were also found in the promoter region of *glnA* in *S. violacea*. From elecrophoretic mobility shift assay studies, we confirmed that the *S. violacea* NtrC protein specifically recognizes the element containing the NtrC consensus sequence on the *S. violacea glnA* operon (Ikegami et al. 2000b, c).

To reconstitute the two-component regulatory system and characterize the autophosphorylation of NtrB and transphosphorylation of phosphorylated NtrB to NtrC in vitro, we purified both of the proteins from the *S. violacea ntrB* and *ntrC* genes using recombinant DNA techniques. The results of the phosphorylation experiments at several temperatures indicated that autophosphorylation of NtrB occurred only at low temperatures (0–10°C), whereas no activity was detected at 37°C. Autophosphorylation activity of NtrB at such low temperatures was not detected in *Escherichia coli*. Furthermore, we have detected transcriptional activity at low temperatures in *S. violacea*. Therefore, this piezophilic bacterium adapts to the psychrosphere (low-temperature environment) and may have evolved a low-temperature-adapted system in the deep-sea environment. The phosphorylated relay between NtrB and NtrC of the two-component system in *S. violacea* could be detected particularly at low temperatures so that this transphosphorylation might also occur under high-pressure conditions (Nakasone et al. 2002).

In transcription of the sigma 54-dependent promoter such as the pressure-regulated and the *glnA* operons, sigma 54-containing RNA polymerase holoenzyme activates transcription at the promoter assisted by the activity of NtrC, which in turn is controlled by NtrB. As mentioned above, these *trans*-acting factors (sigma 54, NtrC, or NtrB) might play an important role in pressure-regulated transcription at the sigma 54-dependent promoter in this piezophilic bacterium. Sigma 54 in *S. violacea* is expressed at a relatively constant level under both atmospheric and high-pressure conditions, suggesting that the level of functional sigma 54 molecules is possibly regulated by the availability of NtrC. We examined the expression of the NtrC protein in *S. violacea* by Western blot analysis under different pressure conditions. The results indicated that the levels of this factor expressed at high pressure were greater than that at atmospheric pressure (Ikegami et al. 2000c).

Consideration of the results of pressure regulation by sigma 54 led us to propose a possible model for the mechanism of regulated expression of the pressure-regulated and the *glnA* operons in the deep-sea piezophilic bacterium *S. violacea* as shown in *Fig. 5.2.2*. Because

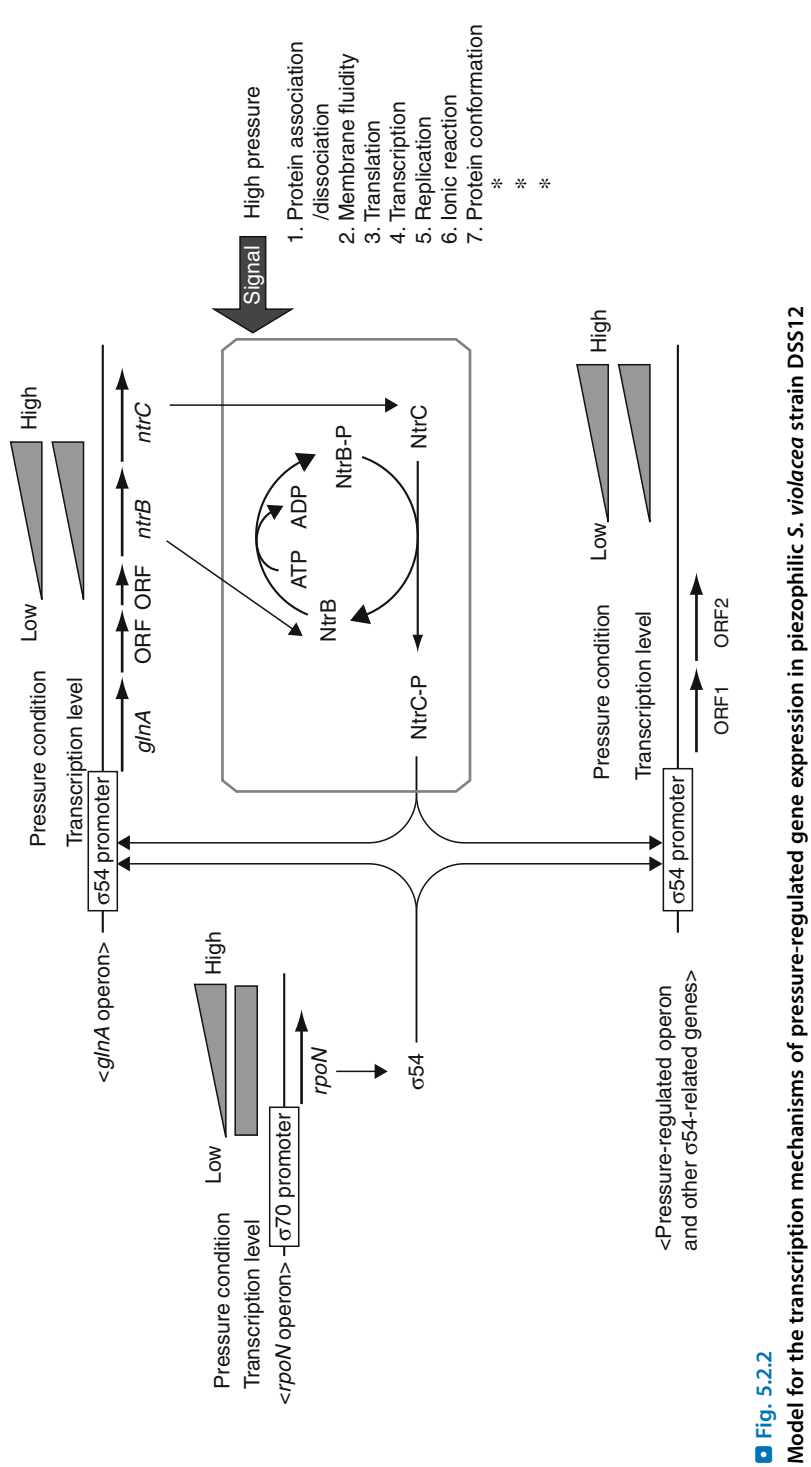

Fig. 5.2.2
Model for the transcription mechanisms of pressure-regulated gene expression in piezophilic *S. violacea* strain DSS12

S. violacea sigma 54 is expressed at consistent levels at both atmospheric and high pressure, it is suggested that the intracellular levels of sigma 54-containing RNA polymerase holoenzyme under both conditions are constant. This observation also strongly suggests that the transcriptional activity at this sigma 54-dependent promoter is proportional to the amount of NtrC factor and that this factor regulates gene expression under pressure conditions. This model suggests that NtrB might function as a pressure sensor and then this protein would be autophosphorylated under high-pressure conditions. Subsequently, the phosphorylated relay affects the NtrC protein followed by phosphorylated NtrC activation of the sigma 54-dependent promoters. This model explaining gene expression at high pressure should be confirmed using molecular-genetic approaches analyzing several pressure-and low-temperature-sensitive mutants in these transcription systems.

Respiratory Systems

As evidence of piezo-adaptation, a pressure-regulated operon was observed in this bacterium as described above. Downstream from this operon, an open reading frame homologous to the *cydD* gene of *E. coli* was found and the significance of the gene in bacterial growth under high pressure has been suggested (Kato et al. 1996). The gene product of *cydD* in *E. coli* is thought to be required for the assembly of respiratory components (Poole et al. 1989, 1993, 1994). Further, the expression of a respiratory system was regulated by hydrostatic pressure in *S. violcea* (Tamegai et al. 1998; Yamada et al. 2000; Nakasone et al. 2001) and in another piezophilic bacterium, *S. benthica* strain DB-172F (Qureshi et al. 1998a, b). These were the first reports concerning respiratory systems in deep-sea bacterium and the first evidence that expression of genes for respiratory components are regulated by physical parameters, such as hydrostatic pressure. Generally, bacteria have branched respiratory chains. Specifically, *Shewanella* strains have many respiratory components for adaptation to environmental changes (Heidelberg et al. 2003, Aono et al. 2010). These observations and the results of previous studies suggest that pressure regulation for expression of respiratory systems in *S. violacea* plays an important role in bacterial adaptation to high pressure.

Cytochrome *bd* is one of the members of the quinol oxidases, distinct from the heme-copper oxidase super family. In *E. coli*, two types of quinol oxidases, cytochrome *bo* and cytochrome *bd*, exist and both share roles in respiration. Cytochrome *bo* is expressed in log phase and cytochrome *bd* is expressed in stationary phase (Kita et al. 1984a, b). Cytochrome *bd* shows higher affinity for O_2 as compared to cytochrome *bo* and the former acts as a terminal oxidase under low oxygen concentration conditions (Kita et al. 1984b). For the biosynthesis of cytochrome *bd*, structural genes (encoded by *cydAB* operon) and genes for assembly of mature enzyme (encoded by *cydDC* operon) (Georgiou et al. 1987; Poole et al. 1989) are required. Expression of *cydAB* in *E. coli* is regulated by ArcA and Fnr, common O_2-regulated transcriptional regulators (Cotter et al. 1997; Govantes et al. 2000), and that of *cydDC* was regulated by NarL (involved in the two-component regulatory system for nitrate respiration) as well as Fnr (Cook et al. 1997). However, in *S. violacea*, no cytochrome *bd* has been detected spectrophotometrically under atmospheric pressure even during the stationary phase. Surprisingly, cytochrome *bd* has been detected only under growth conditions of high hydrostatic pressures (Tamegai et al. 1998). Thus, transcriptional regulation of cytochrome *bd* in *S. violacea* may be different from other organisms and this may be important for bacterial adaptation to high pressure. Furthermore, cytochrome *bd*-encoding *cydAB* genes were identified in *S. violacea*. Transcriptional analysis was carried out for *cydAB* and *cydCD* operons, and it

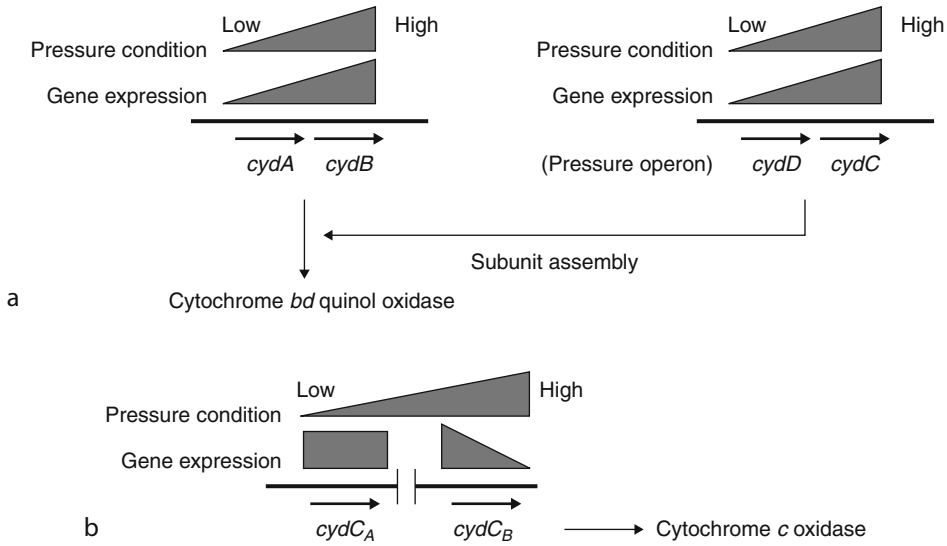

Fig. 5.2.3

Respiration model of *S. violacea* under pressure conditions. (a) Cytochrome *bd* quinol oxidase system. (b) Cytochrome c oxidase system

was observed that transcription of the *cydDC* operon was strongly regulated by hydrostatic pressure (Tamegai et al. 2005).

By the way, there are two types of soluble cytochrome *c* in this bacterium. One of them, cytochrome c_A, was constitutively expressed regardless of growth pressure. On the other hand, the expression of another cytochrome *c* (cytochrome c_B) was repressed under high hydrostatic pressure (Yamada et al. 2000). The pressure-regulated respiratory systems concerning cytochrome *bd* and *c* were concluded in *Fig. 5.2.3*. At atmospheric pressure condition, cytochrome *c* oxidase pathway could be the main pathway; however, at high-pressure condition, cytochrome *bd* oxidase pathway could be major. These results suggested that pressure regulation for expression of the respiratory systems in *S. violacea* plays an important role in bacterial adaptation to high hydrostatic pressure.

Cell Division Protein, Ftsz

Some rod shaped bacteria, including *E. coli*, exhibit cell filamentation without septum formation under high hydrostatic pressure conditions, indicating that the cell division process is affected by hydrostatic pressure (Marquis 1976). We examined effects of elevated pressure on FtsZ-ring formation in *E. coli* cells by indirect immunofluorescence microscopy. Elevated pressure completely repressed colony formation of *E. coli* cell at 40 MPa in our cultivation conditions and the cells exhibited obviously filamentous shapes. In the elongated cells, normal cell division processes appeared to be inhibited, because no FtsZ-rings were observed by indirect immnofluorescense staining. In addition, we also observed that hydrostatic pressure dissociated the *E. coli* FtsZ (ecFtsZ) polymers in vitro (Ishii et al. 2004). These results suggest that the high pressure directly affects cell survival and morphology through the dissociation of cytoskeletal frameworks.

In the piezophilic *S. violacea* DSS12, the growth was occurred at high-pressure conditions (Kato et al. 1995; Nogi et al. 1998). To analyze the transcription upstream from the *ftsZ* gene, Northern blot and primer extension analyses were performed and the results showed that gene expression was not pressure dependent. Western blot analysis also showed that the *S. violacea* FtsZ protein (svFtsZ) was equally expressed under several pressure conditions in the range of atmospheric (0.1 MPa) to high (50 MPa) pressures (Ishii et al. 2002). Using immunofluorescence microscopy, the svFtsZ ring was observed in the center of cells at pressure conditions of 0.1–50 MPa. These results imply that the svFtsZ protein function is not affected by elevated pressure in this piezophilic bacterium.

The cell morphology of *E. coli* under high pressure is, however, quite different from *S. violacea*. The filamentous cells indicate to stop the cell division steps and we expect the hydrostatic pressure affect FtsZ protein function and causes inhibition of cell division. The C-termini of their FtsZ are not conserved in each other and the region is essential for polymerization activity (Ishii et al. 2002). Therefore the characteristic property under high pressure is considered from the variety. Therefore, we expect that characterization of the biochemical features and polymerization activities of the FtsZ and any terrestrial bacteria (e.g., *E. coli*) in vitro help us to understand the effect of pressure on cell division steps in vivo.

Stability of the RNA Polymerase Under Pressure Conditions

A high-pressure electrophoresis apparatus (HPEA) was developed by a modification of the method previously reported by Erijman and Clegg (1995) (*Fig. 5.2.4*, Kawano et al. 2004). The cathode and anode were attached separately above and below the sample chamber. Pressures of up to 200 MPa can be applied within 1 min with silicon oil KF-96-1.5CS (Shin-Etsu Chemical, Tokyo, Japan), using a hand pump. First, native polyacrylamide gel electrophoresis (PAGE) was carried out in capillary glass tubes 75 mm in length and 0.85 mm in inner diameter under pressure conditions using the HPEA. The sample was kept at the desired pressure for 30 min for equilibration before electrophoresis. Electrophoresis was carried out at the constant voltage of 350 V for 1 h. After decompression, the gel was removed and equilibrated in sodium dodecyl sulfate (SDS) buffer. Next, the gel was overlaid onto the SDS gel and subjected to SDS-PAGE using 10% polyacrylamide gels at atmospheric pressure. Proteins were then visualized by silver staining.

Using the HPEA system, the relative stability of the *S. violacea* RNA polymerase (svRNAP) and *E. coli* RNA polymerase (ecRNAP) under pressure conditions were analyzed. The results showed that a pressure of 140 MPa caused dissociation of ecRNAP but not that of svRNAP. On the other hand, the core enzyme of svRNAP, which lacked the sigma factor, was dissociated at 140 MPa (Kawano et al. 2004). These results suggest that the sigma factor is required for stabilization of svRNAP under high-pressure conditions. The sigma subunit is known to change the quaternary structure of ecRNAP (Wu et al. 1976; Greiner et al. 1996). It is likely that the *S. violacea* sigma subunit stabilizes the core enzyme through alteration of the quaternary structure of RNA polymerase resulting in piezotolerance. In this context, the predicted β-sheet domain, which is not observed in the *E. coli* and *S. oneidensis* sigma 70 subunits, may have a role in stabilization of RNA polymerase at high pressure (Kawano et al. 2004). Further experimentation is required to determine the significance of the β-sheet domain by comparing the structure with that of other mesophilic *Shewanella* strains and by analysis of the effects of mutations within the domain on the piezotolerance of RNA polymerase in terms of

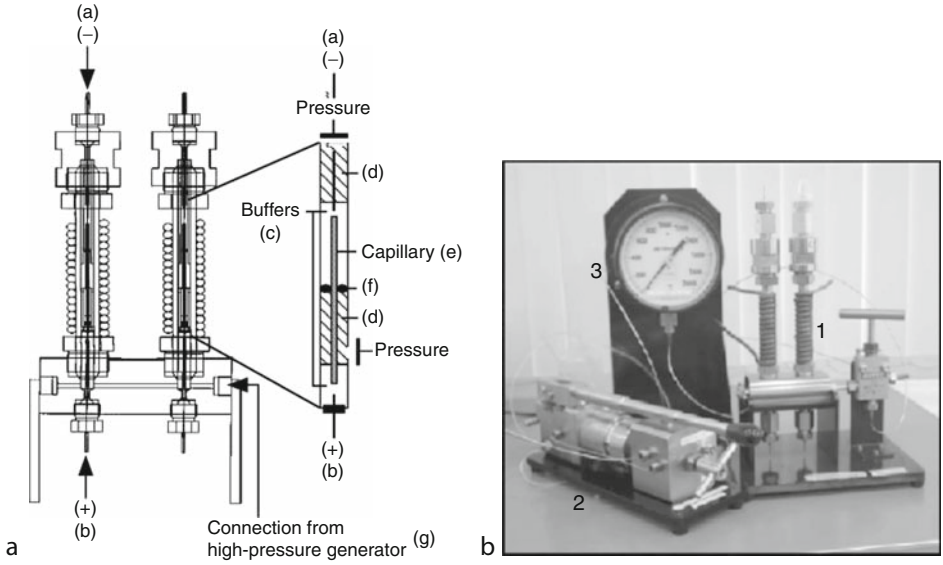

Fig. 5.2.4

Diagram of the high-pressure electrophoresis apparatus (HPEA). (a) High-pressure electrophoresis chamber for HPEA. (a) Connection to the power supply (anode), (b) connection to the power supply (cathode), (c) buffers, (d) silicone oil KF-96-1.5CS, (e) glass microcapillary tube, (f) o-ring to partition a space into the upper and lower spaces, (g) connection to a high-pressure pump. (b) Photograph of the HPEA. 1. High-pressure electrophoresis chamber, 2. high-pressure hand pump, 3. pressure gage

transcriptional activity and subunit association. For investigations of the molecular adaptation of proteins to high hydrostatic pressure, the HPEA is a powerful tool in combination with techniques based on molecular biology and bioinformatics.

Dihydrofolate Reductase (DHFR)

A new dihydrofolate reductase (svDHFR) was purified from a deep-sea bacterium, *S. violacea* DSS12. In contrast with *E. coli* DHFR (ecDHFR), the enzyme activity of svDHFR increased with increasing hydrostatic pressure up to 100 MPa, suggesting that the enzyme kinetics and structural fluctuation of svDHFR are adapted to a high-pressure environment.

The enzyme activity of svDHFR increases with pressure, to at most 30% at 100 MPa, and then gradually decreases although maintains a higher activity at 250 MPa than at atmospheric pressure. This is significantly different from the behavior of ecDHFR, which exhibits a monotonous decrease in the activity even under low pressure. Therefore, it can be expected that svDHFR has distinguished characteristics in enzyme kinetics and structural fluctuation to adapt itself to deep-sea conditions. The activation volume of enzyme reaction, $\Delta V^{\#}$, was calculated (Ohmae et al. 2004). The negative $\Delta V^{\#}$ value of svDHFR under pressures below 100 MPa, as expected from the positive slope, means that the activated state has a smaller volume than the reactant in the catalytic reaction coordinate. At pressures above 100 MPa this is reversed, as shown by the positive $\Delta V^{\#}$ value (also for ecDHFR). These results predict that

svDHFR, being highly flexible at pressures below 100 MPa, changes its conformation to be more rigid (like ecDHFR) at higher pressures although the protein may be denatured at pressures above a few hundred MPa. Determining the volume changes in each process and the rate-limiting process, which is in progress, should provide more detailed understanding of the adaptation mechanism of the enzyme reaction.

Isopropylmalate Dehydrogenase (IPMDH) Enzymes

The isopropylmalate dehydrogenase (IPMDH) enzymes from various *Shewanella* species adapted to different pressure environments have been extensively studied. The 0.1 MPa-adapted *S. oneidensis* MR-1 (Vankateswaran et al. 1999), and the piezophilic species *S. violacea* DSS12, *S. benthica* DB6705, and *S. benthica* DB21MT-2 were isolated from depths of 5,110, 6,356, and 10,989 m, respectively (Kato et al. 1995, 1998; Nogi et al. 1998; Nogi and Kato 1999). All IPMDH genes were cloned and sequenced as well as recombinanty expressed to yield purified protein for further biochemical studies. The greatest variation in amino acid sequences of the IPMDH was found between *S. oneidensis* and the two deep-sea *Shewanella* species, which also possessed more hydrophobic amino acid residues in their proteins (De Poorter et al. 2004).

The activity of IPMDH was also investigated as a function of temperature and pressure. Activity measurements performed between 10–72°C showed a positive correlation between the optimal temperature for enzyme activity and the optimal pressure for the growth of the piezophilic *Shewanella* species. In contrast, *S. oneidensis* IPMDH had a very broad temperature optimum. Also no direct correlation between the temperature-induced denaturation and pressure adaptation was observed (De Poorter et al. 2004).

The application of hydrostatic pressure reduced the activity of enzymes from all species (*Fig. 5.2.5*). Pressure-dependent stability was higher for the IPMDHs from organisms isolated at greater depth. Although *S. benthica* DB6705 IPMDH has a higher relative activity than strain DB21MT-2 for pressures up to 100 MPa, it is rapidly deactivated at higher pressures. DB21MT-2 IPMDH might not be very stable at low pressures because it is naturally adapted to very high pressure. These results suggest that activity of enzymes from piezophilic *Shewanella* species is driven by pressure acclimatization.

S. violacea Genome Analysis

To understand pressure regulation in deep-sea bacteria, we performed genome analysis of the piezophilic bacterium *S. violacea* strain DSS12 as a model deep-sea bacterium (Aono et al. 2010). The genome size of this bacterium is 4,962,103 bp, and 14 ribosomal RNA operons were identified. The number of ribosomal operons might correspond to extreme environmental conditions and it is likely that the ability of *S. violacea* to grow under high pressure is due to the transcriptional efficiency of mumerous ribosomal proteins under such stress conditions. From the results of genome sequencing, 4,346 predicted CDSs were identified. Preliminary analyses of the annotation suggested that almost 50% of the CDSs are hypothetical proteins and unassigned. This result indicates that many genes from deep-sea microbes could be novel and might have also novel functions. Therefore, genomic analysis of marine extremophiles may lead to the discovery of new functions for genes. In the *S. violacea* genome, the following interesting genes were also identified: haloalkane dehalogenase, extracellular proteases, chitinase, chitobibiase, EPA synthesis

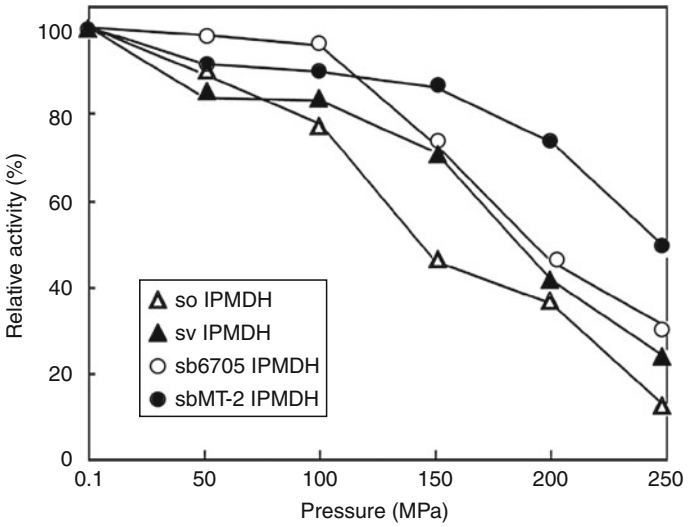

Fig. 5.2.5

Pressure-dependent activity of IPMDH from various bacterial sources. Relative activities were measured in pressurized cells 2 min after the application of pressure. *Symbols* indicate origin of IPMDH: △, *S. oneidensis*; ▲, *S. violacea* DSS12; ○, *S. benthica* DB6705; ●, *S. benthica* DB21MT-2

clusters, thiamine biosynthesis clusters, lipoproteins, vitamin B6 biosynthesis protein, biotin synthetase, cellulases, organic solvent tolerance proteins, etc.

Following *S. violacea* genome analysis, we are planning to initiate a postgenome study called the "PIEZOME project," which will focus on the proteome, transcriptome, and metabolome of piezophiles affected by high-pressure environments. We are looking forward to furthering our understanding of the molecular mechanisms involved in high-pressure life on our planet based on the results of the PIEZOME project.

Acknowledgments

I am very grateful to Prof. Koki Horikoshi for continued support of my extremophile studies. I also thank my colleagues, Drs. Maria Smorawinska, Lina Li, Takako Sato, Yuichi Nogi, Kaoru Nakasone, Hideyuki Tamegai, Mohammad Hassan Qureshi, Eiji Ohmae, Akihiko Ikegami, Akihiro Ishii, and Hiroaki Kawano, for excellent collaboration, and I would like to keep nice relations with them in the future, too. Finally, I appreciate the crews of the research ship and members of the submersible operation division at JAMSTEC for their efforts in collecting samples from the deep-sea environment.

Cross-References

5.1 Distribution of Piezophiles
5.3 Piezophysiology of the Model Bacterium *Escherichia coli*
5.4 High Pressures and Eukaryotes

5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low-Temperature Piezophiles
5.6 Cultivation Methods for Piezophiles
6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens

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5.3 Piezophysiology of the Model Bacterium *Escherichia coli*

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_5.3,

Introduction

Pressure is a thermodynamical parameter that varies greatly throughout the biosphere. From an average of 0.1 MPa at the surface of the earth, pressure increases to 110 MPa at the deepest point of the ocean, the Challenger Deep, located about 11 km below sea level in the Mariana Trench (Abe et al. 1999; Lauro and Bartlett 2008). These deep sea niches are populated by piezophilic (i.e., pressure loving) bacteria which require these high pressures for optimal growth. Conversely, growth of mesophilic with respect to pressure-bacteria such as *Escherichia coli* is accompanied by filamentation and slows down under high pressure up to about 50 MPa, after which it completely halts (Zobell and Cobet 1962, 1964). These simple observations indicate that the cellular machinery in these different microorganisms has acquired specific adaptations to function optimally at the pressure prevailing at the surface of the earth or in the depths of the oceans in the course of evolution (Yayanos 1995; Bartlett 2002; Simonato et al. 2006; Lauro and Bartlett 2008).

Besides the phenomenon of growth at high pressure (<100 MPa), there is an increasing interest in the lethal effects of HP shocks (>>100 MPa for several minutes). This is especially the case in food processing, where exposure to HP is currently considered as the most promising alternative to thermal processing. In this respect, HP shocks of 200–600 MPa can readily inactivate food-borne bacteria while allowing a superior quality retention of the food compared to pasteurization, a process called "pascalisation" (Knorr 1999; Patterson 2005). Interestingly, however, non-sporulating bacteria like *E. coli* or *Listeria monocytogenes* are able to rapidly acquire extreme levels of resistance to HP shock treatment, without apparent fitness costs (Hauben et al. 1997; Karatzas and Bennik 2002), and studies of deep subsurface environments have yielded bacteria that survive and probably proliferate at pressures in the GPa range (Zhang et al. 2005). The exploration of the limits of piezophily and piezoresistance, as well as the underlying cellular and molecular basis, will be a major challenge for high pressure microbiologists in the years to come.

In this chapter, we will focus on the piezophysiology of the model bacterium *E. coli*, by describing the state of knowledge on the cellular impact of both sublethal (<100 MPa) and lethal (>>100 MPa) pressures, together with the physiological responses and possible adaptations to these stresses.

Impact of Sublethal Pressures (< 100 MPa) on Essential Cellular Processes and Structures

Elevated pressures (<100 MPa) primarily and progressively impair the processes of macromolecular synthesis in *E. coli* (Zobell and Cobet 1962). In vitro experiments indicated that translation was the most HP sensitive of these processes, followed by replication and transcription, respectively (Hildebrand and Pollard 1972). Detailed in vivo studies on the effects of HP on nucleic acid synthesis in *E. coli* were conducted by Yayanos and Pollard (1969), by analyzing the incorporation of radioactive labeled thymine, leucine, and uracil during the first hours of incubation at elevated pressures. It was shown that DNA replication, as measured by thymine incorporation, reflected a synchronous replication process at pressures between 25 and 45 MPa. Moreover, at 30 MPa, this synchronous incorporation of thymine seemed to be most evident, while between 50 and 80 MPa only an initial period of thymine incorporation was observed, after which further incorporation stopped (Yayanos and Pollard 1969). Thus,

at higher pressures (50–80 MPa), it was assumed that completion of the initiated DNA replication rounds remained possible, but initiation of new rounds of DNA replication was blocked. Finally, at pressures >95 MPa an immediate cessation of thymine incorporation was apparent. At these pressures, it appears that the DNA replication machinery itself becomes inactive in *E. coli*, although DNA replication slowly recovers after pressure release (Yayanos and Pollard 1969; Aertsen and Michiels 2008).

Transcription became suppressed at 20 MPa, and was completely inhibited at 80 MPa (Yayanos and Pollard 1969). Interestingly, it was demonstrated that at any particular pressure only a small part of the RNA polymerase (RNAP) molecules became inactivated and, in addition, that this inactivation depended on their cellular state at the time HP is applied (Erijman and Clegg 1995). More specifically, when RNAP molecules were actively involved in a stable ternary transcribing complex during HP treatment, transcription was able to resume normally after decompression, and this for up to 180 MPa (Erijman and Clegg 1995, 1998). On the other hand, free RNAP molecules gradually became irreversibly dissociated, as the HP-dissociated subunits underwent conformational changes that disturbed the ability to reform the active enzyme. In this respect, in vitro studies of *E. coli* free RNAP exposed to 100 MPa (30 min) implicated a decrease in RNAP activity of 60% in comparison with the activity at atmospheric pressure (0.1 MPa) (Kawano et al. 2004).

In addition to replication and transcription, also translation is influenced by elevated pressures. In intact *E. coli* cells, pressures of 67 MPa are sufficient to completely inhibit protein synthesis. Nevertheless, inhibition at these pressure levels was reversible and cells rapidly resumed after decompression (Yayanos and Pollard 1969). In vivo studies by Schwarz and Landau (1972) demonstrated that cellular amino acid permeability, amino acid activation, aminoacyl-tRNA formation, and maintenance of the polysomal integrity were not affected by pressures up to 67 MPa, suggesting that translation inhibition occurred during elongation of the growing peptide chain. Further in vitro studies, focusing on ribosome activity, pointed out that the post-translocational complex was the most HP sensitive intermediate of the elongation cycle, with a midpoint dissociation of approximately 70 MPa that corresponds to the pressures needed to block protein synthesis in vivo (Gross et al. 1993).

Effect of Sublethal Pressures on Growth and Motility

The effects of HP on macromolecular synthesis evidently lead to growth suppression of *E. coli* under HP. Zobell and Cobet (1962) discovered that growth and division of *E. coli* cells were retarded at pressures up to 50 MPa, growth ceased, and bacteria started to die upon prolonged exposure. Interestingly, *E. coli* cells exhibit filamentation under growth permissive pressures (<50 MPa) (*Fig. 5.3.1*), indicating that cell division becomes affected earlier than the production of biomass, i.e., macromolecular synthesis (Zobell and Cobet 1962, 1964; Marquis 1976). Using immunofluorescence microscopy, Ishii et al. (2004) proposed that FtsZ plays an important role in this phenomenon. FtsZ is a GTP-hydrolyzing tubulin-like protein that polymerizes into a cytoskeletal ring and localizes at the division site (Erickson and Stoffler 1996; Lockhart and Kendrick-Jones 1998). However, no FtsZ rings were observed in the filamentous cells grown at HP (Ishii et al. 2004). In addition, with increasing cell length, the RNA content also increased, while the DNA content remained approximately the same. The lack of DNA synthesis at elevated pressures (~50 MPa) may be responsible for the inhibition of cell division, resulting in filament formation (Zobell and Cobet 1962, 1964). Zobell and Cobet

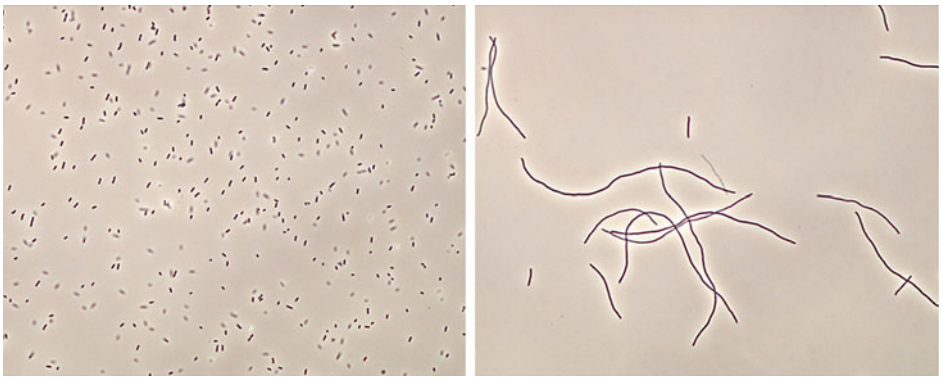

Fig. 5.3.1

Phase contrast images of *Escherichia coli* growing (37°C, 12 h) as normal rods at atmospheric pressure (0.1 MPa; *left panel*) and as filamentous rods at high pressure (35 MPa; *right panel*)

(1964) also discovered that cell filamentation was transient, and within a few minutes following decompression to atmospheric pressure, septum formation and cell division began to take place in all of the pressure-induced filaments.

In order to study the cellular processes that limit growth under high pressure, Marquis and Bender (1980) selected mutants of *Streptococcus faecalis* that could grow at increased pressures (up to ~100 MPa). These mutants had lost their normal catabolite repression, which resulted in the induction of additional ATP-generating catabolic enzymes as well as accumulation of ammonia, due to the increased activity of arginine deiminase, and these physiological changes could play a role in piezotolerance. Indeed, the proton-translocating membrane-bound enzymes, such as F_0F_1-ATPase found in *S. faecalis* but also in *E. coli*, are generally highly sensitive to HP (Marquis and Bender 1987), and this is one of the reasons why HP stress can lead to intracellular acidification (Abe and Horikoshi 1998). The enhanced growth of the mutants at elevated pressures, therefore, could be due to an increased ATP availability for proton pumping (Welch et al. 1993). In addition, an increased acid neutralization ability due to the elevated ammonia production could possibly also contribute to piezotolerance (Bartlett 2002).

Not only growth, but also motility is influenced under HP. Motility is controlled by complex chemosensory networks and a molecular motor that drives the rotation of flagella to allow bacteria to move up- or downstream physical and chemical gradients, and is seen as one of the most pressure-sensitive cellular processes in non pressure adapted microorganisms. It was proposed that HP inhibits the polymerization of flagellin monomers into a functional flagellum (Meganathan and Marquis 1973), but at this moment no further studies have been conducted to define in detail the effects of HP on motility of *E. coli*. Conversely, it has been documented that piezophiles like *Photobacterium profundum* SS9 developed important adaptations to preserve their motility during growth under elevated pressures, up to 150 MPa (Meganathan and Marquis 1973; Simonato et al. 2006; Lauro and Bartlett 2008; Eloe et al. 2008). In fact, Eloe et al. (2008) elegantly demonstrated that SS9 possesses both a polar flagellum and lateral flagella, which are adapted and fully functional for swimming and swarming, respectively, at depth.

Cellular Response to Sublethal Pressures

Interference of HP Growth with Normal Regulation of Specific Genes or Proteins

Several studies have been conducted on the effect of sublethal HP on gene expression and protein production patterns in *E. coli*. An analysis of the abundance of cell surface proteins of *E. coli* cells grown at HP (30 MPa) revealed that the expression of the outer membrane proteins OmpC and OmpF was repressed during growth under HP, although this repression seemed not to be controlled by the EnvZ–OmpR dependent signal transduction cascade normally governing these proteins (Bartlett et al. 1995; Nakashima et al. 1995). Another study found that during growth under HP (30 MPa), plaque formation by phage λ in *E. coli* was attenuated because of repression of the maltose regulon, which also codes for the λ attachment protein *lamB* (Sato et al. 1996).

Finally, induction of the *lac* promotor was also affected in *E. coli* growing at HP. Normally, this promoter is induced by allolactose or gratuitous inducers such as isopropyl-β-D-thiogalactopyranoside (IPTG), which release the LacI repressor from its binding site in the *lac* promoter (Wilson et al. 2007). However, HP seems able to inactivate the lactose repressor or at least interfere with its binding to the *lac* operator sequence, although the exact mechanism is still elusive (Kato et al. 1994; Bartlett et al. 1995). More specifically, an 80- to 90-fold induction of plasmid-borne *lac* promoter activity was demonstrated in *E. coli* cells after growth at 30 MPa in the absence of an inducer (Kato et al. 1994). Importantly, this could not be attributed to an HP effect on the plasmid copy number, as this only increased two- to threefold.

Induction of Cold and Heat Shock Proteins During Growth Under HP

Bacteria are able to mount specific stress responses to maintain or restore their cellular homeostasis and/or to protect key processes in hostile environments. The first insights in the stress response of *E. coli* during growth at HP were provided by using two-dimensional electrophoresis of cellular proteins (Welch et al. 1993). An abrupt shift from atmospheric pressure up to 55 MPa for 60–90 min caused a general decrease in protein synthesis, and perturbed cell division and nucleoid structure (Welch et al. 1993). However, 55 proteins were also found to be transiently induced. While most of these were ribosomal proteins, also four cold shock proteins (CSP, including RecA and H-NS) and 11 heat shock proteins (HSP, including RpoH, DnaK, GroEL, GroES, GrpE, and Lon) were identified (Welch et al. 1993). Induction of both HSPs and CSPs by the same stress seems counterintuitive, as they are normally induced by mutually exclusive stresses (i.e., heat and cold, respectively). Interestingly, however, VanBogelen and Neidhardt (1990) demonstrated that HSPs and/or CSPs could also be triggered by using different ribosome-targeting antibiotics, implicating that ribosomes could function as a prokaryotic sensor for heat and cold shock response networks. These combined observations suggest HP might influence the *E. coli* ribosomes in such a way that both HSP and CSP response networks are triggered (Welch et al. 1993), although this hypothesis would require further evidence.

Presumably, both HSPs and CSPs are mandatory to cope with HP stress during growth of *E. coli*. HSPs comprise several molecular chaperones and proteases, whose actions involve quality control and refolding of proteins, preventing protein aggregation and adjustments of

the translation machinery (Arsène et al. 2000; Rasouly and Ron 2009). CSPs, on the other hand, are able to restore the expression of genes involved in protein translation (Jones et al. 1987), although they may also function to destabilize cold-induced secondary structures in DNA or RNA molecules to facilitate replication and transcription, respectively (Bartlett et al. 1995; Thieringer et al. 1998). Importantly, some CSPs also counteract the decrease in membrane fluidity at low temperatures, which would otherwise result in structural and functional disruption of the cell membrane (Macdonald 1984; Bartlett 2002).

Although H-NS can be cataloged as a CSP, this DNA-binding protein has a predominantly negative effect on gene expression, and thus can be seen as a transcriptional repressor and gene regulator with a global reach (Dorman 2004). Interestingly, and corresponding to the earlier mentioned observation that H-NS induction was observed during HP growth (Welch et al. 1993), it was shown that *hns* mutants of *E. coli* were severely compromised in growth under HP, suggesting a protective role of the DNA-binding protein H-NS (Ishii et al. 2005). However, a precise mechanism for this pressure resistance is still elusive.

Recently, genome wide expression analysis in *E. coli* W3110 after HP exposure showed that many of the 4,300 open reading frames of *E. coli* W3110 were affected by growth at pressures of 30 or 50 MPa (Ishii et al. 2005). While their findings confirmed induction of members of the heat and cold shock response, it is currently unfeasible to explain all differences in gene expression as an adaptation to high pressure growth, underscoring the need for follow-up studies.

Induction of the Heat Shock and SOS Response After a Short Sublethal HP Shock

Expression of HSP genes proved not only up-regulated when *E. coli* is grown under HP (~50 MPa), but also after a sublethal HP shock of 150 MPa for 15 min (Aertsen et al. 2004b). Moreover, the assumption that HSPs are implied in the protection against (protein) damage caused by HP shock was supported by the fact that a sublethal heat shock (50°C), raising the levels of HSPs in the cell, substantiously protected *E. coli* to a subsequent lethal HP challenge (Aertsen et al. 2004b).

Aside the heat shock response, also the SOS response could be triggered by sublethal HP shock (100 MPa) in *E. coli* MG1655. This SOS response was discovered by the RecA and LexA-dependent expression of *uvrA*, *recA*, and *sulA* after HP shock (Aertsen et al. 2004a). Typically, the SOS response of *E. coli* is induced by DNA damage, and subsequently controls a distinct cellular response to repair this damage (Butala et al. 2009). Basically, the induction of SOS-induced genes is controlled by an interplay of two proteins, namely, LexA (locus for X-ray sensitivity A) and RecA (recombinase A). Under normal growth conditions, the LexA transcriptional repressor protein is stable and represses a regulon of more than 40 genes predominantly coding for DNA repair mechanisms (Little 1993; Courcelle et al. 2001; Janion 2008; Van Melderen and Aertsen 2009). On the other hand, aside from being a recombinase, RecA also harbors coprotease activity, which under certain conditions can assist the autocatalytic self-cleavage of LexA. DNA damage typically leads to the exposure of single stranded DNA, which forms a nucleoprotein filament with RecA and, in turn, probes this protein to stimulate the autoproteolytic activity of LexA. Upon self-cleavage, LexA dissociates from its DNA targets, resulting in a derepression of the SOS regulon (Little 1993; Friedberg et al. 1995). Subsequently,

after DNA repair, the absence of single stranded DNA will dampen the coprotease activity of the RecA and allow LexA to re-establish repression of the SOS regulon (Erill et al. 2007; Butala et al. 2009).

An important member of the SOS regulon, which is thus also produced after sublethal HP shock in *E. coli*, is SulA. This protein physically binds to FtsZ, a key protein in septum formation, to inhibit cell division in case of DNA damage (Aertsen and Michiels 2005b; Van Melderen and Aertsen 2009). Normally, SulA is rapidly degraded by the Lon protease, but in *lon* mutants increased SulA accumulation and division inhibition lead to severe filamentation and even inactivation of the cells (Van Melderen and Aertsen 2009). This hyperfilamentation is clearly seen in HP shocked *lon* mutants and can be suppressed by knocking out the *sulA* gene (Aertsen and Michiels 2005b). Interestingly, however, some *sulA* independent cell elongation also appears to occur (Kawarai et al. 2004; Aertsen and Michiels 2005b), although the mechanisms behind this phenomenon are not clear yet.

Another phenomenon linked to the SOS response is the activation of the lytic cycle of lambdoid prophages in *E. coli*. Lysogeny of lambdoid prophages is maintained by the CI repressor of which autocleavage, similar to LexA, can be stimulated by an activated RecA nucleoprotein fragment. This cleavage results in activation of the prophage from a lysogenic into a lytic form (Janion 2008). It was demonstrated that λ prophages in lysogenic *E. coli* MG1655 cells were induced ca. 3 h after exposure to HP shock (100 MPa, 15 min), resulting in a 10^4-fold induction of the infective λ particles. This pattern was similar to λ prophage induction by UV irradiation and as mitomycin C treatment (Aertsen et al. 2004a; Aertsen et al. 2005a).

Interestingly, after a specific genetic screen, mutants of *E. coli* were discovered that proved to be deficient only in HP mediated SOS induction, while not being affected in UV or mitomycin C mediated SOS induction (Aertsen and Michiels 2005a). Studying these mutants in more detail pinpointed an endogenous type IV restriction endonuclease of *E. coli*, encoded by the *mrr* gene, to play a major role as final effector in translating the perception of HP into double strand breaks and concomitant SOS induction (Aertsen and Michiels 2005a). In fact, mutants in *mrr* display a slightly increased survival after HP shock, although they don't show any differences in HP growth (Aertsen and Michiels 2005a). How exactly HP shock triggers Mrr activity remains still elusive.

Cellular Impact of Lethal Pressures (>>100 MPa) on Essential Cellular Processes and Structures

High hydrostatic pressure is viewed as one of the most promising non-thermal food preservation techniques, and pressures in the range of 200–600 MPa are applied to destroy food-borne pathogens and spoilage microorganisms (Patterson 2005; Considine et al. 2008). Nevertheless, only fragmentary knowledge exists on the mechanisms of pressure inactivation. Following-up on sublethal pressures, we will now survey the impact of lethal HP shocks on *E. coli*.

Effects of Lethal HP Shock on Membranes and Proteins

The cell membrane of microorganisms is believed to be a major target affected by lethal HP treatment. Based on the observation that HP (>>100 MPa) causes leakage of cytoplasmic contents and can promote entry of compounds from the extracellular environment, it has been postulated that cellular membranes are breached under HP. Physical damage of the cell

membrane was observed by using fluorescent dyes, e.g., propidium iodide, which can only penetrate the cell membrane if it is damaged (Smelt 1998; Ritz et al. 2000). Complementary evidence of cell leakage stems from measuring the extracellular ATP or UV-absorbing material from the bacterial cells after HP treatment (Smelt 1998; Pagán and Mackey 2000). Additionally, the relationship between membrane damage and cell death after HP treatment was also examined in different *E. coli* strains. Important differences in the way that pressure affects the membranes of exponential- and stationary-phase cells were described, as well as evidence that the nature of membrane damage may depend on the intensity of HP treatment. Interestingly, stationary-phase cells seem able to reveal their membrane after decompression, while exponential-phase cells permanently lost their membrane integrity. These findings were in support with previous observations that membrane damage is an important event in the inactivation of bacterial cells by HP (Pagán and Mackey 2000).

Besides loss of functional barrier properties, biological membranes appear also to be severely affected in their architecture after HP shock. Normally, biological membranes are composed by a bilayer of phospholipids, embedded with functional proteins that play an important role in transporting ions and other substances across the membrane (Smelt 1998; San Martín et al. 2002). HP, however, was demonstrated to convert the liquid-crystalline state of the membrane to a highly ordered gel state as a result of a straightening of the acyl chains (Braganza and Worcester 1986). Moreover, due to differences in compressibility between lipid species on the one hand and between lipids and membrane proteins on the other, phase separations are induced (Macdonald 1984; Braganza and Worcester 1986). Additionally, membrane proteins can also be displaced from the membrane as a result of the phase changes (Casadei et al. 2002). Together, these structural effects will lead to a functional destruction of the cell membrane by HP (Cheftel 1992; Heremans 2001; San Martín et al. 2002).

In this respect, it should be noted that fatty acids of piezophilic microorganisms show a higher degree of unsaturation with increased growth pressure (DeLong and Yayanos 1985). This unsaturation compensates for the increased melting point of the lipids under HP, and helps the cells to preserve their membrane fluidity and viscosity, a phenomenon called homeoviscous adaptation (Abe et al. 1999). The same phenomenon explains why exponential-phase cells grown at lower temperatures typically display an elevated HP resistance: since these cells incorporate an increased proportion of unsaturated fatty acids during growth at lower temperatures (Casadei et al. 2002). Conversely, in the presence of cholesterol, the fluidity of prokaryotic cell membranes diminishes and cells become more pressure sensitive (Smelt 1998; Mañas and Mackey 2004).

Not only cellular membranes are affected by HP, but also protein denaturation seems to play a decisive role in bacterial inactivation after HP shock. This denaturation takes place from 200 to 300 MPa because HP differentially affects the bonds that stabilize protein tertiary and quaternary structure, such as intramolecular salt bridges, hydrogen bonds and Vanderwaals interactions, and protein solvent (i.e., water) interactions (Balny and Masson 1993; Balny et al. 2002). Depending on the pressure, denaturation can be reversible or irreversible (Rastogi et al. 2007). As a result, many cellular processes (translation, transcription, etc.) are also affected by lethal HP shock, because they are typically carried out by large protein complexes (e.g., ribosomes) (Niven et al. 1999; Mañas and Mackey 2004).

In this context, a correlation was shown between loss of viability and decrease in ribosome-associated enthalpy in cells subjected to pressures of 50–250 MPa for 20 min (Niven et al. 1999), but it remains of course difficult to prove that ribosome disintegration is the cause of cell death (Niven et al. 1999; Abe 2007).

Induction of Oxidative Stress by Lethal HP Shock

Recently, using a leaderless alkaline phosphatase probe, cytoplasmic oxidative stress as a result of HP shock treatment could be demonstrated in both exponential- and stationary-phase cells of *E. coli* MG1655 (Aertsen et al. 2005b). Correspondingly, HP treatment also made the cells hypersensitive to sublethal doses of oxidative stress generated by t-butylhydroperoxide and plumbagin, and knock-out mutants (*katE*, *katF*, *oxyR*, *sodAB* and *soxS*) impaired in oxidative stress management showed a higher sensitivity towards HP (Aertsen et al. 2005b). Furthermore, pressure shock-resistant mutants of *E. coli* (Hauben et al. 1997) were found to be more resistant towards plumbagin, and to incur only marginal levels of cytoplasmic oxidation in response to HP. Consequently, these findings provide convincing evidence that HP triggers an endogenous oxidative burst which may be an additional cause of cell death by HP (Aertsen et al. 2005b).

In this context, it is interesting to note that some time ago a so-called suicide hypothesis was postulated to explain microbial inactivation by a number of stresses when applied at mild doses (Aldsworth et al. 1998; Aldsworth et al. 1999). According to this hypothesis, mild stress creates a metabolic imbalance in the cell, leading to an excess of reducing power and the derailing of electron transfer reactions, which in turn results in the generation of reactive oxygen species. As such, this intrinsic suicide mechanism could in part explain the higher resistance of stationary-phase cells compared to exponential-phase cells because this oxidative burst is expected to be more prominent, and thus destructive, in actively respiring cells.

Intrinsic Microbial Factors Affecting Resistance or Sensitivity to Pressure Shock

Generally, the resistance of microorganisms to HP is very variable. Vegetative cells of molds and yeasts and of most bacteria are relatively sensitive to HP, being rapidly inactivated at pressures >200–400 MPa. Bacterial spores, on the other hand, are, perhaps not surprisingly, the most pressure resistant organisms known so far (Farkas and Hoover 2000), together with the ascospores of heat-resistant molds, although there are only a few studies about the latter (Reyns et al. 2003; Dijksterhuis and Samson 2006). It is often stated that, among gram-positive vegetative bacteria are more HP resistant than gram-negative ones, because of the more complex cell envelope of gram-negative bacteria, which would render them more prone to damage caused by HP (Patterson 2005). However, the major differences in cell envelope architecture between both types of bacteria are the greater thickness of the peptidoglycan layer and the lack of an outer membrane in gram-positive bacteria, both of which are not considered as lethal targets of HP. Furthermore, cocci seem generally more resistant than rod-shaped bacteria, but it is not clear whether there is really a causative link between cell morphology and HP resistance.

However, an increasing number of exceptions to these general trends are reported. Most notably, substantial variability in HP resistance between different species of vegetative bacteria and even within one species is observed (Patterson et al. 1995; Benito et al. 1999; Alpas et al. 1999). To illustrate this, a comprehensive set of literature data concerning the HP inactivation at ambient temperature (20–25°C) and at pH7.0 of different strains of *E. coli* (gram-negative) and *L. monocytogenes* (gram-positive) were collected and plotted in a single graph representing

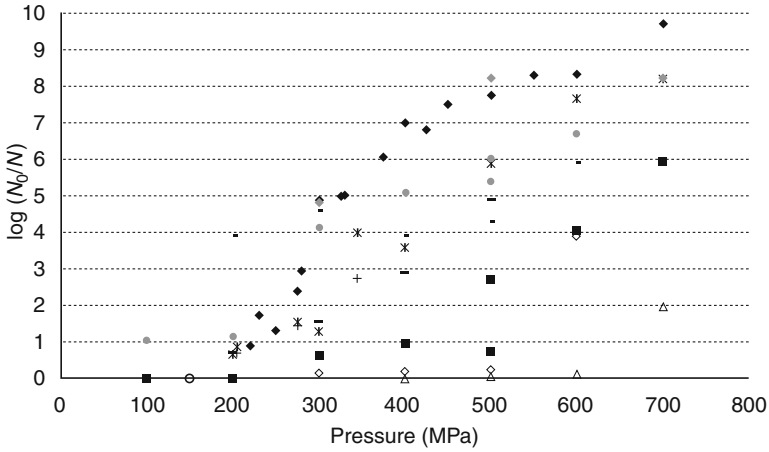

Fig. 5.3.2

Compilation of published data ($n=79$) on pressure sensitivity of different *E. coli* strains in buffer (pH 7.0) at room temperature. Strains were pressurized during 15 min. Symbols represent different strains: MG1655 (◆), ATCC43888 (_), ATCC43892 (◈), NCTC12079 (■), 931 (*), 933 (+), J1 (◇), NCTC8003 (●), H1071 (-), C9490 (Δ), NCTC9001 (○), W2-2 (●), 30-2C4 (■). N_0 and N refer to cell numbers before and after HP treatment, respectively. (Patterson et al. 1995; Hauben et al. 1997; Hauben 1998; Garcia-Graells et al. 1998; Benito et al. 1999; Pagán and Mackey 2000; Alpas et al. 2000; Casadei et al. 2002; Mañas and Mackey 2004)

inactivation (log N_0/N) versus pressure for a constant exposure time of 15 min. Representative data of *E. coli* (Fig. 5.3.2) and *L. monocytogenes* (Fig. 5.3.3) correspond to 13 and 12 different strains, respectively. Since all data have been obtained under similar process and environmental conditions, except for the pressure, the distribution of points in the graphs gives an indication of the intrinsic variation in resistance among strains of the same species, which appears to be very large.

It has been well documented that, besides structure and morphology, cell physiology plays an important role in HP resistance (Mañas and Mackey 2004). In this context, a finding that has been confirmed in many other bacteria is that exponential-phase cells are more sensitive to HP treatments than stationary-phase cells (Pagán and Mackey 2000). Upon entering the stationary phase of growth, bacteria become genetically reprogrammed and become more protected against a variety of adverse conditions including high temperature, oxidative stress, high salt concentrations, and, as reported by several authors, also against HP (Hirsch and Elliot 2002). Recently, it was also demonstrated that the increased pressure resistance in stationary phase is partly controlled by the alternative sigma factor RpoS (σ^s) (Robey et al. 2001), which regulates the expression of over 50 genes in *E. coli* (Casadei et al. 2002). In *L. monocytogenes*, a comparable alternative sigma factor, σ^b, was induced after exposure of cells to several stresses, including HP (Casadei et al. 2002; Wemekamp-Kamphuis et al. 2004).

Further, Malone et al. (2006) recently discovered genes of *E. coli* O157:H7 that are involved in HP resistance by interpreting transcriptional changes in response to HP. As such, it was

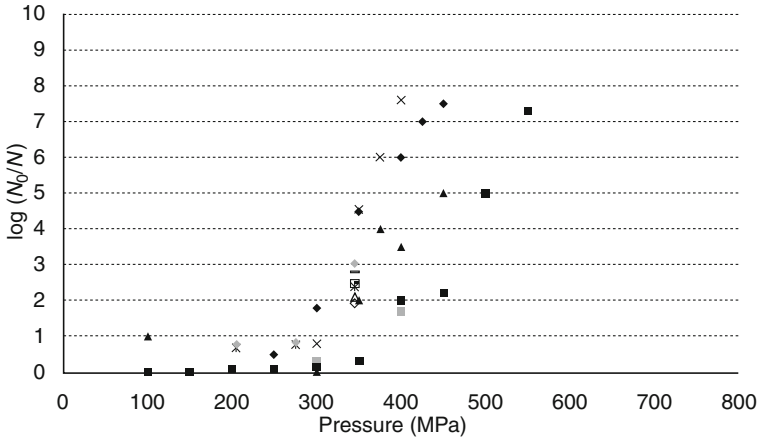

Fig. 5.3.3

Compilation of published data (n=43) on HP inactivation of different *Listeria monocytogenes* strains in buffer (pH 7.0) at room temperature. Strains were pressurized during 15 min. Symbols represent different strains: Scott A (◆), 2,433 (x), NCTC11994 (▲), CA (*), V7 (◇), 35,091 (Δ), 117 (□), 103 (-), OhiO2 (_), NCTC7973 (■), AK01 (■), OH2 (◆). N_0 and N refer to cell numbers before and after HP treatment, respectively (Mackey et al. 1994; Simpson and Gilmour 1997; Kalchayand et al. 1998; Alpas et al. 1999; Alpas et al. 2000; Tholozan et al. 2000; Karatzas et al. 2001; Karatzas and Bennik 2002)

demonstrated that HP shock affected the expression of thiol-disulfide redox systems and the Fe–S cluster status, suggesting a destabilization of the cell's redox homeostatic balance (Malone et al. 2006). Moreover, subsequent analysis of specific mutants compromised in Fe–S assembly displayed pressure resistance. It was therefore hypothesized that HP is able to destabilize the Fe–S clusters of some proteins, resulting in the release of Fe, triggering the formation via the Fenton reaction of hydroxyl radicals which are highly reactive and cause cell damage.

One of the most remarkable findings related to bacterial inactivation under HP is that *E. coli* MG1655, which is originally HP sensitive, could easily acquire extreme levels of HP resistance after alternating cycles of exposure to HP and regrowth of the surviving cells (Hauben et al. 1997). High level HP resistance can be stably achieved already after 25 successive cycles (*Fig. 5.3.4*). While the parental strain was completely inactivated (i.e., $>10^9$-fold reduction) at 800 MPa (15 min), up to 0.1% of the pressure resistant mutants survived (i.e., $\sim10^3$-fold reduction) (Hauben et al. 1997) (*Fig. 5.3.4*). Based on these observations, and the high HP resistance of some natural *E. coli* isolates (Alpas et al. 1999; Benito et al. 1999; Malone et al. 2006), it appears that *E. coli* can be considered as genetically predisposed to acquire extreme levels of HP resistance.

In accordance with the earlier mentioned role of HSPs in the HP shock response, the extremely HP resistant mutants isolated by Hauben et al. (1997) displayed constitutively increased expression level of HSPs (*Fig. 5.3.5*), providing a possible explanation for their resistance (Aertsen et al. 2004b). Moreover, increased levels of HSPs were also demonstrated in

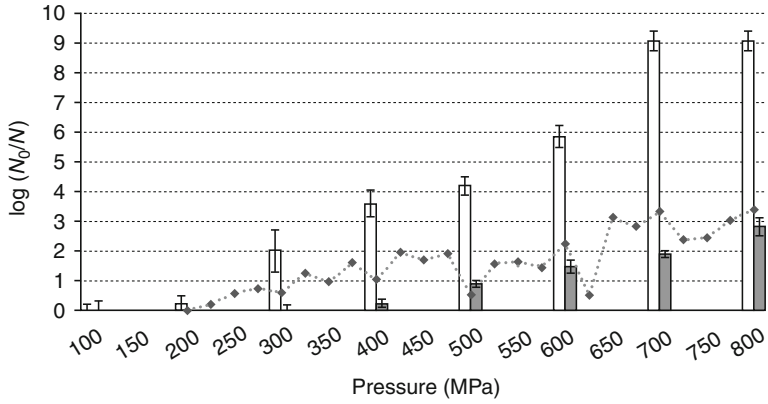

Fig. 5.3.4

Logarithmic viability reduction after high pressure treatment (15 min, 20°C) of *E. coli* MG1655. *Line graph* (-◆-) presents the inactivation during the stepwise selection regime with 25 MPa increments. *Bars* represent inactivation of the original parental strain (□) and the finally selected HP mutant (▯) at different pressures. Results are expressed as mean values ± standard deviations of three experiments. Initial cell numbers (N_0) were approximately 1×10^9 CFU/ml. N indicates the number of survivors recovered after treatment. At log (N_0/N) = 9, detection limit was reached

piezotolerant mutants of *L. monocytogenes* (Karatzas et al. 2003). On the other hand, however, also cold shock seems to increase HP resistance in *Staphylococcus aureus* and *L. monocytogenes* (Wemekamp-Kamphuis et al. 2002; Noma and Hayakawa 2003).

Extrinsic Parameters Affecting Pressure Resistance or Sensitivity

HP resistance is not only caused by hereditary mutations in the chromosome of the bacteria (Hauben et al. 1997; Karatzas and Bennik 2002), but it can also be transiently induced by specific physicochemical characteristics of the environment which might affect cellular homeostasis and resistance. In this context, it was demonstrated that the growth temperature and the composition of the pressure medium (Gervilla et al. 1997; Linton et al. 1999; García-Graells et al. 1999; Masschalck et al. 2000; Van Opstal et al. 2005) can affect the rate of inactivation of bacteria.

One of the environmental parameters that exerts the strongest protective effect against bacterial inactivation by HP is reduced water activity as a result of high salt or sugar concentration (Oxen and Knorr 1993; Van Opstal et al. 2003). The resistance to inactivation at reduced water activity is not completely understood. An effect that may contribute is the stabilization of proteins at reduced water activity because less water molecules are available to compete with the intramolecular protein–protein bonds. The same effect underlies the increased heat tolerance of proteins at low water activity. Another suggestion is that HP resistance may be related to the reduced membrane permeability and fluidity caused by the thickening of the cell membrane when cell shrinkage occurs at low water activity, although this has not been directly proven (Palou et al. 1997). While the previous mechanisms are passive,

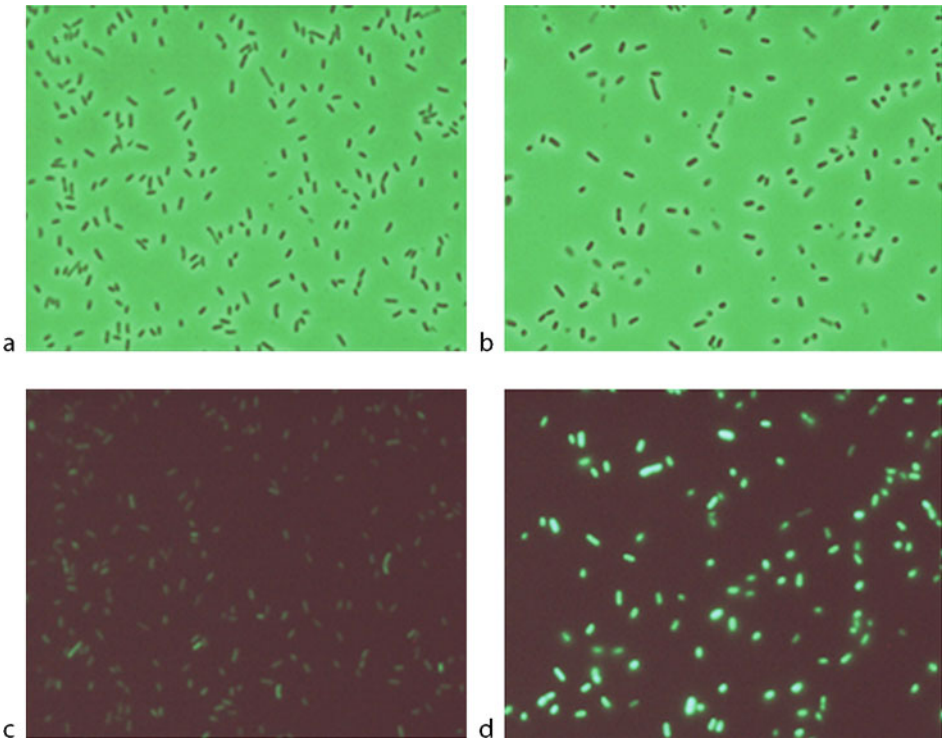

Fig. 5.3.5

Phase contrast (a and b) and epifluorescence (c and d) images of stationary phase cells of *E. coli* MG1655 wild-type (a and c) and an extremely HP resistant derivative (b and d), not subjected to HP shock. Fluorescence intensity is a measure of expression of the heat shock chaperone gene *dnaK* from a plasmid-borne *dnaK-gfp* reporter fusion. It can be seen that the HP resistant mutant displays higher constitutive levels of *dnaK* expression

active mechanisms may also be involved when the cells are pre-exposed to reduced water activity for some time before HP treatment. Microorganisms are able to accumulate compatible solutes, such as trehalose, during the osmotic stress response, and these may protect the cellular targets affected by elevated pressures (Molina-Höppner et al. 2004; Smiddy et al. 2004). In this respect, many in vitro and in vivo studies demonstrated that trehalose stabilizes proteins at high temperatures in their native state and preserves the integrity of membranes during stresses (Crowe et al. 1984; Colaco et al. 1994; Singer and Lindquist 1998). Interestingly, pressure-resistant *E. coli* mutants (Hauben et al. 1997) still increased their HP resistance in the presence of sucrose (Van Opstal et al. 2003).

Finally, it was shown that very low concentrations of particular cations substantially increase the survival of *E. coli* MG1655 upon HP treatment. Specifically, the addition of 10 mM Ca^{2+} to the pressure treatment medium resulted in about a 1,000-fold increased resistance of *E. coli* MG1655 at 270 MPa (Hauben et al. 1998). Moreover, this effect was ion specific, since Mg^{2+}, Mn^{2+}, and Fe^{2+} also protected *E. coli* against HP inactivation, while Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} had an opposite effect. Conversely, the addition of the chelator EDTA resulted in

a decreased viability after HP. Thus, these results may indicate that the accumulation of certain bivalent cations like Ca^{2+} protect the cell against HP inactivation.

Conclusions

It can be concluded that the mesophilic model bacterium *E. coli* displays a remarkable piezophysiology, and possesses various mechanisms to respond and adapt to HP stress. Further, molecular and genetic elucidation of the responses induced by HP and the genes necessary to grow under HP and to withstand HP inactivation will help to understand the microbial perception of HP stress and the cellular targets it affects, and will eventually lead to a better insight in the behavior of bacteria during and after exposure to HP. Furthermore, studies on the high pressure physiology of mesophilic bacteria like *E. coli* may also prove rewarding to generate novel insights in non pressure-related aspects of *E. coli* physiology.

Acknowledgments

D.V. holds a Ph.D. grant from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). A.A. acknowledges financial support from Research Foundation – Flanders (FWO-Vlaanderen; Grant 1.5258.08N).

Cross-References

5.1 Distribution of Piezophiles
5.2 High Pressure and Prokaryotes
9.1 Sub-seafloor Sediments – An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)

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5.4 High Pressures and Eukaryotes

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General Effects of High Hydrostatic Pressures in Biological Systems

The effects of high hydrostatic pressure in biological systems have been mainly investigated from the following perspectives: (1) structural perturbation of macromolecules such as proteins and lipids, and kinetic analysis of biochemical reactions; (2) microbial adaptation to high pressure in mesophiles and piezophiles; and (3) inactivation of food-spoiling microbes, and applications in nonthermal food processing. During the past decades, an increasing number of innovative high-pressure studies on biological processes have been performed by applying advanced techniques of genetics and molecular biology in bacteria and yeasts as model organisms (Horikoshi 1998; Abe et al. 1999; Abe and Horikoshi 2001; Bartlett 2002; Abe 2004, 2007a; Aertsen et al. 2009). Recent studies in this field have revealed the potential of a broad range of microbes to adapt and develop resistance to increasing hydrostatic pressure and have shown unexpected outcomes in microbial growth, physiology, and survival. More recently, high hydrostatic pressure has begun to be applied in the medical sciences to interesting areas such as the engineering of multicellular matrices or tissues (Diehl et al. 2003; Frey et al. 2004, 2008; Liu et al. 2009).

The application of pressure yields a fundamental physical parameter in any reaction, that is, volume change. The following two equations describe the effect of hydrostatic pressure on the equilibrium A $\Leftrightarrow$ B and the reaction A $\rightarrow$ B, respectively:

$$(\partial \ln K/\partial p)_T = -\Delta V/RT \tag{5.4.1}$$

$$(\partial \ln k/\partial p)_T = -\Delta V^{\neq}/RT \tag{5.4.2}$$

where K is the equilibrium constant, k the rate constant, p pressure (in megapascals [MPa]), T absolute temperature (K), and R the gas constant (ml MPa/K · mol). For the pressure unit definition, MPa is used throughout this chapter (atmospheric pressure ~0.1 MPa = 1 bar = 0.9869 atm = 1.0197 kg of force/cm^2). ΔV is the difference between the final and initial volumes in the entire system at equilibrium (reaction volume), including the solute and the surrounding solvent. $\Delta V^{\neq}$ is the apparent volume change in activation (activation volume), representing the difference in volume between the reactants and the transition state. The direction and degree of pressure effects on any reaction are governed by the sign and magnitude of the volume change. When a reaction is accompanied by a volume increase, it is inhibited by increasing pressure. When a reaction is accompanied by a volume decrease, it is enhanced by increasing pressure. A number of excellent reviews are available detailing the physicochemical basis of pressure effects on biological macromolecules (Heremans and Smeller 1998; Balny et al. 2002; Gekko 2002; Royer 2002).

Theoretically, the effects of high pressure on living cells are also dependent on the sign and magnitude of volume changes associated with any intracellular chemical reaction. Nevertheless, it is difficult to predict how the complex metabolic pathways are modified in response to increasing hydrostatic pressure and whether the cell fate commits to continuing growth or arrest of the cell cycle. Explorations in piezophysiology are being undertaken to discover whether the physiological outcome of living cells exposed to high pressure is relevant to their growth and survival and to identify the genes responsible. In this chapter, the effects of high hydrostatic pressure on the model eukaryote yeast *Saccharomyces cerevisiae* are described and the findings that can be generalized from the study of those effects are discussed. A number of outstanding challenges are being addressed in emerging biotechnology applications in medical

sciences. For example, high pressure is applied for the inactivation of viruses to produce vaccines (Ishimaru et al. 2004; Otake et al. 2005), induction of apoptosis in tumor cells (Frey et al. 2004), elimination of host corneal cells in corneal transplants (Sasaki et al. 2009), and sterilization of bone in orthopedic surgery (Diehl et al. 2005). However, this chapter does not go into detail on such applications; other excellent reviews should be referred to (Frey et al. 2008; Aertsen et al. 2009).

Effects of Lethal Levels of Hydrostatic Pressure on Yeast

The occurrence and significance of the effects of high pressure on *S. cerevisiae* are listed in *Table 5.4.1*. High hydrostatic pressure greater than 100 MPa is considered to be lethal, with a marked decrease in viability that depends on the magnitude and duration of the pressure treatment. In an earlier study, Rosin and Zimmerman reported that a pressure treatment of 100 MPa for 4 h increased the occurrence of cytoplasmic petite mutants, which were characterized by small colony size and respiration deficiency of the cells, reflecting the susceptibility of the mitochondrial function (Rosin and Zimmerman 1977). At pressures of 100–150 MPa, the spindle pole bodies and microtubules are lost (Kobori et al. 1995). Pressures of 200–250 MPa induce tetraploids and homozygous diploids, potentially providing industrial uses for strains with increased growth rates (Hamada et al. 1992). A short-duration heat-shock treatment (e.g., 42°C, 30 min) increases the viability of the cells at 140–180 MPa 100- to 1,000-fold compared with untreated cells (Iwahashi et al. 1991). Among the numerous heat-shock proteins (Hsps), the molecular chaperone Hsp104 (Singer and Lindquist 1998) plays an essential role in heat-induced piezotolerance by unfolding denatured intracellular proteins in an ATP-dependent manner. After high-pressure treatment at 140 MPa, Hsp104 is associated with an insoluble protein fraction (Iwahashi et al. 1997; Kawai et al. 1999). Hence, Hsp104 is likely to contribute to the unfolding of proteins that have been denatured by high pressure in the cells. The deletion mutant for *HSP104* fails to acquire heat shock-inducible piezotolerance, and hence unfolding of cellular proteins could be one of the reasons for high pressure-induced loss of yeast cell viability (Iwahashi et al. 1997). The types of intracellular proteins that are denatured by high pressure are still unclear. Treatment of cells with cold shock, or a moderate concentration of H_2O_2 or ethanol, also increases their piezotolerance (Palhano et al. 2004). Similarly, a moderate pressure treatment of 50 MPa for 1 h increases the viability of the cells at 200 MPa (Domitrovic et al. 2006). Pressure-induced piezotolerance is governed by the two transcription factors Msn2 and Msn4, which are induced by various types of stress. The loss of both *MSN2* and *MSN4* genes results in susceptibility to high pressure. How high pressure regulates Msn2 and Msn4 following the transcription of their downstream genes remains to be clarified. Trehalose is a nonreducing disaccharide known to protect proteins, membranes, and other macromolecules in cells against various stresses. This disaccharide has a role in the piezotolerance of yeast by preventing the formation of protein aggregates and promoting the refolding activity of Hsp104. While yeast mutants defective in the accumulation of trehalose are susceptible to increasing pressure (Iwahashi et al. 1997), a mutant lacking neutral trehalase Nth1 that catalyzes the hydrolysis of one trehalose molecule producing two glucose molecules also displays susceptibility to high pressure (Iwahashi et al. 2000). Neutral trehalase is required for recovery after high-pressure treatment but is not required during the treatment. Accordingly, glucose is likely to be required as an energy source for recovery from pressure-induced damage at atmospheric pressure.

Table 5.4.1

Effects of high pressure and occurrences in yeast *Saccharomyces cerevisiae*

Pressure (MPa)	Effect and occurrence	Description	References
0.1–50	Arrest of cell growth	Cell cycle arrest in G_1 phase in Trp^- strains (15–25 MPa and 24°C)	Abe and Horikoshi (2000)
		Trp^+ or HPG strains, (50 MPa and 24°C)	Abe and Horikoshi (2000), Abe and Iida (2003)
		80 genes required for growth at 25 MPa and 24°C, and/or 0.1 MPa and 15°C	Abe and Minegishi (2008)
	Inhibition of amino acid uptake	Severity, Trp>Lys>His>Leu	Abe and Horikoshi (2000)
	Pressure-upregulated transcription	Stress-inducible genes (30 MPa and 25°C)	Iwahashi et al. (2005)
		Stress-inducible genes (40 MPa and 4°C)	Iwahashi et al. (2003)
		DAN/TIR family genes (25 MPa and 24°C)	Abe (2007b)
	Enhancement of esterase activity	Nonspecific esterases (30–50 MPa)	Abe (1998)
50–	Inhibition of ethanol fermentation	Internal ATP level is unchanged	Abe and Horikoshi (1997), Abe and Horikoshi (1998)
	Cytoplasmic and vacuolar acidification	Fluorescence analysis; Internal pH decreases by 0.3−0.5 units	Abe (1995), Abe and Horikoshi (1997), Abe and Horikoshi (1998)
100–	Reduction in viability	Colony-forming unit measurement	Rosin and Zimmerman (1977), Hamada et al. (1992), Shimada et al. (1993), Kobori et al. (1995), Sato et al. (1996), Perrier-Cornet et al. (1999), Sato et al. (1999)
	Disruption of microtubules	Electron microscopy	Kobori et al. (1995), Sato et al. (1996)
	Depolymerization of F-actin	Electron microscopy	Sato et al. (1996, 1999)
	Nuclear membrane perturbation	Electron microscopy	Kobori et al. (1995), Sato et al. (1996, 1999)
	Acquired piezotolerance	Heat shock (42°C, 30 min); Hsp104 and trehalose have a role	Iwahashi et al. (1991, 1997), Kawai et al. (1999), Iwahashi et al. (2000, 2001)
	Acquired piezotolerance	Msn2/Msn4 transcription factors	Domitrovic et al. (2006)
	Pressure-upregulated transcription	After pressure release (180 MPa and 4°C)	Iwahashi et al. (2003)

Table 5.4.1 (Continued)

Pressure (MPa)	Effect and occurrence	Description	References
200–	Acquired piezotolerance	H_2O_2, ethanol or cold-shock treatment	Palhano et al. (2004)
	Induction of petite mutation	Strain and growth phase dependent	Rosin and Zimmerman (1977)
	Induction of homozygous diploids	Dye-plate method	Hamada et al. (1992)
	Shrinkage of cells	Direct microscopic observation	Perrier-Cornet et al. (1995)
	Leakage of internal substrates	Amino acids and ions	Shimada et al. (1993)
	Pressure-upregulated transcription	After pressure release (200 MPa, 30 min)	Fernandes et al. (2004)

Direct Measurement of Intracellular pH Reveals Cytoplasmic and Vacuolar Acidification in Response to High Pressure

The maintenance of neutral cytoplasmic pH is important for the optimal activity of cytoplasmic enzymes. In yeast, the plasma membrane H^+-ATPase, Pma1, plays an essential role in cytoplasmic pH homeostasis by pumping out protons that are accumulated in the cytoplasm as a result of ethanol fermentation (Serrano 1993). The yeast cell has a large acidic compartment, the vacuole, with an internal pH of around 6.0. Acidic vacuolar pH is maintained through the function of vacuolar H^+-ATPase (V-H^+-ATPase) on the vacuolar membrane by pumping in protons from the cytoplasm (Kakinuma et al. 1981). The cytoplasmic pH and vacuolar pH of living yeast cells were individually analyzed using pH-sensitive fluorescent dyes, 5- (and 6-) carboxy SNARF-1 and 6-caroboxyfluorescein, respectively, in a hydrostatic pressure chamber with transparent windows. The fluorescence spectra of these dyes depend on the external pH, making it possible to monitor the intracellular pH of labeled cells. Pressure of 40–60 MPa decreased both cytoplasmic and vacuolar pH by about 0.3–0.5 units (Abe and Horikoshi 1995, 1997, 1998). Internal acidification of the cytoplasm and vacuoles is attributable to the production of carbon dioxide (CO_2) through ethanol fermentation. CO_2 is easily soluble in water; at 0.1 MPa more than 99% of aqueous CO_2 exists as dissolved gas and less than 1% exists as carbonic acid, H_2CO_3, which partly dissociates to give H^+, HCO_3^-, and CO_3^{2-}. The reaction volume (ΔV) of the reaction $H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$ is negative (-26.0 ml/mol), which means that the equilibrium shifts toward the production of protons by increasing hydrostatic pressure. Accordingly, the cytoplasm is likely to be acidified under high pressure. The decrease of 0.3–0.5 pH units appears small but is sufficient to slow the catalysis of fructose-6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase, the allosteric enzyme in glycolysis. To maintain a favorable cytoplasmic pH, the yeast vacuole is assumed to serve as a proton sequestrant under high hydrostatic pressure by taking up protons from the cytoplasm (Abe and Horikoshi 1998). Accordingly, chemical reactions involving abundant intracellular low

molecular-weight compounds such as CO_2 should be elucidated with respect to the physiological responses of living cells to high pressure.

Tryptophan Availability Limits Cell Growth Under Nonlethal Levels of High Pressure

While high pressure greater than 100 MPa is lethal, pressures of less than 50 MPa do not affect the survival rate significantly even when yeast cells are pressurized for more than several hours. Nevertheless, the cells undergo growth arrest at less than 50 MPa (Abe and Horikoshi 2000). In *S. cerevisiae*, the availability of tryptophan is one of the most high pressure-sensitive processes in yeast physiology. Experimental wild-type strains (e.g., YPH499 and W303-1A) usually have several nutrient auxotrophic markers such as *trp1* (tryptophan), *leu2* (leucine), *lys2* (lysine), *his3* (histidine), *ade2* (adenine), or *ura3* (uracil) for the selection of plasmid-bearing transformants. Regardless of other forms of auxotrophy, *trp1* cells undergo arrest of the cell cycle in the G_1 phase when cells are exposed to pressure of 15–25 MPa (Abe and Horikoshi 2000). This is due to reduced tryptophan uptake activity (see below). This property offers a unique method to synchronize the cell cycle of *S. cerevisiae* using moderate high-pressure treatment. In contrast, tryptophan-prototrophic strains are capable of growth under the same conditions (Abe and Horikoshi 2000). Any factors that lead to increased tryptophan availability enable *trp1* cells to grow at high pressure. The addition of excess tryptophan to the medium (e.g., 1 g/L in comparison with 20–40 mg/L in normal medium), introduction of the *TRP1* gene to confer tryptophan synthesis, or overexpression of the gene encoding tryptophan permease Tat1 or Tat2 confers the ability to grow at 25 MPa on *trp1* cells (Abe and Horikoshi 2000, Abe and Iida 2003). The growth of *trp1* cells is also inhibited at low temperatures of 10–15°C. In a manner analogous to high pressure, the growth defect at low temperature can be compensated for by increased tryptophan availability. The similarity between high pressure and low temperature is related to the physicochemical properties of the lipid bilayer. The structure of the lipid bilayer is easily perturbed by hydrostatic pressure and temperature (Winter and Dzwolak 2004). Particularly, membrane fluidity is decreased either by increasing hydrostatic pressure or decreasing temperature. In this sense, tryptophan uptake by yeast cells is sensitive to decreases in membrane fluidity caused by either high pressure or low temperature.

Isolation of High-Pressure Growth Mutants

Mutants capable of growth at high pressure were isolated from a *trp1* strain. They are designated high-pressure growth (*HPG*) mutants (Abe and Iida 2003). The identification of the mutation sites and phenotypic characterization allow us to elucidate the complex intracellular mechanism that regulates tryptophan permeases. The experimental procedure for the isolation of the *HPG* mutants is described in Chap. 5.7 Isolation Methods for High-Pressure Growth Mutant in Yeast. All *HPG* mutants have acquired the ability to grow at low temperatures of 8–15°C and at pressures of 25–30 MPa (Abe and Iida 2003). The *HPG* mutants are classified into four semidominant linkage groups designated *HPG1*, *HPG2*, *HPG3*, and *HPG4*. The *HPG1* and *HPG2* genes were identified (see below) (Abe and Iida 2003; Nagayama et al. 2004).

Regulation of Tryptophan Permeases by the Ubiquitin System in Response to High Pressure

HPG1 appears to be allelic to *RSP5*, which encodes Rsp5 ubiquitin ligase (Abe and Iida 2003). Ubiquitination is a selective degradation system of cellular proteins in eukaryotes. After ubiquitin molecules are covalently bound to target proteins, the ubiquitinated proteins are delivered to the proteasome or the vacuoles for degradation. Rsp5 is an E3 enzyme and plays a central role in selecting target proteins to be ubiquitinated (Hicke 1999; Katzmann et al. 2001). The four *HPG1* mutation sites are located in the catalytic HECT (homologous to E6-AP *C*-terminus) domain of Rsp5 (Abe and Iida 2003). When the wild-type cells are exposed to pressure of 25 MPa, the tryptophan permeases Tat1 and Tat2 are degraded in a ubiquitination-dependent manner. However, both permeases become resistant in the *HPG1* mutant and are stabilized in the plasma membrane. Consequently, the *HPG1* mutant is capable of growth under pressure due to the stable uptake of tryptophan (Abe and Iida 2003). Deletion of *BUL1* that encodes an Rsp5-binding protein (Yashiroda et al. 1996) also results in the stabilization of tryptophan permeases. Bul1 has the Pro-Pro-X-Tyr (PPXY) motif that interacts with the WW domain of Rsp5 and supports the function of Rsp5 upon the ubiquitination of Tat2. Another high-pressure growth gene, *HPG2*, appears to be allelic to *TAT2* (Nagayama et al. 2004). When yeast cells are deprived of nutrients, Tat2 is degraded following the ubiquitination of its 29th and/or 31st lysine at the *N*-terminal cytoplasmic tail (Beck et al. 1999). The three *HPG2* mutation sites are located in the cytoplasmic domains of the *N*- and *C*-terminal tails of the Tat2 protein (Nagayama et al. 2004). These amino acid substitutions are likely to interfere with ubiquitination on the lysines by Rsp5 ubiquitin ligase. Consequently, the mutant forms of Tat2 are stabilized, leading to high-pressure growth of the *HPG2* mutant.

Ubiquitinated proteins undergo deubiquitination for the recycling of ubiquitin prior to breakdown by the proteasomes or in the vacuoles. Deubiquitination is catalyzed by ubiquitin-specific proteases (Ubps). Of the 17 potential Ubps encoded by the yeast genome, Doa4 (Ubp4), Ubp6, and Ubp14 are involved in the degradation of Tat2. Disruption of one of the three *UBP* genes stabilizes Tat2 at high pressure, and consequently the disruptants exhibit high-pressure growth at 25 MPa (Miura and Abe 2004). It is still unclear whether these ubiquitin-specific proteases directly or indirectly affect the ubiquitinated state of Tat2 in the plasma membrane or within the endocytic pathways.

A small protein (~15 kDa) of unknown function encoded by *SNA3* appeared to render high-pressure growth ability on the *trp1* strain when it was overexpressed (Hiraki and Abe 2010). Sna3 possesses two transmembrane domains and is targeted to the vacuolar lumen (Reggiori and Pelham 2001; McNatt et al. 2007; Stawiecka-Mirota et al. 2007). This protein has the PPXY motif that interferes with the interaction between the WW domain of Rsp5 and the PPXY motif of Bul1. As a result of deficiency in Rsp5-dependent ubiquitination, Tat2 is stabilized in the Sna3-overexpressing cells, leading to the efficient uptake of tryptophan (Hiraki and Abe 2010). Factors that affect the stability of tryptophan permeases and high-pressure growth are listed in *Table 5.4.2*.

In this manner, if tryptophan-auxotrophic strains are used to isolate high-pressure growth mutants, the corresponding mutations will frequently occur in proteins involved in the degradation of Tat2. Using this approach, any factor affecting the stabilization of Tat2 through ubiquitination can be evaluated by examining high-pressure growth in the genetic background of tryptophan auxotrophy.

Table 5.4.2

Factors conferring stabilization of tryptophan permeases and high-pressure growth on Trp⁻ strains of *Saccharomyces cerevisiae*[a]

Overexpression or mutation	Description	References
TAT1 overexpression	Low-affinity type tryptophan permease	Abe and Iida (2003)
TAT2 overexpression	High-affinity type tryptophan permease	Abe and Horikoshi (2000), Abe and Iida (2003)
HPG1 mutation	HECT domain of Rsp5 ubiquitin ligase	Abe and Iida (2003)
HPG2 mutation	Cytoplasmic tails in Tat2	Nagayama et al. (2004)
BUL1 deletion	Rsp5-binding protein with the PPXY motif	Abe and Iida (2003)
DOA4 deletion	Ubiquitin-specific protease (deubiquitinating enzyme)	Miura and Abe (2004)
UBP6 deletion	Ubiquitin-specific protease	Miura and Abe (2004)
UBP14 deletion	Ubiquitin-specific protease	Miura and Abe (2004)
VPS27 deletion	Endosomal protein that interacts with ubiquitin	Abe and Iida (2003)
SNA3 overexpression	Integral membrane protein with the PPXY motif	Hiraki and Abe (2010)

[a]The growth rate was measured at 25 MPa and 24°C.

Probing for Dynamic Tryptophan Import Using Hydrostatic Pressure

The effects of hydrostatic pressure on an enzymatic reaction are interpreted within the framework of the simplest kinetic mechanism in which the transition state presents the highest energy barrier, and the chemical transformation of substrate to product is considered to be a single rate-limiting step. Equation 5.4.2 represents a quantitative estimation of the effects of pressure. The activation volumes associated with tryptophan import through the tryptophan permeases Tat1 and Tat2 are relatively large, at 89.3 ml/mol and 50.8 ml/mol, respectively (Abe and Iida 2003). This means that increasing hydrostatic pressure dramatically impairs tryptophan uptake mediated by both permeases. There is a significant difference in the activation volume for tryptophan import between Tat1 and Tat2, which means that the defect is more severe with Tat1 than with Tat2. In other words, Tat1 undergoes a more dramatic conformational change than Tat2 upon tryptophan import. The different sensitivity of the two tryptophan permeases to high pressure is related to the lipid domain where they localize. Although these permeases have high homology in their primary structures, Tat1 exists in the tight and ordered lipid microdomain, the raft, whereas Tat2 exists in the disordered fluid domain, the nonraft (Abe and Iida 2003). The raft is characterized by its sphingolipid and ergosterol (cholesterol in the case of animal cells) constituents (Simons and Ikonen 1997). The remarkable difference between the two permeases in the activation volume (89.3 ml/mol and 50.8 ml/mol, respectively) is accounted for by the difference in the volume of the initial states, that is, the initial volume of Tat1 is likely to be smaller than that of Tat2. This explanation agrees

well with the finding that Tat1 is localized in the highly ordered lipid phase in which the volume is likely to be small, whereas Tat2 is localized in the disordered phase in which the volume is likely to be large (Abe and Iida 2003). A pioneering work demonstrated that the order of lipid bilayers affected the hydrolytic activity of pig kidney Na^+,K^+-ATPase, although the lipid raft model had not yet been proposed (de Smedt et al. 1979). The ATPase activity is accompanied by a smaller activation volume ($\Delta V^{\neq} = 53$ ml/mol) when the membrane is less ordered under low-pressure conditions (0.1–24 MPa). Meanwhile, the activity is accompanied by a higher $\Delta V^{\neq}$ (83 ml/mol) when the membrane structure is more ordered under higher-pressure conditions (pressure $>$ 24 MPa) (de Smedt et al. 1979). Therefore, to elucidate membrane protein dynamics in living cells, hydrostatic pressure is an attractive tool for probing lipid microdomains and the proteins resident in membranes by measurement of their activation volumes.

Global Analysis of Transcription in Response to High Pressure

Genome-wide expression profiles under high pressure were characterized in *S. cerevisiae* in some laboratories using DNA microarray hybridization, but the results are controversial. This is probably because experimental conditions such as yeast strains, pressure (25–200 MPa), temperature (4–30°C), and duration of pressurization (10 min to overnight) differed among laboratories. According to a report by Iwahashi et al., high-pressure treatment at 30 MPa and 25°C, which is a growth-permissive pressure for tryptophan-protrophic strains, upregulates 366 genes by more than twofold and downregulates 253 genes by more than twofold. Many of the upregulated genes are in the categories of stress response and metabolism of carbohydrates, lipids, and amino acids (Iwahashi et al. 2005). The upregulation of *INO1*, *OPI3*, *PST1*, *RTA1*, *SED1*, *PRM5*, and *POX1*, which are involved in biosynthesis and the formation of membrane structure, was confirmed using RT-PCR. The same group analyzed the genome-wide gene expression profiles in cells exposed to nongrowth-permissive pressure treatment at 180 MPa and 4°C briefly or 40 MPa and 4°C for 16 h. During recovery after the treatments, the transcription of genes categorized into energy metabolism, cell defense, and protein metabolism functions was markedly upregulated (Iwahashi et al. 2003). Upon exposure of the cells to a lethal pressure of 200 MPa for 30 min, genes involved in stress defense and carbohydrate metabolism were markedly upregulated, while genes involved in cell cycle progression and protein synthesis were downregulated (Fernandes et al. 2004). In our study, we compared the global transcriptional profile in cells grown under normal growth conditions (0.1 MPa, 24°C), high pressure (25 MPa, 24°C for 5 h), and low temperature (0.1 MPa, 15°C for 5 h) (Abe 2007b). A comparison of their transcriptional profiles revealed that the majority of genes were transcribed at similar levels between the normal growth conditions and high pressure, and between the normal growth conditions and low temperature. Notably, there was greater similarity in transcription levels between high pressure and low temperature. Of the 6,337 genes, 561 were concurrently upregulated by high pressure and low temperature, while 161 were downregulated. In particular, expressions of the *DAN*/*TIR* cell wall mannoprotein genes, which are generally expressed under hypoxia, were markedly upregulated by high pressure and low temperature (Abe 2007b). In support of the role of the mannoproteins in cell wall integrity, cells acquire resistance against a short-term treatment with SDS (0.05%), Zymolyase 100T (500 μg/ml), or a lethal level of pressure at 125 MPa for 1 h after cells have been preincubated under high pressure (25 MPa, 5 h) or low temperature (15°C, 5 h). Although transcriptional

regulation in response to high pressure and low temperature is apparently different from that in response to hypoxia, the finding implies fascinating crosstalk in the regulatory networks of *DAN/TIR* transcription. The central issue to be addressed is how seemingly unrelated environmental factors induce *DAN/TIR* gene expression in yeast. Abramova et al. speculated that cells exhibited reduced membrane fluidity under hypoxia as a possible outcome of anaerobiosis and at low temperature as a result of reduced lateral diffusion and increased microviscosity (Abramova et al. 2001a, b). Their findings could be consistent with the transcriptional responses to high hydrostatic pressure in terms of the reduction in membrane fluidity. In this sense, a membrane sensor(s) is likely to exist in the membrane and may transduce the changes in membrane fluidity into intracellular signals that stimulate transcription of the *DAN/TIR* genes. Despite the clear transcriptional activation, however, no single deletions of the genes cause susceptibility of the cells to high pressure in terms of growth and viability. This suggests that not all pressure-activated genes have a physiological relevance to growth and survival. Otherwise, their functionally redundant genes could be encoded by the yeast genome.

A subset of *HSP* genes was systematically analyzed to understand their roles in high-pressure growth in terms of expression and disruption. Of the 17 *HSP*s and their related genes, *HSP104*, *HSP10*, and *HSP78* are upregulated by three- to fourfold when cells are exposed to 25 MPa for 5 h, whereas *HSP26* and *HSP31* are downregulated by about twofold (Miura et al. 2006). The mechanism by which high pressure activates the transcription of these genes and their roles in the growth and/or survival of cells at high pressure remains unclear. The loss of *HSP31* renders a slow-growth phenotype in a tryptophan-prototrophic strain at 25 MPa (Miura et al. 2006), suggesting a role of this protein in high-pressure growth. Hsp31 is a 25.5-kDa protein and a possible chaperone and cysteine protease with similarity to *Escherichia coli* Hsp31 (Malki et al. 2005). Accordingly, some proteins might be misfolded during de novo protein synthesis in the endoplasmic reticulum at 25 MPa, and such misfolded proteins could be catalyzed by the presumed Hsp31 chaperone.

Global Screening of Genes Required for Growth Under High Pressure

A PCR-generated deletion strategy (Baudin et al. 1993; Wach et al. 1994) was used to replace each yeast open-reading frame from its start- to stop-codon systematically in a tryptophan-prototrophic strain (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html#intro). High-throughput screening of the library consisting of 4,828 deletion strains was performed to search for novel mutants that are defective in a variety of cellular functions (Giaever et al. 2002). Considering the similarity of the effects of high pressure and those of low temperature, screening of the library was performed to obtain mutants defective in growth under either high pressure or low temperature (Abe and Minegishi 2008). The screening revealed a number of unpredicted genes that are unrelated to tryptophan biosynthesis and anticipated to have a variety of functional roles responsible for growth under high pressure and low temperature. The details of the screening procedure are described in Chap. 5.7. The first gross screening in our study anticipated 1,022 candidate strains including 779 high pressure-sensitive mutants and 560 low temperature-sensitive mutants with a significant overlap of 317 strains. Those strains were quantitatively examined in liquid culture to evaluate their ability to grow at 25 MPa and 24°C or 0.1 MPa and 15°C, and finally 80 genes including 71 genes responsible for high-pressure growth and 56 genes responsible

for low-temperature growth with a significant overlap of 47 genes were obtained (Abe and Minegishi 2008) (Table 5.4.3). These genes are involved in a broad range of cellular functions such as amino acid biosynthesis, mitochondrial function, the actin cytoskeleton, membrane trafficking, transcription, ribosome biogenesis, chromatin structure, and unknown roles. Therefore, factors with various cellular functions support cell growth under high pressure and low temperature. As expected from studies on the *trp1* strain, the losses of tryptophan-synthetic genes such as *ARO1*, *ARO2*, *TRP1*, *TRP2*, *TRP4*, and *TRP5* resulted in marked sensitivity to high pressure and low temperature. Deletion of amino acid-biosynthetic genes such as *HOM3*, *THR4*, and *SER1* resulted in auxotrophy for methionine (*hom3*Δ), threonine (*thr4*Δ), and serine (*ser1*Δ), and thus the growth of these mutants depends on external amino acids. Because the three mutants also exhibit growth defects, high pressure and low temperature impair the ability to take up these amino acids, potentially inactivating their permeases. The EGO complex, a vacuolar membrane-associated protein complex (Dubouloz et al. 2005), is required to maintain the activity of amino acid uptake under high pressure and low temperature, probably due to the appropriate cell surface delivery of amino acid permease proteins. The loss of any one of the EGO complex members causes marked sensitivity to high pressure and low temperature (Abe and Minegishi 2008). Among 11 deletion strains for mitochondrial proteins, the *aco1*Δ and *caf17*Δ mutants require either glutamine or glutamate for growth. Taking the results together, permease-dependent uptake of amino acids generally becomes compromised

Table 5.4.3

Genes required for growth under high pressure and/or low temperature in a Trp$^+$ strain of *Saccharomyces cerevisiae*[a]

Category	Gene name[b]
Amino acid biosynthesis	*TRP1, TRP2, TRP4, TRP5, ARO1, ARO2, LEU3, THR4, HOM3, SER1*
Microautophagy/ permease trafficking	*EGO1, EGO3, GTR1, GTR2*
Mitochondrial function	*MRPL22, MRF1, CAF17, ACO1, MRP51, MRPL38, ATP15, MDJ1, MSY1*
Actin organization/bud formation	*LTE1, HOF1, SLM3, CLA4, CDC50, SLM6*
Membrane trafficking	*VID24, VPS34, SEC22, PEP3, CHC1, PEP5, VPS45, ERG24, VPS54, AKR1, SAC1*
Inositol phosphate metabolism	*PLC1, ARG82, PHO88, KCS1*
Transcription/mRNA degradation	*SNF6, MOT2, POP2, SHE3, CDC73, RPB4, HFI1, PAF1, ELF1, SNF1, SRB1, TAF14, CCR4, SAP155*
Ribosome function	*RPL1B, RPL21A, RPS30B*
Chromatin maintenance	*NBP2, YAF9, IES2, CGI121, ARD1*
Stress response	*HSP31, YDJ1*
Unknown genes	*AVL9, DLT1, CSF1, YDR008C, YKL098W, YHR151C, YPR153W, YBR255W, YGL218W, YDL172C, YDL173W, YDR442W*

[a]The growth rate was measured at 25 MPa and 24°C (high pressure) or 0.1 MPa and 15°C (low temperature). Gene functions are described in Abe and Minegishi 2008.

[b]Gene names are given by *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

by increasing hydrostatic pressure and decreasing temperature. The mitochondrial proteins of which the loss does not affect amino acid synthesis are still required for growth under high pressure and low temperature, but the reason for the sensitivity is not yet known.

Oligomerization of subunit proteins and assembly of regulatory components are high pressure- and low temperature-sensitive processes in the cell. *CDC50* encodes a protein required for actin cytoskeleton organization and trafficking of proteins between the Golgi complex and the endosome/vacuole. The lack of *CDC50* confers sensitivity to high pressure (Abe and Minegishi 2008) and low temperature (Misu et al. 2003; Saito et al. 2004), which is attributable to a defect in the establishment of actin networks resulting from improper localization of regulator proteins for polarized growth. Cdc50 associates with Drs2, a P-type ATPase of the aminophospholipid translocase that functions in phospholipid asymmetry (Chen et al. 1999; Natarajan et al. 2004; Saito et al. 2004). The lack of *DRS2* also results in growth defects under high pressure and low temperature (Abe and Minegishi 2008).

Numerous genes involved in membrane trafficking are responsible for growth under high pressure and low temperature. Delivery of newly synthesized proteins to appropriate locations, for example, the bud neck, cell surface, or cell wall, might be diminished at high pressure and low temperature when any one of the genes listed in *Table 5.4.3* is lost. *ERG24* encodes the C-14 sterol reductase that catalyzes a step in ergosterol biosynthesis (Lorenz and Parks 1992). The *erg24* mutants are viable but accumulate an abnormal, ergosta-8,14 dienol (Parks et al. 1995). Genes encoding the latter five steps are nonessential. Among them, deletions of *ERG6*, *ERG2*, *ERG3*, and *ERG5* result in growth defects under high pressure and low temperature (our unpublished observations). Therefore, the specific structural motif of ergosterol is required for the function and/or trafficking of membrane proteins under high pressure and low temperature. Kishimoto et al. demonstrated that the *cdc50* mutation is synthetically lethal with the *erg* mutations. In the *cdc50*Δ*erg3*Δ mutant, sterol was predominantly detected either diffusely within the cytosol or as punctate dots with a concomitant decrease in the plasma membrane resulting from a possible defect in recycling sterols to the plasma membrane (Kishimoto et al. 2005). Therefore, high pressure and low temperature could lead to a situation analogous to ergosterol deficiency in which the Cdc50-Drs2 complex is unable to function in membrane trafficking and the organization of the actin cytoskeleton. Excessive ordering of biological membranes would have an adverse impact on membrane-mediated functions in cells that have been adapted to atmospheric pressure and moderate temperature. It is likely that proteins encoded by those membrane-trafficking genes facilitate steps in vesicle budding and/or vesicle fusion in the endocytic pathway when the membrane is ordered.

Genes involved in transcription and mRNA degradation comprise a major class of genes required for growth at high pressure and low temperature. The Ccr4-Not complex is a global regulator of transcription consisting of five Not proteins (Not1–Not5), Pop2, Caf40, Caf130, and Ccr4 (Liu et al. 1998; Tucker et al. 2001; Collart 2003). Among them, Not1 is the scaffold of the complex and is essential for viability (Maillet et al. 2000). Ccr4 and Pop2 associate with a central portion of the *N*-terminal domain of Not1 and function as the major yeast deadenylases that play a role in mRNA degradation (Tucker et al. 2001). Meanwhile, Not4 associates with the *C*-terminal domain of Not1 and functions as an E3 ubiquitin ligase upon ubiquitination (Albert et al. 2002). Deletion of *CCR4*, *POP2*, or *NOT4* results in substantial growth defects at high pressure and low temperature. Accordingly, the loss of one of the components destabilizes the Ccr4-Not1 complex at high pressure and low temperature, or alternatively the assembly of the complex is impaired by high pressure and low temperature. Oligomerization of proteins is a typical high pressure-sensitive process. The ribosome has an

enormous structure, and its assembly is likely to be sensitive to high pressure. The loss of the ribosomal subunit Rpl1b or Rpl21a confers high-pressure and low-temperature sensitivity. These proteins could function to stabilize ribosomal structures and subunit associations at high pressure and low temperature.

Future Perspectives

Utilizing genetic databases and applying techniques for molecular biology in studies of the yeast *S. cerevisiae* have improved our fundamental understanding of the effects of high hydrostatic pressure in living cells in a broad range of experimental fields including genetics, physiology, biochemistry, and biotechnology. Further understanding of the effects of high pressure will be achieved by introducing high-throughput techniques to traditional biochemical and genetic studies. However, numerous questions remain to be answered: What is the cellular sensor that responds to changes in hydrostatic pressure? How could the hypothetical pressure sensor transduce signals into downstream gene expression? How do proteins encoded by the high-pressure-growth genes fulfill their functions under high pressure? More biophysical approaches will provide evidence for actual structure–function relationships. We have not yet understood how the complex metabolic network is optimized to maintain life under a given pressure condition. Breakthroughs will come based on detailed descriptions of experimental conditions and results, and cooperative interaction among investigators in high-pressure biosciences regardless of their objectives or types of target organism.

Cross-References

- 5.1 Distribution of Piezophiles
- 5.2 High Pressure and Prokaryotes
- 5.3 Piezophysiology of the Model Bacterium *Escherichia coli*
- 5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low Temperature Piezophiles
- 5.6 Cultivation Methods for Piezophiles
- 5.7 Isolation Methods for High-Pressure Growth Mutant in Yeast

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5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low Temperature Piezophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_5.5,

Introduction

Pressure is an important environmental thermodynamic parameter that influences chemical equilibria and kinetics both inside and outside of organisms. Within the known biosphere, pressure extends three orders of magnitude from atmospheric pressure (1 atmosphere = 0.101325 megapascals [MPa]) at sea level to 90–110 MPa in the deepest ocean trenches. In stratified waters, deep-sea microbial communities display optimal productivity at in situ pressure (Tamburini et al. 2009). However, pressure effects on microbes are complex. Seawater microcosm studies indicate that even moderate pressures (40 MPa) can effect growth and cell size in a species-specific fashion (Grossart and Gust 2009). In this chapter, the focus is on high-throughput sequence analyses of microbial communities and species with a particular focus on the ribosome. Those interested in learning more about the phylogenetics and adaptations of low-temperature, piezophilic (high-pressure-adapted) microbes should consult the chapters by Kato et al. (5.1 Distribution of Piezophiles; 5.2 High Pressure and Prokaryotes) in this handbook and other references (Bartlett 2002b; Bartlett et al. 2007; Michiels et al. 2008; Prieur et al. 2009).

Who Lives Down Deep?

Table 5.5.1 lists some of the large-scale sequencing publications that have addressed aspects of the distribution and adaptations of deep-sea microbes to deep low-temperature environments. A useful starting point for considering the "deep" sequencing of deep-sea microbes is ribosomal tag pyrosequencing. These massively parallel sequencing approaches have generated tens to hundreds of thousands of partial ribosomal RNA gene sequences for taxonomic assignment, including members making up a very small fraction of the total rank abundance profile, present at depths down to about 4 km in both the North Atlantic and North Pacific Oceans (Brown et al. 2009; Sogin et al. 2006). An important take-home message from this work is that in general deep, low-temperature pelagic waters are enriched with members of the phyla *Verrucomicrobia*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Acidobacteria*, and *Firmicutes*, and with very high proportions of *Proteobacteria* whose subphylum distributions vary with locale (Brown et al. 2009; Lauro and Bartlett 2008). Additional phylogenetic data on deep-sea pelagic microbes has been derived from the shotgun sequencing of DNA recovered from samples. Phylogenetic analyses of metagenomic data from the North Pacific (4 km depth) and from the Mediterranean Sea (3 km depth) have reinforced some of the trends derived from tag sequencing and have also noted high levels of *Pelagibacter ubique* strains within the *Alphaproteobacteria* (Martín-Cuadrado et al. 2007; Pham et al. 2008). Archaeal diversity is much less than that of the bacteria at depth and is particularly high in Marine Group I *Crenarchaeota* (Brown et al. 2009; DeLong et al. 2006; Martín-Cuadrado et al. 2007). The least is known about eukaryal microbe taxonomic distributions with depth, but more recent efforts indicate high proportions of flagellate species affiliated with the two major uncultured diplonemid clades and some radiolarian classes (Brown et al. 2009; Lara et al. 2009). Statistical analyses of community structures verify that deep-sea communities tend to be more similar to one another than to the assemblages present in their overlying water masses (Brown et al. 2009). Taken together these data are consistent with the view that the depth contours of the world oceans correlate with distinct microbial assemblages and that one of the environmental parameters driving this distribution is adaptation along the pressure continuum.

Table 5.5.1

Selected papers contributing to high-throughput sequencing analyses of low-temperature deep-sea microbes

Year	Project	Reference
2005	First genome sequence and transcriptome analysis of a low-temperature piezophile (*Photobacterium profundum* SS9)	Vezzi et al. (2005)
2005	Comparative genomics of piezophilic and non-piezophilic *P. profundum* strains	Campanaro et al. (2005)
2006	Tag pyrosequencing of deep-sea bacterial communities in the North Atlantic	Sogin et al. (2006)
2006	Metagenomic analysis of North Pacific pelagic Bacteria and Archaea down to 4 km depth	DeLong et al. (2006)
2007	Metagenomic analysis of Mediterranean pelagic Bacteria and Archaea down to 3 km	Martín-Cuadrado et al. (2007)
2008	Genome analysis of the low-temperature piezotolerant bacterium *Shewanella piezotolerans* WP3	Wang et al. (2008)
2008	Comparative genomics of additional low-temperature piezophiles	Lauro and Bartlett (2008)
2008	Rapidly evolving genes in two piezophiles	Campanaro et al. (2008)
2008	Phylogenetic analyses of fosmid clones of the 4 km depth pelagic Bacteria in the North Pacific	Pham et al. (2008)
2009	Further metagenomic analysis of the 4 km depth pelagic Bacteria and Archaea in the North Pacific	Konstantinidis et al. (2009)
2008	Comparative genomics of shallow and deep ecotypes of *Alteromonas macleodii*	Ivars-Martinez et al. (2008)
2009	Tag pyrosequencing of deep-sea bacterial communities in the North Atlantic	Brown et al. (2009)

How Are Microbes Adapted to Life at Depth?

Many clues to the nature of bacterial and archaeal life at depth, and in particular life at high pressure, have come from genome analyses. The path to whole genome sequencing began with the sequencing of individual genes of interest from cultured piezophiles, such as those whose expression changes with pressure or that encoded enzymes or structural proteins or interest. This list includes pressure-regulated outer membrane protein-encoding genes, pressure-regulated cytochrome-related genes, enzymes for the production of saturated and monounsaturated fatty acids, malate dehydrogenase, isopropylmalate dehydrogenase, single-stranded DNA-binding protein, and the tubulin-like cytoskeletal protein FtsZ (Bartlett 2002a).

A further step-up in complexity was the examination of a 33-kilobase-pair locus from the moderate piezophile *Photobacterium profundum* strain SS9 that governs the production of an omega-3 polyunsaturated fatty acid (PUFA). A hallmark of many low-temperature deep-sea bacteria is the production of large amounts of omega-3 PUFAs in their membrane lipids. The nature of the large multidomain proteins that catalyze omega-3 PUFA production in *P. profundum* strain SS9 was determined by construction and partial sequencing of

an SS9 genomic fosmid library (Allen and Bartlett 2002). This indicated that PUFA production in deep-sea bacteria proceeds via a novel polyketide synthesis mechanism.

Photobacterium profundum SS9 was also the first deep-sea microbe adapted to low temperature and high pressure to have its entire genome completely sequenced (Vezzi et al. 2005). This species has also been the subject of intraspecific comparisons of its piezophilic and piezosensitve members (Campanaro et al. 2005). Various degrees of whole genome sequence information are now also available for the psychrophilic or psychrotolerant piezophiles *Shewanella benthica* KT99, *Psychromonas* sp. CNPT3, *Moritella* sp. PE36, and *Carnobacterium* sp. AT7 (Goldberg et al. 2006; Lauro and Bartlett 2008), and these have also been used for certain comparisons. Analyses of COG (Clusters of Orthologous Groups) distributions among these piezophiles and their closely related comparison strains (listed in *Table 5.5.2*) indicate an increased fraction of genes devoted to translation, ribosome structure, secretion, and motility within the piezophiles (Lauro and Bartlett 2008; Prieur et al. 2009). It also appears that versatility in both the front end and back end of metabolism is important for many piezophiles. These organisms appear to possess an enhanced capacity for transporting and catabolizing a variety of organics including various xenobiotics, and chitin, pullulan, cellulose, and starch, while also possessing a large repertoire of cytochromes at their disposal (Prieur et al. 2009). The indications of catabolic diversity are consistent with the fact that in many deep-sea settings organic substrates are low in abundance, diverse, and highly recalcitrant (Nagata et al. 2010). The presence of diverse cytochromes could reflect adaptations to different redox environments or to pressure effects on electron transport (Chikuma et al. 2007).

By far the most dramatic and universal genomic characteristic of low-temperature piezophiles is the possession of increased numbers of mobile elements. This trend is also evident in comparisons of shallow and deep ecotypes of *Alteromonas macleodii* from the Mediterranean Sea (Ivars-Martinez et al. 2008). Detailed analyses of metagenomic data from the Hawaii Ocean Time Series Station ALOHA indicate that deep-sea communities are characterized by a higher content of "selfish" genetic elements, such as transposases and prophages, whose propagation is apparently favored by more relaxed purifying (negative) selection in deeper waters (Konstantinidis et al. 2009).

Table 5.5.2

Selected piezophile genome sequences and comparison genome sequences. Most of these genomes are described or referenced in the cited references (Campanaro et al. 2008; Campanaro et al. 2005; Goldberg et al. 2006; Lauro and Bartlett 2008; Lauro et al. 2007; Vezzi et al. 2005), with the exception of *Colwellia* MT41 whose genome description is unpublished (Methé et al. in preparation)

Piezophile	Piezophile depth of isolation[a] (pressure)	Comparison strain
Colwellia sp. MT41	10,476 (105 MPa)	*Colwellia Psychrerythraea* 34H
Shewanella KT99	9,856 (99 MPa)	*Shewanella frigidimarina* NCIMB 400
Psychromonas CNPT3	5,782 (58 MPa)	*Psychromonas ingrahamii* 37
Moritella PE36	3,584 (36 MPa)	*Moritella vicosa*
Photobacterium profundum SS9	2,551 (26 MPa)	*Photobacterium profundum* 3TCK

[a]In meters.

These community genomic analyses also indicate that deep-ocean microbes encode proteins that have a slight preference for hydrophobic and smaller volume amino acids, perhaps reflecting optimization of protein function at elevated pressure. However, these changes are very subtle. By contrast, amino acid compositional biases that correlate with low-temperature adaptation tend to be more readily apparent (Methé et al. in preparation.; Riley et al. 2008).

Statistical analyses of the most rapidly evolving genes in two species of piezophiles, the moderate piezophile SS9 and the extreme piezophile *S. benthica* KT99, have highlighted the importance of motility and transport for life at depth (Campanaro et al. 2008). Both transport and motility have received additional study. In the former case, comparative genomics microarray hybridization experiments utilizing one piezosensitive and two piezophilic *P. profundum strains* indicate that the piezosensitive *P. profundum* strain lacks a number of transporters (Campanaro et al. 2005). The expression of many *P. profundum* strain SS9 transporter genes is also highly pressure responsive (Campanaro et al. 2005).

In the case of motility, genome analyses have provided the hint that deep-sea bacteria may be enriched in lateral flagella systems, which are used for movement on surfaces. This includes the microbes *P. profundum* SS9, *Shewanella* WP3, and *Moritella* PE36 (Eloe et al. 2008; Lauro and Bartlett 2008; Wang et al. 2008). This is consistent with the large influence of particulate organic carbon on deep-sea microbial abundance and activity (Nagata et al. 2010), and the metagenomic data that suggest that microbial life at depth includes a high proportion of genes associated with surface colonization (DeLong et al. 2006). SS9 lateral flagella development requires both high pressure and high viscosity, the latter correlating with the condition of a bacterial cell on a surface (*Fig. 5.5.1*). An intact bacterial flagellum is also required for transmitting the high-pressure/high-viscosity signal. Direct liquid swimming velocity measurements obtained using a high-pressure microscopic chamber indicate that the SS9 polar flagellum system is amazingly pressure resistant compared to that of the mesophile *Escherichia coli*. It is operational for at least short periods well above the known pressure limit for life: 150 MPa (Eloe et al. 2008).

The Ribosome as a Possible Target of Pressure Adaptation

As a case study in one aspect of the comparative genomics of piezophiles we now consider the ribosome. The ribosome is a ubiquitous macromolecular machine found in all modern living cells.

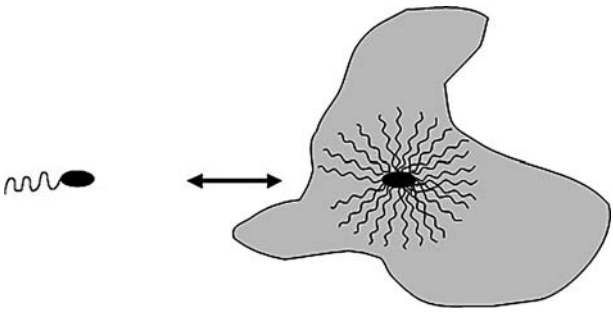


Fig. 5.5.1

Many deep-sea bacteria have adaptations for life on surfaces. *Photobacterium profundum* SS9 turns on a lateral flagella system when it encounters both high pressure and high viscosity (Eloe et al. 2008)

It is composed of both RNA (known as ribosomal RNA or rRNA) and proteins. Although proteins are typically associated with catalyzing reactions in the cell (as enzymes), the ribosome is in fact a ribozyme, with the rRNA catalyzing the formation of the peptide bond between amino acids (Steitz and Moore 2003).

The ribosome and the translation of proteins are affected in various ways by both low temperature and high hydrostatic pressure. Three proteins produced in response to cold shock are known to associate with the ribosome specifically (Thieringer et al. 1998). There are also a number of cold shock proteins that bind mRNA, which are thought to destabilize problematic secondary structures in the mRNA and thereby facilitate translation (Thieringer et al. 1998). Under high hydrostatic pressure, equilibria and reactions causing an overall increase in the volume of the system are disfavored (Somero 1990). High-hydrostatic-pressure effects on macromolecules can stem from effects on hydration and internal cavities (Mozhaev et al. 1996).

There is a considerable potential for the ribosome to be negatively affected by the environmental conditions encountered by piezophiles. It has been shown that mesophile ribosomes are sensitive to hydrostatic pressure (Gross and Jaenicke 1990; Landau 1967), and the piezosensitivity of the *E. coli* ribosome has been linked to the dissociation of the ribosome (Niven et al. 1999). The ribosome relies extensively on the secondary structure of its rRNA components, which could be affected by the low temperature as evidenced by the proteins of the cold shock response. The ribosome's superstructure has internal cavities where translation takes place and there is potential for these to be compressed by the high hydrostatic pressure. Additionally, the proper folding of the rRNA and ribosomal proteins (RPs) into their respective three-dimensional structures could be inhibited at high pressure.

Data from piezophiles also exists suggesting a linkage between the ribosome and pressure adaptation. As previously mentioned, many piezophile genomes possess an increased fraction of genes devoted to translation and ribosome structure. Also, conditional pressure-sensitive mutants have been obtained in SS9 that have transposon insertions in the translation-associated genes *suhB*, a DEAD box helicase family gene and *spoT* (Lauro et al. 2008). SuhB modulates rRNA processing, DEAD box helicases can be involved in ribosome biogenesis, and *spoT* mutants exhibit an increased stringent response and repression of ribosome production. But, perhaps the most striking evidence of a link between ribosome structure and pressure adaptation has come from rRNA structural modeling (Lauro et al. 2007). There is a correlation between piezophily in some genera and elongation of helices 10 (*E. coli* positions 184–193) and 11 (*E. coli* positions 198–219) within the small subunit 16S rRNA. Piezophilic *Colwellia* and *Photobacterium* species harbor insertions in helix 10, whereas piezophilic *Shewanella* exhibit an insertion in helix 11. Helices 10 and 11 have been implicated in interactions with ribosomal protein S20, which is necessary for ribosome assembly. Insertions within the rRNA genes are not universal among piezophiles. Other piezophiles, such as *Moritella* PE36 and *Psychromonas* CNPT3 lack these or other sizable insertions within the 16S rRNA.

Further Bioinformatics Analyses of the Piezophile Ribosome

In order to more broadly assess the importance of the ribosome to piezophily, we have performed a more extensive bioinformatics analysis of all of the ribosomal RNAs and of the ribosomal proteins. The initial analyses were performed with all of the organisms listed in ▶ *Table 5.5.2*. The alignment of piezophile and mesophile 23S rRNA sequences reveal four regions where the piezophilic sequence differs considerably from the paired mesophilic

sequence. These differences are all in the form of insertions into the RNA ranging from 10 to 17 bp. They were found to occur in the same three piezophiles possessing 16S rRNA insertions: *Colwellia* sp. MT41, *S. benthica* KT99, and *P. profundum* SS9.

For the analysis of these insertions, the conservation diagrams from the Comparative RNA Web Site and Project (CRW) were used (Cannone et al. 2002). Sequence data of γ-proteobacteria for the determination of insertion uniqueness was also obtained from the CRW and was supplemented with sequence from version 2.4 of the Integrated Microbial Genomes (IMG) system (Markowitz et al. 2006). Helix numbering is the same as in Mueller et al. (Mueller et al. 2000). Three-dimensional structure analysis was done with the *E. coli* PDB structure 2AW4 (Schuwirth et al. 2005).

Helix 25

The insertions in SS9 and KT99 are 14 bp and 13 bp in length, respectively. Relative to their mesophile pairs, the SS9 insertion is at position 537 and the KT99 insertion is at 534. This places the insertion in helix 25. In the γ-proteobacteria, helix 25 is 20–28 bp long (10–14 bp per strand) capped off with a tetraloop. The *E. coli* structure is 28 bp long with a helical length of 12 (*Fig. 5.5.2*). Helix 25 in *P. profundum* 3TCK has a length of 27 bp with a helical length of 11. In *Shewanella frigidimarina*, helix 25 is 29 bp long and has a helical length of 12. This is in contrast to SS9 and KT99, whose helices have lengths of 41 and 42 bp, respectively. In an alignment with 281 γ-proteobacteria, insertions of this length in helix 25 are found only in KT99 and SS9. Insertions in other species were either much shorter (1–4 bp) or much larger ($\sim$100 bp).

As shown in *Fig. 5.5.2a*, both of the piezophiles' helices are elongated and internal loops have been introduced in both structures. Interestingly, there is a single unpaired cytosine residue in the same position relative to the terminal loop in both structures. This residue is part of the internal loop found on both structures, though the loops are of different sizes.

Helix 25 projects from the top 50S subunit and is surrounded by a number of other helices and proteins. *Figure 5.5.2b* shows the special arrangement of helix 25 relative to helices 2, 46, and 98 and RPs L13, L20 and L21. Through cross-linking studies, helix 25 has been shown to interact with L13 and L21 (Brimacombe 1995).

Insertion #1 is found in two piezophiles and is a unique or mostly unique structural feature within the γ-proteobacteria. It has potential to cause increased interaction with other ribosomal components. It is also possible, because it will stick out beyond the surface of the ribosome, that the insertion may interact with other cellular components increasing stability or efficiency of the ribosome.

A New Helix

The second insertion occurs only in KT99 among the selected piezophiles and mesophiles, and in fact, is a unique feature among 281 γ-proteobacteria examined. The insertion is 13 bp long, and is at position 655, relative to *S. frigidimarina*. KT99's insertion is predicted to form a new helix between helices 27 and 30. Though this feature is not found in the other 280 γ-proteobacteria examined, similar structures are found in other phyla. For example, in 431 bacterial 23S sequences, 36.2% have an insertion at this same point with a maximum length of 25 bp. It is unlikely that this insertion in KT99 is a piezophilic adaptation.

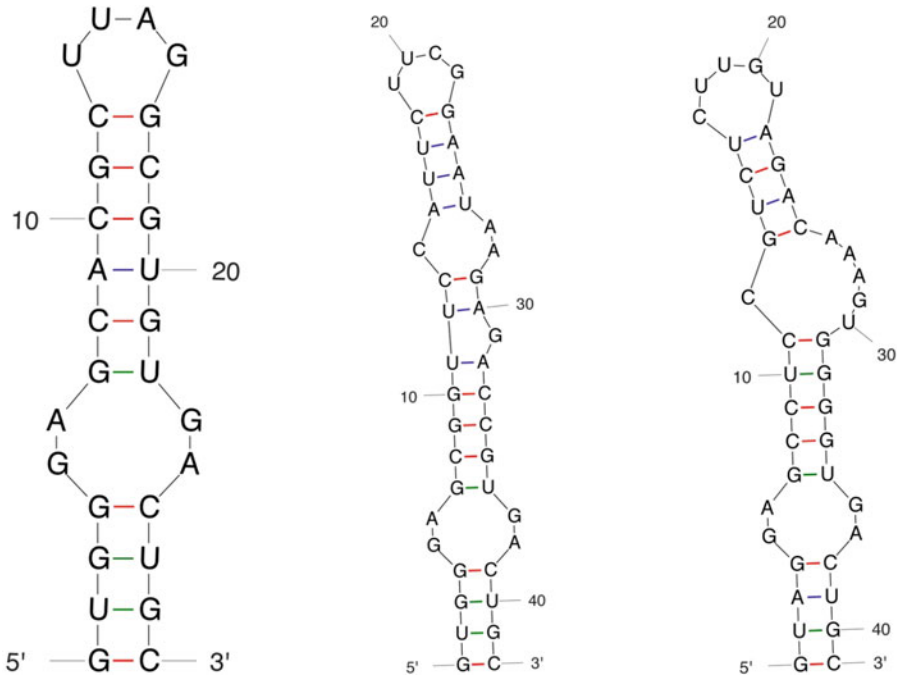


Fig. 5.5.2

(a) Predicted structures of helix 25 in *Escherichia coli*, *Shewanella benthica* KT99, and *Photobacterium profundum* SS9, as predicted by UNAFold v3.5. (b) Ribbon diagram of the region of the 50S ribosomal subunit surrounding helix 25, rendered by the UCSF Chimera software package. Helices and proteins of interest are colored. The point of insertion on helix 25 is colored green

Helix 45

Insertion #3 only occurs in SS9 and is an insertion of 17 bp relative to its comparison strain *P. profundum* 3TCK. This insertion occurs on helix 45 and results in the elongation of the helix. Helix 45 is a hypervariable region whose length is known to vary by as much as 129 bp in the γ-proteobacteria. The insertion into SS9 falls well within this range, and though its insertion is not found in its comparison strain, *P. profundum* 3TCK, it is unlikely that this mutation is a piezophilic adaptation.

Helix 58

Insertion #4 occurs at position 1507 (*E. coli*) and is found in three piezophiles (MT41, KT99 and SS9) and one of the comparison mesophiles (*S. frigidimarina*). It is also found in two other species of *Shewanella*: *S. baltica* and *S. denitrificans*. The insertion is 14 bp long when aligned to the *E. coli* sequence. This occurs in helix 58 at the beginning of a two residue bulge (*Fig. 5.5.3*). The new sequence is predicted to cause the formation of a new helix, shown in the predicted structures in *Fig. 5.5.3*. The helical base ranges from 3 to 5 bp long (per strand) and the terminal loops are either 4 or 6 bp in length.

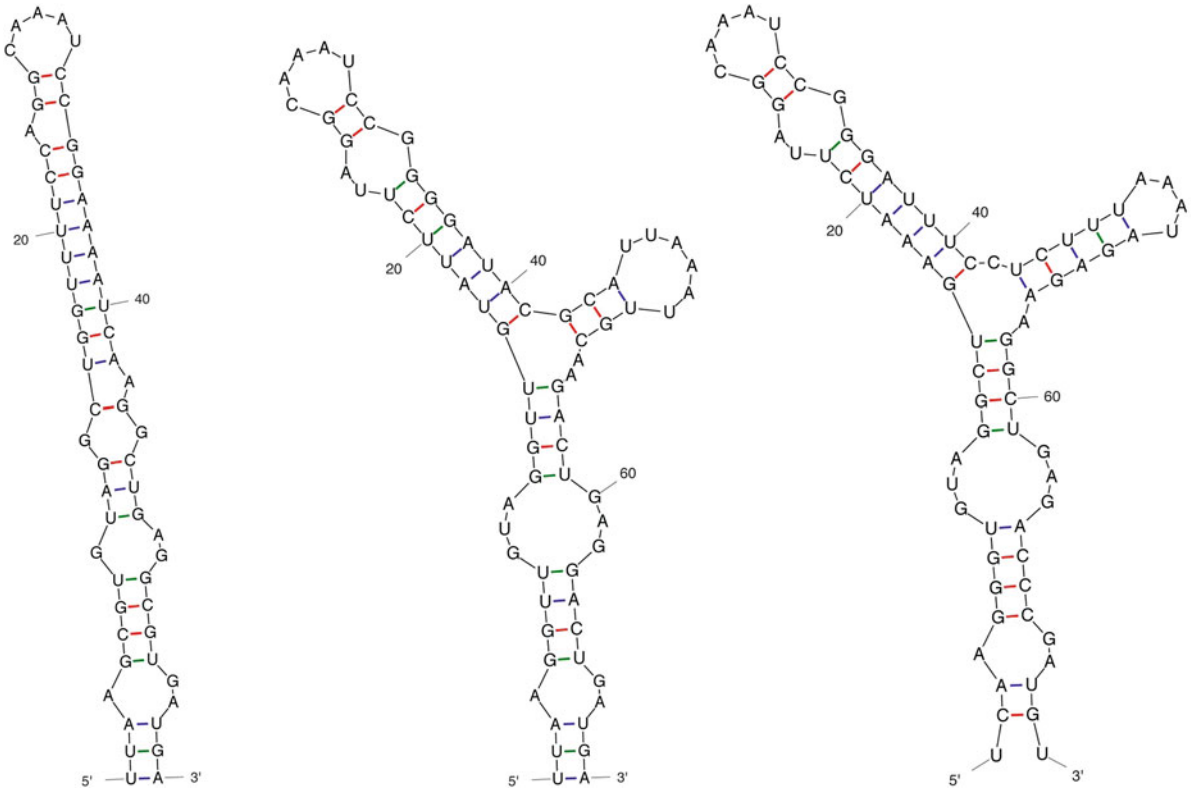
On visual examination of the crystal structure, helix 58 is located on the side of the 50S subunit, toward the bottom near the 50S–30S interface (Schuwirth et al. 2005). Helix 58 has been shown to interact with L9 (Brimacombe 1995), however the crystal structure places considerable distance between these two structures. Instead, we find that RP L2 is in close proximity to helix 58 (*Fig. 5.5.4*). Helix 58 is also in close proximity to helices 53, 54, 55, and 63.

Insertion #4 has the strongest piezophilic signature of the four large insertions analyzed. This insertion is found in the same three genera as were the insertions noted in the 16S sequence (Lauro et al. 2007). Insertion #4 is also the only insertion found in all 15 of SS9's 23S rRNA sequences. Also similar to the insertions into the 16S sequence, the analysis of the three-dimensional models indicates that the insertion would likely alter existing interactions with other components in the ribosome.

Evidence against this insertion being a piezophilic adaptation is its presence in various non-piezophilic *Shewanella* species. However, this insertion (and the resulting extra helix) is only found in the *Shewanella*, except for those found in piezophiles MT41 and SS9.

Additional Translation-Related RNAs

The smallest of the ribosomal RNAs, the 5S is part of the large ribosomal subunit. The piezophilic 5S is relatively unchanged compared to its mesophilic counterparts. The number of copies of 5S genes is not significantly different between piezophiles and mesophiles. Transfer RNAs function is to translate the genetic code into amino acids. Their structure is important for them to properly function, and like the ribosomal RNAs, may be under selective pressure at high hydrostatic pressure. In all of the sequence alignments, no significant variations were found between piezophiles and mesophiles. When an Average Distance phylogenetic tree is built from the alignments, the tRNAs cluster by genera. The lack of sequence diversity between piezophiles and mesophiles suggests that tRNAs are not significantly affected by high hydrostatic pressure. The prevalence of tRNAs was also investigated, but also did not lead to



a Helix 58, E*scherichia coli* 23S rRNA *Shewanella benthica* KT99 *Photobacterium profundum* SS9

Fig. 5.5.3 (Continued)

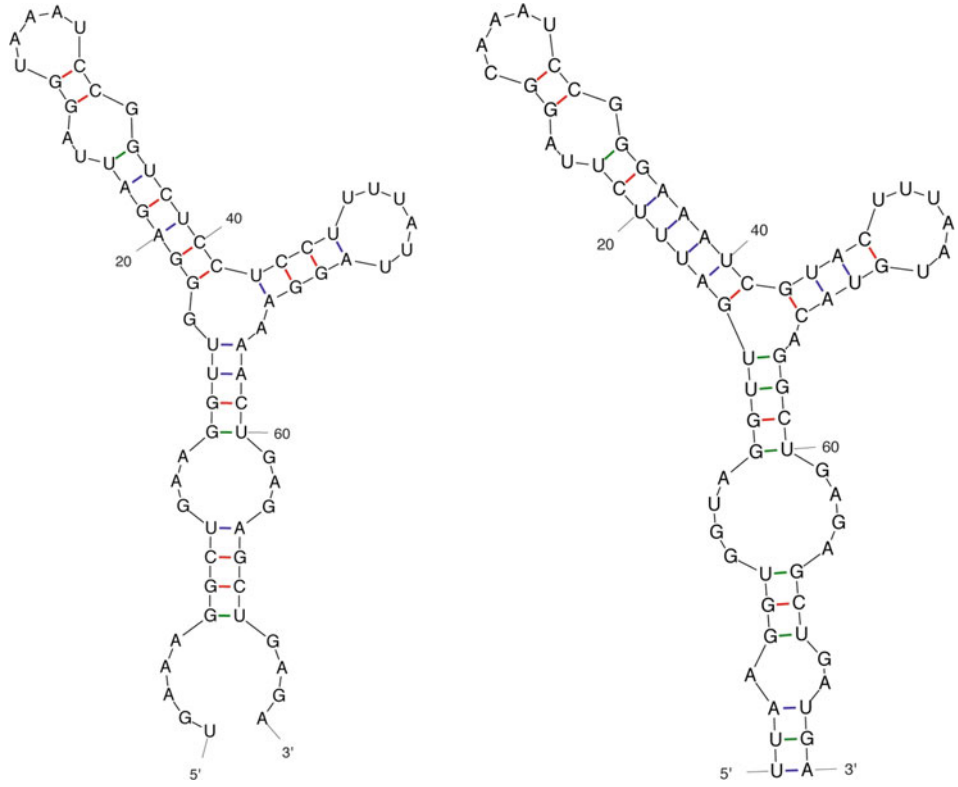


Fig. 5.5.3

Predicted structure helix 58 in *Escherichia coli* and helix 58 with Insertion #4 in *Shewanella benthica* KT99, *Photobacterium profundum* SS9, *Colwellia* sp. MT41, and *Shewanella frigidimarina*

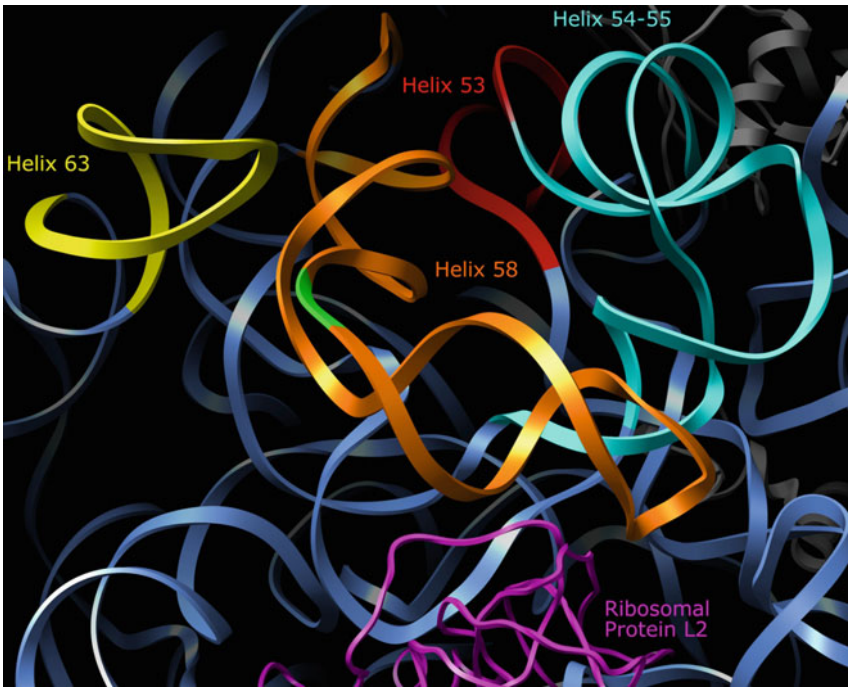


Fig. 5.5.4

Ribbon diagram of the region of the 50S ribosomal subunit surrounding helix 58, rendered by the UCSF Chimera software package. Helices and proteins of interest are colored. The point of insertion on helix 58 is colored green

a suggestion that tRNA count is affected by high hydrostatic pressure. Although there is variation between piezophiles and mesophiles, there is no conclusive pattern to suggest a piezophilic trait. This conclusion does not change if amino acids are grouped by physical or chemical properties, such as side-chain size and hydrophobicity (data not shown). The 4.5S RNA is part of a complex that binds to the ribosome and directs protein translation to the cell membrane (Gu et al. 2003). Within this complex the signal recognition particle (SRP), binds to ribosomal protein L23 near the exit point of the new peptide (Gu et al. 2003). Sequences for the 4.5S RNA were found through homology searching using query sequences from *S. frigidimarina*, *E. coli*, and *Psychromonas ingrahamii*. This revealed significant matching sequences in all five γ-proteobacteria piezophiles and their mesophile comparison strains. An alignment of the sequences shows significant diversity between genera, but relatively little diversity within. Based on this analysis, it is unlikely that the 4.5S RNA contributes to an organism's piezotolerance.

Ribosomal Proteins

An overview alignment was done on all ribosomal proteins in the piezophiles and mesophiles. These indicate that there are no major differences between the piezophile proteins and the mesophile proteins. Of the 56 or so ribosomal proteins, nine were identified as having

Table 5.5.3

Piezophile-biased amino acid changes within ribosomal proteins

Protein location	*Colwellia* MT41	*Shewanella* KT99	*Psychromonas* CNPT3	*Colwellia psychrerythraea*	*Shewanella frigidimarina*	*Psychromonas ingrahamii*	P-value
L2:65	I	I	V	V	V	V	0.2
L7:47	G	G	G	A	A	A	0.05
L9:73	V	N	N	V	T	A	0.169
L13:34	S	S	L	T	T	L	0.257
L21:70	D	D	K	P	E	N	0.085
L28:69	A	A	A	T	K	T	0.175
S1:324	S	S	S	N	N	N	0.05
S1:460	I	I	L	T	S	S	0.126
S3:198	V	V	V	I	I	V	0.2
S19:80	F	F	F	Y	Y	Y	0.05
S21:64	S	T	N	N	N	N	0.38

piezophile-biased substitutions. These are L2, L7, L9, L13, L21, L28, S1, S3, S19, and S21. This is most evident in the comparisons of the most piezophilic bacterial proteins with their mesophile counterparts (*Table 5.5.3*).

Summary

High-throughput sequencing efforts, both tag 16S rRNA sequencing and metagenomics analyses, have revealed considerable information about the distribution of pelagic life down to ~4 km depth, but much more information is needed on deeper-dwelling microbes and on deep-sea microbial life in the benthos. Likewise, genome studies of isolated piezophiles have provided a wealth of information on five genera of γ-proteobacteria, but the vast diversity present remains essentially untouched.

The ribosome possesses a number of targets that could be affected by high hydrostatic pressure. Piezophile-biased insertions are present within both the 16S and 23S rRNA, particularly within members of the genera *Colwellia*, *Shewanella*, and *Photobacterium*. Experimental testing of the importance of the rRNA insertions will be necessary to determine if a role in piezophily actually exists. SS9, with its different ribotypes, provides a unique system in which this can be done by knocking out different ribotypes. SS9 has 15 ribosomal RNA operons and possesses a number of ribotypes; five 16S ribotypes and four 23S ribotypes. Paired together, there are nine unique combinations of 16S and 23S ribotypes found within SS9's rRNA operons. In addition, to these insertions other rRNA modifications could exist and influence ribosome function at high pressure such as pseudourilyation (Wang et al. 2008). The post-transcriptional modification of other RNA species such as tRNAs could also be important (Phizicky and Alfonzo 2010; Wang et al. 2008).

The piezophilic trends continue to the ribosomal proteins where ten proteins exhibit potential piezophilic adaptations. These substitutions included both changes in residue

chemistry and size. As with the rRNA differences their significance must be experimentally evaluated. A first step in this direction could be in vitro translation (Giuliodori et al. 2004) using different ribosome sources as a function of pressure and temperature.

Genome-based analyses of cultured and uncultured piezophiles will continue to guide their evolutionary, biochemical, and physiological characterization. It is clear that Moore's law (Moore 1975) applies equally well to sequencing power as to computational power, and that both will be ever more important tools in extremophile research. We are far removed from 1996 when the first deep-sea extremophile sequence (*Methanocaldococcus jannaschii*) was reported (Bult et al. 1996).

Acknowledgments

D.H. Bartlett is grateful to the National Science Foundation for support (EF0827051 and EF0801973).

Cross-References

- 5.1 Distribution of Piezophiles
- 5.2 High Pressure and Prokaryotes
- 5.3 Piezophysiology of the Model Bacterium *Escherichia coli*
- 5.4 High Pressures and Eukaryotes

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5.6 Cultivation Methods for Piezophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_5.6,

Some extremophiles, living under the deep-ocean floor, are microorganisms that have adapted to a high-pressure environment and can grow more easily under high hydrostatic pressure conditions than at atmospheric pressure. We call such deep-sea extremophiles "piezophiles," meaning pressure- (piezo- in Greek) loving (-phile) organisms (Yayanos 1995). In the laboratory, piezophiles could be isolated and cultivated under high-pressure conditions, and therefore special high-pressure equipments, such as pressure vessels, hydrostatic pumps, etc., are necessary. When sampling was performed from deep-sea sediments or seawater to obtain living piezophiles, we also need a pressure-retaining sampler to maintain the environmental pressure and temperature, because some piezophiles are sensitive to drastic pressure and temperature changes. The Japan Agency for Marine-Earth Science and Technology (JAMSTEC) has been developing a "*deep*-sea *ba*ro-piezophile and *th*ermophile isolation and cultivation system," referred to as the "DEEPBATH" system, for handling piezophiles under study (Kyo et al. 1991). In this chapter, the traditional methods for the handling of piezophilic microorganisms are described along with novel systems.

The Procedures of Piezophiles Isolation and Cultivation

In 1957, Zobell and Morita were first considering how to handle piezophilic microorganisms living under the deep-sea floor. They developed a titanium pressure vessel that could produce pressure of up to 100 MPa for the handling and cultivation of such microbes (Zobell and Morita 1957). They attempted to isolate piezophiles from deep-sea samples several times, but they were able to isolate only piezotolerant deep-sea microbes, which showed better growth under atmospheric pressure condition but also grew at high pressure. One reason for their inability to isolate piezophiles was that such extremophiles can be sensitive to drastic changes in pressure and temperature, and it may be very difficult to maintain the microbes at atmospheric pressure. However, Zobell and Morita made a great contribution to the handling of microbes in high-pressure microbiology, and many researchers began to study biological physiology under pressure conditions (Marquis 1976).

In 1979, Yayanos and coworkers succeeded in isolating piezophilic microorganisms from amphipods recovered from a depth of 5,782 m in the Philippine Trench using pressure-retaining traps. This was the first report on the isolation of a piezophilic microbe (Yayanos et al. 1979). They modified their pressure vessels for easier handling, using a pin system to retain the pressure instead of the original screw system. This modification solved the problem of pressure leakage due to wearing and metal fatigue of the screw, and the new system did not require much power for operation. Using those pressure vessels, the author's JAMSTEC group isolated numerous piezophiles during the past 20 years (Kato et al. 2000, 2004, 2008). The procedures for the sampling of deep-sea sediment to the isolation of piezophiles are described below.

Sampling of Deep-Sea Sediments

Deep-sea submersible systems are available in JAMSTEC. The manned submersible *Shinkai 6500* (maximum depth 6,500 m, accommodating one scientist and two pilots during operation) and the unmanned submersible *Kaiko* (maximum depth 11,000 m, containing launcher and vehicle systems) are used for sampling from the deepest ocean (*Fig. 5.6.1*, see the

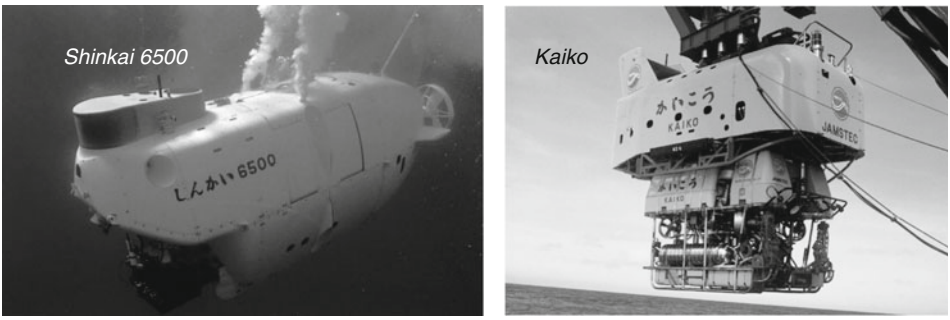

Fig. 5.6.1

Deep-sea research submersibles operated by JAMSTEC. (a) Manned submersible, *Shinkai 6500*, which can dive to 6,500 m depth of ocean, and it is deepest manned submersible. (b) Unmanned submersible, *Kaiko*, which means trench in Japanese, and could dive to 11,000 m depth of the world deepest trench, Mariana Trench Challenger Deep. But this submersible was missing during the operation at Nankai Trough on 2002, because of rough ocean

JAMSTEC home page, http://www.jamstec.go.jp). The sterilized sediment (SS) sampler and/or core sampler is controlled by the manipulators of the submersible, and the desired samples can be obtained. Then the samples are carried to the mother ship laboratories, where they are placed under environmental pressure and temperature conditions as soon as possible.

Mixed Cultivation of Microorganisms Under Environmental Pressure and Temperature Conditions

Deep-sea sediment samples obtained using the submersible are mixed with culture medium (e.g., Marine broth medium 2216, Difco Co.) and high-pressure cultivation initiated using pressure vessels under environmental pressure and temperature conditions. After several days of cultivation, the mixed cultivations are transferred to fresh media to continue culture under different conditions, if necessary. This mixed cultivation procedure could eliminate the atmospheric adapted microorganisms which could be delivered from the pelagic water, and consequently only the high-pressure adapted microorganisms could be concentrated. The procedure of the isolation methods for deep-sea piezophiles is shown in *Fig. 5.6.2*. Plate isolation method is suitable to isolate moderate piezophiles and piezotolerant microbes, and the dilution-to-extinction method is good for thermophilic piezophiles isolation. Since the pressure bag method was discovered, many obligate piezophiles have been isolated using this method, which will be described in detail below.

Isolation of Piezophilic Microorganisms Using the Pressure Bag Method

The mixed cultivation is diluted and mixed with low melting-point agar (0.7% agar) medium in sterilized plastic bags (10 cm in diameter, approximately 20 ml of agar medium)

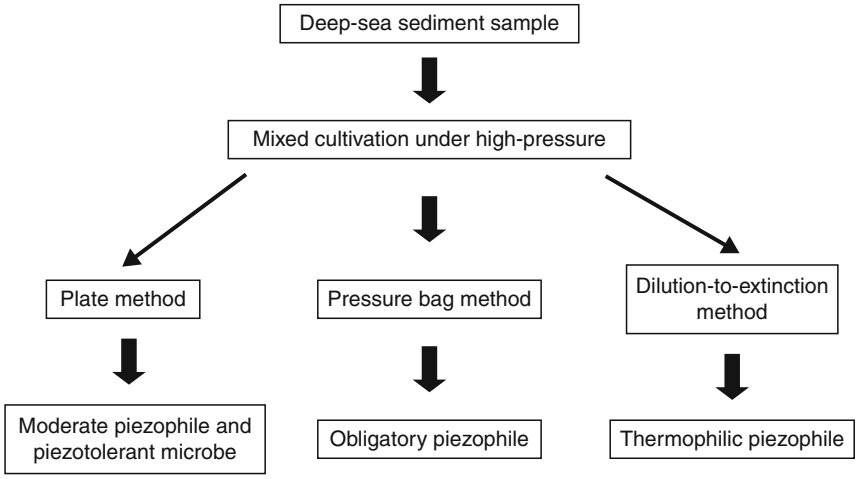

Fig. 5.6.2
Procedure of the purification steps for deep-sea piezophiles

at lower than 20°C. Then the bags are sealed and transferred to ice-cold water for solidification of the medium. After solidification, the bags are placed into the pressure vessels, pressurized to environmental pressure, and kept at environmental temperature for several days. After pressure cultivation, several microbial colonies can be identified in the bag. Each colony is placed into a separate disposable syringe under a stereoscopic microscope, and cultivation under both high-pressure and atmospheric pressure conditions are initiated. If the isolates grow better under elevated pressure conditions than at atmospheric pressure, they are termed piezophiles.

Characterization of Isolates

To determine the optimal growth conditions of the isolates, multiple (20–30) pressure vessels are used to avoid the effects of repeated pressurization and decompression on the cells. If the pressure of a vessel is released, the same vessel is not repressurized to ensure that the results are reproducible. In the case of cultivation under aerobic conditions, oxygenated fluorinert (FC-72, Sumitomo-3M Co., Japan) is added to the cultures (25% of total volume; Kato et al. 1995). To study the physiological and molecular properties of the isolates, 200-ml scale cultivation is carried out using the large type of pressure vessel.

Using the above methods, we have performed several studies on the taxonomy of piezophiles, gene expression controlled by pressure conditions, and genomic analysis of piezophilic microorganisms (Nakasone et al. 1998, 2002; Kato et al. 2000, 2001, 2004). However, the 200-ml culture scale is sometimes insufficient to yield the desired amounts of protein, DNA, lipids, etc., because the maximum cell density of piezophile cultures is not very high (ca. 10^8 cells ml^{-1}). Therefore, we developed a large high-pressure cultivation system, the DEEPBATH system, as described in the next section.

The DEEPBATH System

The DEEPBATH system consists of four separate devices: (1) a pressure-retaining sampling device, (2) dilution device under pressure conditions, (3) isolation device, and (4) cultivation device (Kyo et al. 1991). The system is controlled by central regulation systems, and the pressure and temperature ranges of the devices are from 0.1 to 65 MPa and from 0°C to 150°C, respectively. The scale of the cultivation devices (two sets) is 1.5 l each, and therefore cultures of up to 3 l can be obtained. The construction of the system and the sample stream are shown in *Fig. 5.6.3*. The principle of the isolation of piezophiles is the dilution-to-extinction procedure, although in some cases microbes may be attached so firmly to the sediment that it is difficult to obtain a pure culture using the dilution-to-extinction procedure. Thus, we combined the traditional procedure explained above and the DEEPBATH system protocol for isolation studies.

Other Equipments for High-Pressure Studies

To study the morphology of piezophilic microorganism and their molecular habitat under high-pressure condition, several high-pressure equipments were discovered and developed. High-pressure microscopic system (*Fig. 5.6.4a*) is a powerful tool to observe the morphology change and swimming habitat under pressure condition (Miwa et al. 2002). To analyze the protein–protein interaction and their activities, high-pressure florescent spectrophotometer (*Fig. 5.6.4b*), high-pressure photo spectrometer (*Fig. 5.6.4c*), and high-pressure electrophoresis apparatus (*Fig. 5.6.4d*) were constructed. Using those systems, piezophilic proteins'

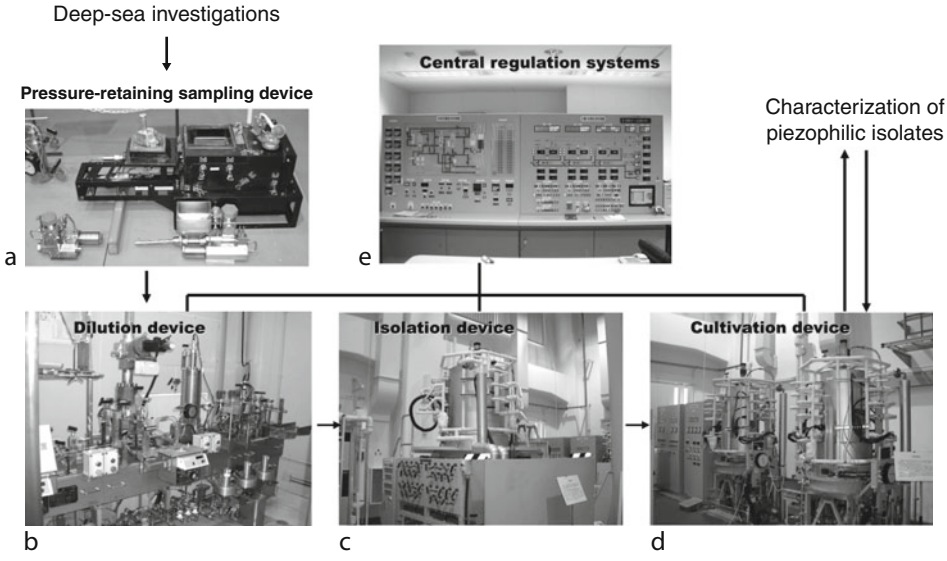

Fig. 5.6.3

The DEEPBATH system. The system is composed of four devices: (a) pressure-retaining sampling device, (b) dilution device under pressure conditions, (c) isolation device, and (d) cultivation device. The system is controlled by Monitoring and Control Console (e)

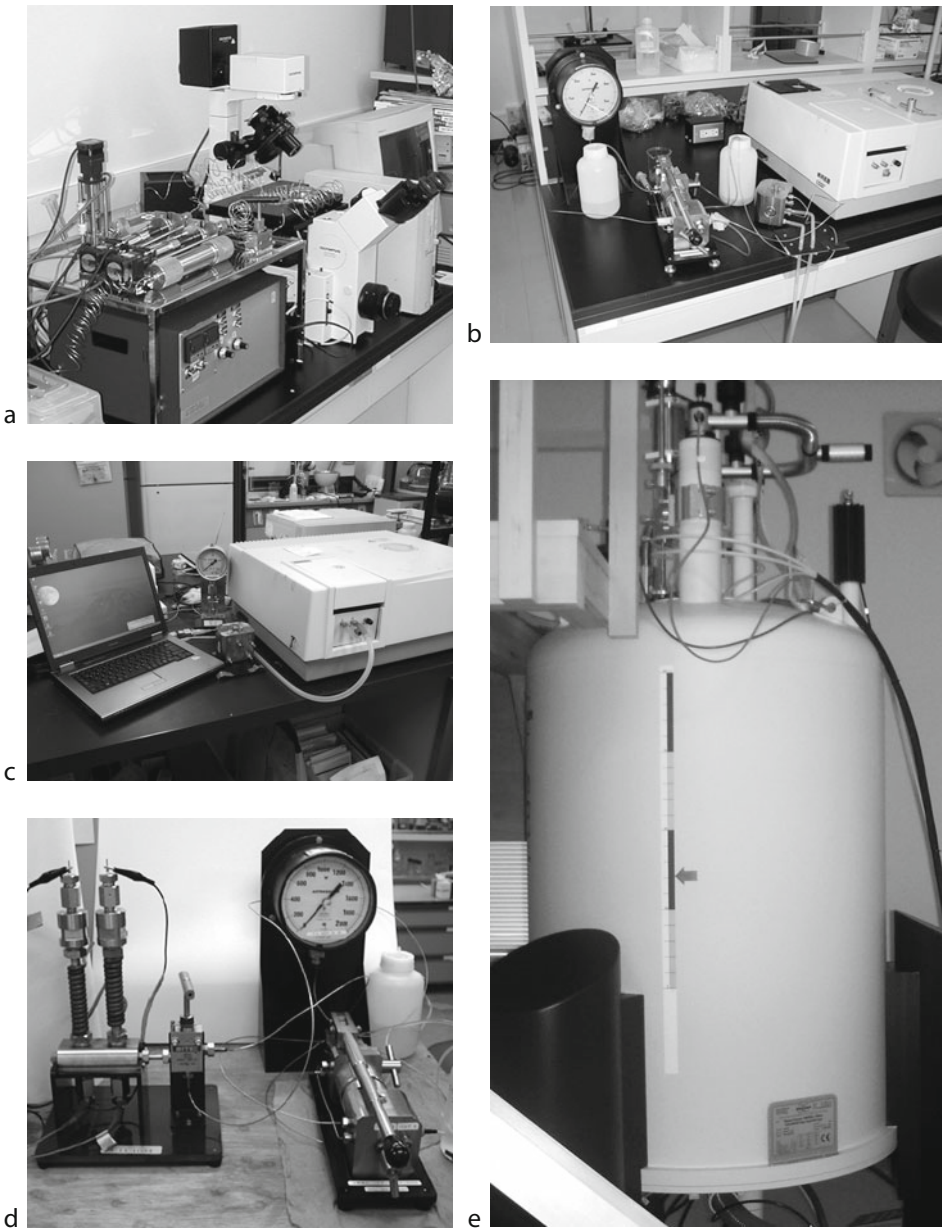

Fig. 5.6.4

Other high-pressure equipments. (a) High-pressure microscopic system (HPMS), (b) high-pressure florescent spectrophotometer (HPFSP), (c) high-pressure spectrophotometer (HPSP), (d) high-pressure electrophoresis apparatus (HPEA), and (e) high-pressure NMR systems (HP-NMR)

properties have been elucidated (Abe and Horikoshi 1998; Kawano et al. 2004). Akasaka (2006) discovered the high-pressure NMR systems (*Fig. 5.6.4e*) to analyze the protein dynamics and structural changes under pressure conditions. On the basis of those discoveries and studies, the field of high-pressure bioscience and biotechnology became one important and promising field of biosciences. Piezophilic microorganisms are certainly one of most important materials to understand how the pressure does affect the life development in this planet.

Acknowledgments

I am very grateful to Prof. Koki Horikoshi for continued support of my extremophile studies. I also thank my colleagues for excellent collaboration. I especially thank Prof. Richard Y. Morita for providing the valuable traditional pressure vessels to study piezophiles, and for his encouragement during the course of high-pressure research. I also thank the submersible operation teams and the DEEPBATH operation staffs for making it possible to undertake piezophilic studies.

Cross-References

5.1 Distribution of Piezophiles
5.2 High Pressure and Prokaryotes
5.3 Piezophysiology of the Model Bacterium *Escherichia coli*
5.4 High Pressures and Eukaryotes
5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low Temperature Piezophiles

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5.7 Isolation Methods for High-Pressure Growth Mutant in Yeast

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Isolation of High-Pressure Growth Mutants from Tryptophan-Auxotrophic Strains of the Yeast *Saccharomyces cerevisiae*

As mentioned in Chap. 5.4, wild-type strains of *S. cerevisiae* with tryptophan auxotrophy (Trp^-) are unable to grow under high pressure (e.g., 15–25 MPa, 25°C) and low temperature (e.g., 0.1 MPa, 10–15°C). This is because high pressure and low temperature compromise tryptophan uptake by inhibiting the permease activity and promoting ubiquitin-dependent degradation of the permeases (Abe 2007). Mutants capable of growth under high pressure, designated *HPG* (high-pressure growth) mutants (referred to as the HPG phenotype, hereafter), are isolated from tryptophan-auxotrophic strains (Abe and Iida 2003). The characterization of the *HPG* mutants and identification of relevant genes allow us to unravel the complex regulatory mechanism of the yeast tryptophan permeases Tat1 and Tat2 with respect to ubiquitination, deubiquitination, and endocytic trafficking in the cell. In this section, the method for the isolation and use of the *HPG* mutants are described for the elucidation of ubiquitin-dependent degradation of the tryptophan permeases.

Any haploid Trp^- strain such as YPH499 and W303-1A can be used to isolate the *HPG* mutants. High-pressure vessels of 100–1,500 ml in volume that can withstand pressure of 100 MPa are made with titanium or stainless alloy (Syn Corporation, Kyoto, Japan). A hand pump is used to apply high hydrostatic pressure to the sample in the vessel. *Figure 5.7.1* summarizes the procedure for isolating *HPG* mutants. Cells are precultured in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% D-glucose) medium at 0.1 MPa and 25°C with shaking and are treated with or without 2% ethylmethanesulfonate for 10–30 min to increase the frequency of mutation in the genome, so that the surviving fraction of the cells decreases to 10–60%. After washing with 0.1 M sodium phosphate buffer (pH 7), the cells are spread on YPD agar plates. The plates are maintained under high pressure (10–50 MPa and 25°C) or low temperature (4–10°C and 0.1 MPa) for 1–2 months to allow colony formation of the mutants. In our experience, all of the *HPG* mutants have been able to grow at low temperatures and vice versa. In other words, the *HPG* mutants can be isolated as low-temperature growth (*LTG*) mutants (referred to as the LTG phenotype, hereafter). In our rough estimation, the occurrence rate of *HPG* or *LTG* mutants was approximately on the order of 10^{-7}–10^{-8}. The appropriate conditions to obtain the *HPG* mutants are 8°C at 0.1 MPa and 25°C at 30 MPa. To confirm the HPG phenotype, the colonies are transferred to fresh YPD agar plates with toothpicks, and the agar is covered with low-temperature melting-point YPD agar. The plate is covered with plastic wrap and incubated at 18 or 25 MPa at 25°C in high-pressure vessels for 2 days to determine the difference in colony size. In our experiments, 34 strains among 400 *HPG* mutants were subjected to genetic analysis. Diploid strains, which were formed by the cross between each of the *HPG* mutants and the mating strain YPH500, grew at 18 MPa but were unable to grow at 25 MPa, showing that the HPG phenotypes were semidominant. Next, 20 of the 34 *HPG* mutants that showed a clearer phenotype than the others were crossed with each other, and the segregants were subjected to high-pressure growth assay. *Figure 5.7.2* shows a typical example segregating as the parental ditype:nonparental ditype:tetra type at 3:3:11 (nearly 1:1:4). Finally, those 20 mutants were classified into four semidominant linkage groups, *HPG1*, *HPG2*, *HPG3*, and *HPG4*, consisting of 10, 8, 1, and 1 mutants, respectively (Abe and Iida 2003).

To identify the *HPG* mutation, a genomic DNA library containing 10- to 20-kb DNA fragments is constructed from each *HPG* strain. After partial digestion with *Sau*3AI, the DNA fragments are inserted into the *Bam*HI site of a low copy-number plasmid such as YCplac111 (*ARS1 CEN4 LEU2*). The wild-type Trp^- strain is transformed with the library, and the

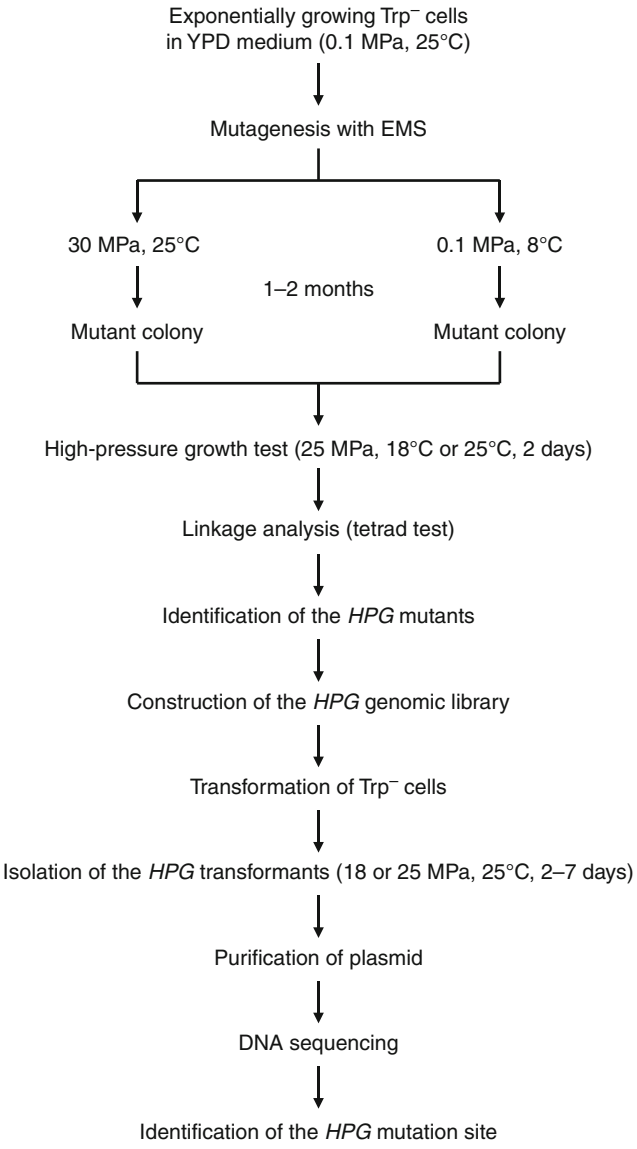

Fig. 5.7.1

Isolation of high-pressure growth (*HPG*) mutants from Trp⁻ strains and the identification of corresponding genes. The *HPG* mutants can also be isolated as low-temperature growth mutants (Abe and Iida 2003). EMS, ethylmethanesulfonate.

transformant cells are mixed with SD (0.67% yeast extract nitrogen base without amino acids, 2% D-glucose, 20 mg/L adenine, 20 mg/L uracil, 30 mg/L leucine, 20 mg/L histidine, 30 mg/L lysine, 20 mg/L tryptophan) low-temperature melting-point agar. After solidifying in sterilized plastic bags, they are exposed to high pressure at 18 or 25 MPa and 25°C for 2–7 days to select transformant colonies. Plasmids are purified from the transformants and reintroduced into the

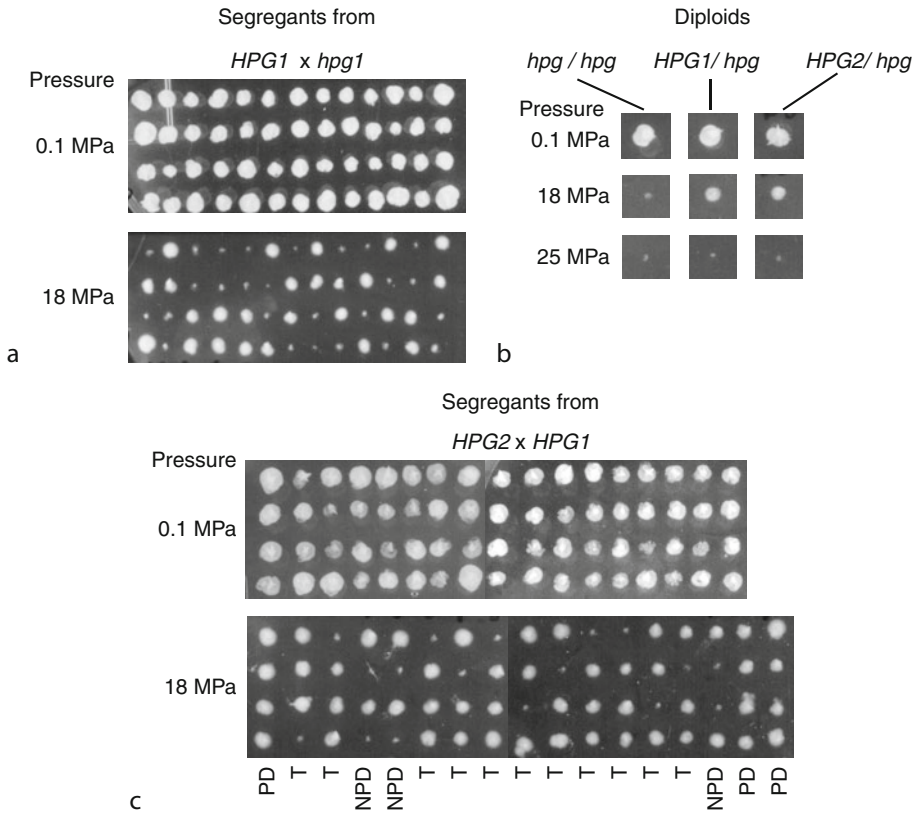

Fig. 5.7.2

Isolation and classification of semidominant *HPG* mutants. Typical examples of high-pressure growth assays are shown. (a) The HPG phenotype of each tetrad derived from the *HPG1*/*hpg1* heterozygous diploid segregated 2 Hpg^+: 2 Hpg^- on YPD agar at 18 MPa. The YPD plates were incubated for 2 days at 0.1 or 18 MPa. Note that the semidominant mutant allele is denoted by uppercase letters and the wild-type alleles by lowercase letters. (b) Heterozygous diploids (*HPG1*/*hpg1* and *HPG2*/*hpg2*) were grown on YPD agar at 0.1, 18, and 25 MPa for 2 days. (c) Tetrad distribution of segregants derived from an *HPG1*/*HPG2* diploid strain. Segregants were grown on YPD agar at 0.1 and 18 MPa for 2 days (Abe and Iida 2003). PD, parental ditype; NPD, nonparental ditype; T, tetra type.

Trp^- strain to confirm the ability to grow under high pressure. To identify the base substitution in the plasmid, the entire region is sequenced and verified on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). In our study, we identified *HPG1* and *HPG2* to be allelic to *RSP5* and *TAT2*, respectively. Four *HPG1* mutation sites are located in the catalytic HECT domain of Rsp5 ubiquitin ligase (Abe and Iida 2003). Three *HPG2* mutation sites are located in the cytoplasmic tails of Tat2 (Nagayama et al. 2004). Genomic DNA is purified from the mutants, and the corresponding mutations are confirmed by sequencing the region.

Tat1 and Tat2 protein levels are analyzed using the common Western blotting method. Plasmids containing genes encoding Tat1 or Tat2 tagged with the influenza hemagglutinin (HA) antigen at the *N*-terminus are constructed. These plasmids are introduced into the

wild-type strain and *HPG1* or *HPG2* mutant, and whole-cell extracts are prepared from cells that have been cultured under high-pressure conditions to detect HA-tagged proteins using the specific monoclonal antibody.

Identification of High-Pressure-Sensitive Mutants from *S. cerevisiae* Tryptophan-Prototrophic Strains

While Trp^- strains of *S. cerevisiae* are sensitive to high pressure and low temperature, tryptophan-prototrophic (Trp^+) strains are relatively resistant and capable of growth at pressure of 25–35 MPa or temperature of 10°C. Because industrial strains such as brewing yeasts are Trp^+, they are expected to show HPG and LTG phenotypes. Ethanol fermentation is typically performed at 10–15°C, and the growth of yeasts at low temperature and the underlying mechanism have been focuses of industrial attention. High-throughput screening of the library consisting of 4,828 deletion strains is performed to search for novel mutants that are defective in a variety of cellular functions. The identification of genes responsible for the growth of Trp^+ strains is successfully performed using the EUROSCARF yeast-deletion library (Giaever et al. 2002), which is commercially available (Invitrogen, Carlsbad, CA, USA). Considering the similarity of the effect of high pressure and that of low temperature, screening of the library is performed to obtain mutants defective in growth under either high pressure or low temperature (Abe and Minegishi 2008). *Figure 5.7.3* summarizes the procedure for isolating these mutants. Small aliquots from 4,828 mutant cell cultures are transferred to fresh YPD medium in 96-well plates, followed by incubation at 25°C and 0.1 MPa overnight. Then, 3 µl of each preculture is transferred to fresh YPD medium in 96-well plates. After sealing the plates with sterilized plastic film, the cells are subjected to pressure of 35 MPa at 25°C in hydrostatic chambers using a hand pump or to low temperature at 0.1 MPa and 6°C. After

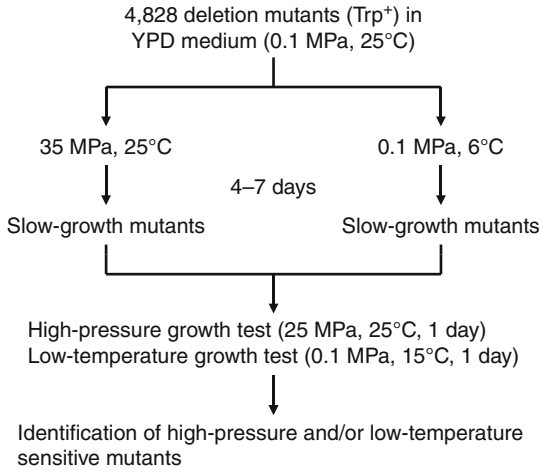

Fig. 5.7.3

Identification of genes responsible for growth in a Trp^+ strain under high pressure and low temperature. The yeast-deletion library consisting of 4,828 gene-deletion mutants was exposed to high pressure and low temperature (Abe and Minegishi 2008).

4–7 days, the cell growth yields are checked visually. For more quantitative analysis, the candidate mutants are grown in SC medium (0.67% yeast extract nitrogen base without amino acids, adenine 20 mg/L, uracil 20 mg/L, tryptophan 40 mg/L, histidine 20 mg/L, leucine 90 mg/L, lysine 30 mg/L, arginine 20 mg/L, methionine 20 mg/L, tyrosine 30 mg/L, isoleucine 30 mg/L, phenyalanine 50 mg/L, glutamic acid 100 mg/L, aspartic acid 100 mg/L, threonine 200 mg/L, serine 400 mg/L, 2% D-glucose) at 0.1 MPa and 25°C with vigorous shaking (150 rpm) in the exponential phase of growth ($OD_{600} < 1.5$). Then the culture is diluted with SC medium to an OD_{600} value of 0.15. The diluted cultures are placed in sterilized tubes, and the tubes are sealed with Parafilm. The culture tubes are subjected to high pressure of 25 MPa at 25°C in hydrostatic vessels or to low temperature of 0.1 MPa at 15°C for 20 h. At the end of the culture period, the pressure is released and optical density is measured at 600 nm using a spectrophotometer. In our experiments, a broad range of cellular functions appeared to be involved in the HPG and LTG phenotypes: amino acid biosynthesis, mitochondrial function, actin cytoskeleton, membrane trafficking, transcription, ribosome biogenesis, chromatin structure, and functionally unknown genes (Abe and Minegishi 2008). Therefore, factors in various cellular functions support cell growth under high pressure and low temperature.

Measurement of Intracellular pH Under High Pressure

The effect of high pressure on cytoplasmic pH and vacuolar pH of living yeast cells is optically analyzed in a hydrostatic chamber with transparent sapphire windows (Fig. 5.7.4).

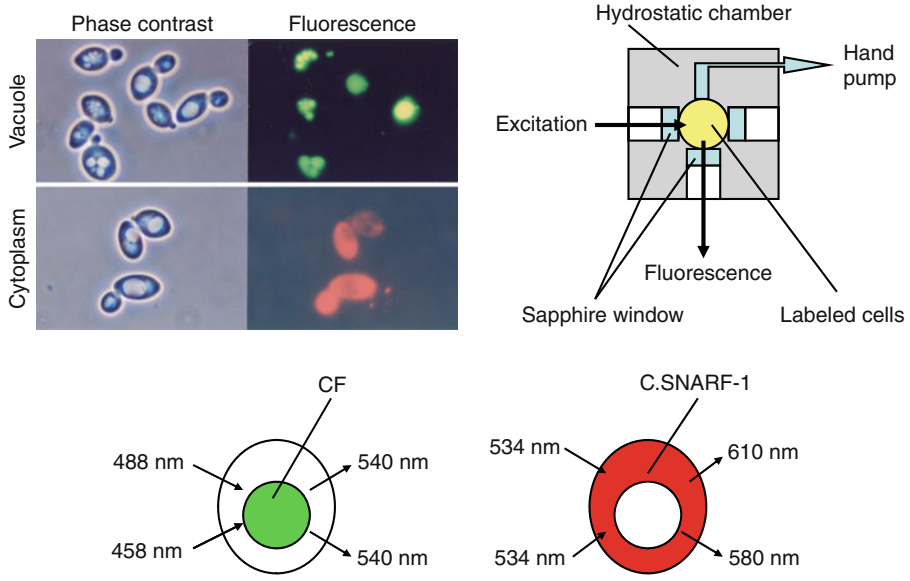

Fig. 5.7.4

Measurement of cytoplasmic pH and vacuolar pH in living cells under high pressure. Cells are labeled with 5-(and 6-)carboxy SNARF-1 acetoxymethyl ester or 6-caroboxyfluorescein diacetate to stain the cytoplasm or vacuoles, respectively. The labeled cells are excited, and fluorescence is emitted through the sapphire window. Hydrostatic pressure is applied using a hand pump (Abe and Horikoshi 1997, 1998).

pH-sensitive fluorescent dyes are used to monitor internal pH values. To label the cytoplasm, membrane-permeable 5-(and 6-)carboxy SNARF-1 acetoxymethyl ester is added to the cell suspension in a buffer at a concentration of 5–10 μM. After hydrolysis by intracellular nonspecific esterases, the fluorescent form of 5-(and 6-)carboxy SNARF-1 (C.SNARF-1) is accumulated in the cytoplasm (Haworth et al. 1991). The ratio of fluorescence intensity (I) emitted at 610 nm to 580 nm with an excitation wavelength of 534 nm ($I_{610/534}/I_{580/534}$) correlates with pH of around 7, and therefore this dye is suitable to measure cytoplasmic pH. It should be noted that the labeling must be done in the absence of glucose, because incubation of the cells with glucose for more than 10 min promotes the accumulation of the dye in the vacuole in an energy-dependent manner (Abe and Horikoshi 1998). To label the vacuole, membrane-permeable 6-caroboxyfluorescein diacetate (6-CFDA) is added to the cells in YPD containing 50 mM citrate (pH 3) at a concentration of 5–10 μM (Preston et al. 1989; Abe and Horikoshi 1995). After hydrolysis by intracellular nonspecific esterases, the fluorescent form of 6-caroboxyfluorescein (CF) is accumulated in the vacuole. The ratio of fluorescence intensity emitted at 540 nm with dual excitation wavelengths of 458 and 488 nm ($I_{458/540}/I_{488/540}$) correlates with pH of around 6, and therefore this dye is suitable to measure vacuolar pH. It should be noted that the labeling must be done at lower pH of around 3, because higher pH causes automatic hydrolysis of the 6-CFDA to give CF in the medium. Tris and Goods' buffer (e.g., Mes, Hepes, or Tes) are preferable for the pH measurement at high pressure because high hydrostatic pressure does not significantly change the pH of these buffers (Kitamura and Itoh 1987). In contrast, buffers based on weak acids such as phosphate, carbonate, or succinate are inadequate because high pressure lowers their pH value. The fluorescence of the labeled cells is emitted through the sapphire window of the hydrostatic chamber that has been placed in a spectrofluorometer under high-pressure conditions. The fluorescence ratio ($I_{610/534}/I_{580/534}$ or $I_{458/540}/I_{488/540}$) is slightly altered by increasing hydrostatic pressure even though the buffer pH is constant. Therefore, it is necessary to plot the fluorescence ratio against the calibration curve. The calibration curve can be obtained under various pressure conditions by determining the fluorescence ratio for free dyes C.SNARF-1 and CF in Goods' buffer, which is adjusted at a series of pH values. In our study, pressures of 40–60 MPa decreased both cytoplasmic and vacuolar pH by about 0.3–0.5 units (Abe and Horikoshi 1997; Abe and Horikoshi 1998) if the buffer contained glucose. Because a longer incubation time (>10 min) in the presence of glucose promotes vacuolar accumulation of C.SNARF-1, the measurement should be done in less than 5 min to monitor accurate internal pH values (Abe and Horikoshi 1998).

Assay of Amino Acid Uptake Under High Pressure and Determination of the Activation Volume

Hydrostatic pressure affects any chemical reaction depending on the sign and magnitude of the activation volume, $\Delta V^{\neq}$. $\Delta V^{\neq}$ represents the difference in the volume between the initial state and activated state of a reaction and can only be obtained by measuring the rate constants of the reaction as a function of hydrostatic pressure. When $\Delta V^{\neq}$ is positive, the reaction is inhibited by increasing pressure. When $\Delta V^{\neq}$ is negative, the reaction is accelerated by increasing pressure. The uptake of amino acids is mediated by specific or broadly specific permeases in the plasma membrane in *S. cerevisiae*. The yeast genome encodes 24 amino acid permeases and their homologues, but the substrate specificity is not always defined (Nelissen et al. 1997). Tryptophan is imported through one of the tryptophan permeases Tat1 and Tat2 (Schmidt et al. 1994).

To determine $\Delta V^{\neq}$ for tryptophan import through Tat1 or Tat2, gene-deletion mutants lacking one of the *TAT1* and *TAT2* genes are constructed. The activation volume associated with the overall process of tryptophan uptake is calculated using the following equation:

$$(\partial \ln k/\partial p)_{\mathrm{T}} = -\Delta V^{\neq}/RT$$

where k is the rate constant of uptake, p is pressure (MPa), T is absolute temperature (Kelvin), R is the gas constant (ml MPa/K · mol), and $\Delta V^{\neq}$ is the apparent volume change of activation (ml/mol). While Tat2 imports only tryptophan, Tat1 imports tyrosine as well as tryptophan. To eliminate potential complexity in calculating $\Delta V^{\neq}$ for Tat1 activity, a synthetic medium that lacks tyrosine and phenylalanine is used in the assay. Cells ($0.5–1.0 \times 10^8$) from an exponentially growing culture are collected by centrifugation and resuspended in 15 ml of fresh medium in a polypropylene tube. One milliliter of the cell suspension is placed on ice and used for determination of the cell count. Then, 14 µl of the radiolabeled tryptophan (e.g., L-[5-^{3}H]tryptophan) is added to 14 ml of the cell suspension, immediately mixed, and the mixture is split into seven equal aliquots in sterilized microtubes. Six tubes are carefully sealed with Parafilm. Each tube is placed in a pressure vessel and then subjected to pressure of 0.1, 25, or 50 MPa for 30 or 60 min. The cells in the tube are collected by filtration on a glass filter, washed with cold distilled water, and the radioactivity on the filter is counted using a liquid scintillation counter. Usually, the amount of time involved from the time of the addition of the labeled tryptophan to the cell suspension until the first filtration is 10 min. Therefore, the 10-min time point is taken to be the starting point in the assay of tryptophan uptake under high pressure. The mean values are shown as picomoles of tryptophan incorporated per 10^7 cells. In our measurement, the activation volume associated with tryptophan uptake through Tat1 and Tat2 was 89.3 and 50.8 ml/mol, respectively, which means that the effect of pressure on Tat1 activity is more severe than that of Tat2 activity (Abe and Iida 2003).

Cross-References

- 5.1 Distribution of Piezophiles
- 5.2 High Pressure and Prokaryotes
- 5.3 Piezophysiology of the Model Bacterium *Escherichia coli*
- 5.4 High Pressures and Eukaryotes
- 5.5 Contributions of Large-Scale DNA Sequencing Efforts to the Understanding of Low-Temperature Piezophiles
- 5.6 Cultivation Methods for Piezophiles

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5.8 Versatile Solidified Media for Growth of Extremophiles

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_5.8,

Introduction

Solidified media are indispensable in various aspects of microbiological research (Codner 1969; Madigan et al. 1997; Zengler 2009). For solid cultures of mesophilic microorganisms, agar is commonly used as a solidifying agent. It is a nonionic polysaccharide consisting mainly of D-galactose and 3,6-anhydro-L-galactose and is produced by sea-weeds (Lahaye and Rochas 1991). Ease of the handling, resistance to enzymatic degradation by most microorganisms, and transparency of solidified plates make agar an ideal solidifying agent (Hashsham 2007), and agar-supported media have been used essentially unchanged since they were first introduced in the late nineteenth century (Codner 1969; Madigan et al. 1997; Zengler 2009).

The situation is very different when it comes to culturing extremophiles on solidified media. For example, agar media are not suitable for culturing thermophiles and hyperthermophiles because the solidification of agar is thermoreversible at around 50–60°C (Watase and Nishinari 1987), and the media are unstable at temperatures much above 70°C for extended periods (Baross 1995). Culturing extremophiles on solidified media under acidic or alkaline conditions presents a similar problem of instability.

Solidified Media for Extremophiles

Any porous materials can substitute agar in principle, as long as the pore size is smaller than the microbial cells so that the cells stay on the surface when they are inoculated (Codner 1969). Various attempts have been made to provide solidified media that remain solid even under extreme culture conditions. Probably the most important progress ever made for extremophiles research is the use of gellan gum (often referred to by its trade name, Gelrite) as a solidifying agent (Lin and Casida 1984; Deming and Baross 1986; Robb and Place 1995). Gellan gum is composed of a unit consisting of 2 β-glucose, β-glucuronic acid, and rhamnose, and is produced by *Sphingomonas elodea* (formerly known as *Pseudomonas elodea*) (Moorhouse et al. 1981). Like agar, gellan gum is soluble in hot water, but forms clear and stiff gel upon cooling, which shows better stability than agar at high temperatures. Various formulations have been proposed to prepare solidified media for hyperthermophiles using gellan gum (Moorhouse et al. 1981; Kang et al. 1982; Shungu et al. 1983).

However, the gel-forming properties of gellan gum are very sensitive to the composition of the medium to which gellan gum is added. It requires large amounts of divalent cations (Moorhouse et al. 1981; Kang et al. 1982) and is also sensitive to other additives such as sugars (Miyoshi et al. 1998; Miyoshi and Nishinari 1999). Because of this sensitivity, one has to carefully design the formulation of the medium employing gellan gum not because it is necessary for the target microorganisms to grow, but because it is necessary to make the plate remain solid at the desired culture conditions (Lin and Casida 1984; Deming and Baross 1986; Baross 1995).

Nanofibrous Cellulose

Kuga reported preparation of porous matrices made of thin crystalline fibers of cellulose (Kuga 1980b), and exploited their application for chromatography (Kuga 1980a). Cellulose is a linear polysaccharide made of glucose connected via β-1-4 linkage, and is the most abundant organic polymer on earth with an estimated annual production of 15 trillion tons (Klemm et al. 2005).

We found that porous matrix made of nanofibrous cellulose could be used as a solid support for microbial culture (Deguchi et al. 2007). Due to extensive hydrogen bonding networks that are formed between the cellulose chains in the crystal, crystalline structure of cellulose is extremely robust and remains intact even when it is heated in water to ~300°C under pressure (Deguchi et al. 2006; Deguchi et al. 2008a, b). Because of this stability, nanofibrous cellulose can be used as a versatile platform for developing solidified media that support the growth of extremophiles under a wide variety of extreme culture conditions.

Preparation of Nanofibrous Cellulose Plates

For preparation of nanofibrous cellulose plates for microbial culture, we modified the literature method (Kuga 1980b) so that a large number of plates can be prepared without difficulty (Deguchi et al. 2007). Cellulose is first dispersed in a saturated aqueous solution of $Ca(SCN)_2$ at a concentration of 1.5 or 3 wt%, and the mixture is stirred at least for 1 h to allow complex formation between the cellulose chains and the calcium thiocyanate ions (Hattori et al. 1998b, c). Twenty mL aliquots of the dispersion are placed in glass culture dishes, and cellulose is allowed to dissolve by heating to 121°C for 1 min in an autoclave. The clear and viscous solution is then left at room temperature overnight.

Cooling a hot solution of cellulose in a saturated solution of $Ca(SCN)_2$ induces a microscopic phase separation into a cellulose-rich phase and a salt-rich phase (Hattori et al. 1999). Recrystallization of cellulose is induced in the cellulose-rich phase, which provides cross-linking points and solidifies the whole solution (Hattori et al. 1999). The solidification is thermoreversible at around 80°C, but is fixed once the salt is removed (Kuga 1980b). Removal of $Ca(SCN)_2$ is accomplished by washing the plate with methanol and then a copious amount of water. It is important to note that washing a solidified plate immediately with water leads to disintegration (Kuga 1980b). Washing is continued until the conductivity of the water in the washing bath decreases below 10 $\mu S\ cm^{-1}$.

A saturated aqueous solution of $Ca(SCN)_2$ dissolves a diverse range of celluloses such as conifer pulp, cotton, bacterial cellulose, tunicate cellulose, and rayon (Hattori et al. 1998a). However, when cellulose is allowed to dissolve by heating in an autoclave, insoluble cellulose is often found when the solution is taken out of the autoclave, probably because the dispersion is not stirred during heating. Longer heating does not help. Rather, the strong smell of sulfur compounds becomes evident when the plate is taken out of the autoclave after longer heating, possibly due to thermal decomposition of the SCN ion (Kuga 1980b). The resulting plates are also yellow, and the color remains even after the salt removal.

The success of preparation by the autoclave depends strongly on the type of cellulose used. Funacel SF (Funakoshi, Tokyo, Japan) and cellulose microcrystalline for thin layer chromatography (Merck, Darmstadt, Germany) are free from the incomplete dissolution issue at concentrations up to 20 wt%, while CF1 fibrous cellulose powder (Whatman, Brentford, UK), CC41 microgranular cellulose powder (Whatman, Brentford, UK), and amorphous-like cellulose (Sigmacell cellulose type 101, Sigma, St. Louis, MO) do not work well (Deguchi et al. 2007).

Typical agar plates for solid culture contain 1.5 wt% of agar, but the optimal concentration for cellulose is between 2 and 3 wt%. The plate containing less than 1 wt% of cellulose is too fragile, and spreading a dispersion of microorganisms on the surface is not possible. When the cellulose concentration is increased above 3 wt%, the plate after the salt removal is no longer flat and so is not suitable for solid culture.

Structural Characteristics

White plates thus obtained consist of fine fibers of crystalline cellulose (*Fig. 5.8.1a, b*). The typical width of the individual fibers is 20–50 nm (*Fig. 5.8.1b*). The fibers are entangled and form a porous structure, whose typical pore size is on the order of several hundreds of nm

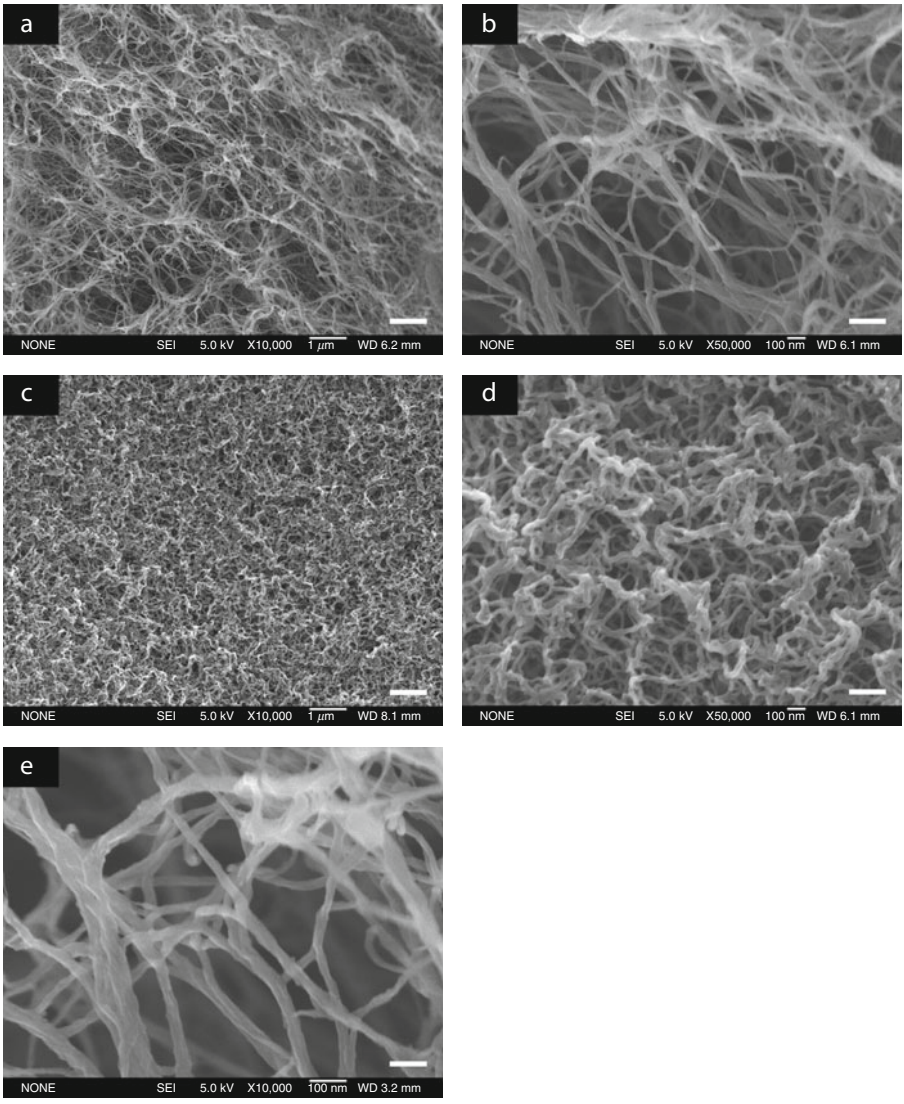

Fig. 5.8.1

SEM images showing surface morphology of nanofibrous cellulose (a and b) and agar hydrogel (c and d). A close-up of fibers making up nanofibrous cellulose is shown in (e). *Scale bars*: a and c, 1 μm; b and d, 200 nm; e, 100 nm. The concentrations of the polysaccharides are 1.5 wt% (Taken from Deguchi et al. 2007. Reproduced by permission of The Royal Society of Chemistry [RSC])

(Fig. 5.8.1a, b). The agar plate shows similar surface morphology (Fig. 5.8.1c, d). However, the fibers seem to be thinner, and the average pore size seems smaller due to a higher fiber density. The pore size seems to agree with the values reported for the agar hydrogel of the same concentration (148.1 nm) (Chui et al. 1995).

Figure 5.8.2 shows *Escherichia coli* cells on the surface of the nanofibrous cellulose plate. It is clearly seen that the pores are small enough that the *E. coli* cells stay on the surface and are not able to go into the pores. In addition, the *E. coli* cell has several flagella extending from the cell surface (Macnab 1996). The flagella are typically 5–10 μm long (Macnab 1996). In liquid media, *E. coli* moves rapidly by rotating a bundle of the flagella. It is plain to see that rotation of the long flagella on the surface of the nanofibrous cellulose results in entanglement that also helps in keeping the cell on the surface.

The molecular weight of cellulose does not change by the plate preparation (Table 5.8.1), indicating that dissolution of cellulose in a saturated solution of $Ca(SCN)_2$ by heating is not accompanied with a significant degradation of the cellulose chains. On the other hand, the

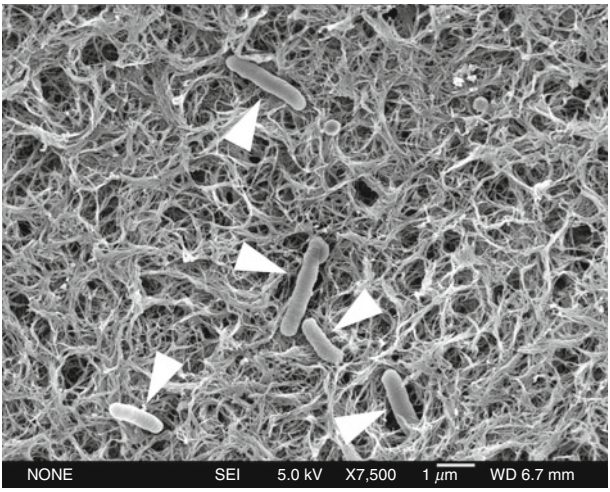

Fig. 5.8.2

SEM image of *Escherichia coli* cells (indicated by *white triangles*) on the surface of a nanofibrous cellulose plate. The cellulose content of the plate is 3 wt% (Taken from Deguchi et al. 2007. Reproduced by permission of The Royal Society of Chemistry [RSC])

Table 5.8.1

Characteristics of cellulose before and after conversion to the nanofibrous form. Taken from Deguchi et al. 2007 (Reproduced by permission of The Royal Society of Chemistry [RSC])

	Molecular weight	DP[a]	Crystallinity	Crystalline form
Before	3.6×10^4	2.2×10^2	58.2 %	Cellulose-I
After	4.2×10^4	2.6×10^2	44.5 %	Cellulose-II

[a]Degree of polymerization.

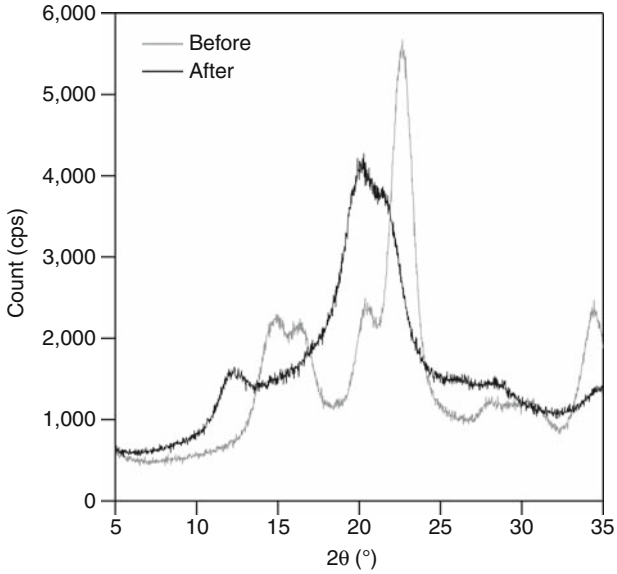

Fig. 5.8.3

Powder X-ray diffractograms of cellulose before and after conversion to the nanofibrous form (Taken from Deguchi et al. 2007. Reproduced by permission of The Royal Society of Chemistry [RSC])

crystallinity drops from 58.2% to 44.5% by X-ray diffraction (XRD, *Fig. 5.8.3*). XRD analysis also shows that the crystalline form changes from cellulose-I, which is a characteristic crystalline form for natural cellulose, to cellulose-II, which is a characteristic crystalline form for regenerated cellulose.

Structural Robustness Under Extreme Culture Conditions

One of the obvious advantages of the cellulose plate over the agar plate is that the cellulose plate does not lose its integrity even at harsh physicochemical conditions such as high temperatures. Despite its seemingly fragile structure (*Fig. 5.8.1*), nanofibrous cellulose plate shows structural robustness comparable to that of highly crystalline cellulose in hydrothermal conditions (Deguchi et al. 2007; Deguchi et al. 2008b).

Figure 5.8.4 shows a series of optical microscopic images of a small piece of the nanofibrous cellulose plate in water at high temperatures and at a constant pressure of 25 MPa. The observation was made on an optical microscope equipped with a high-temperature and high-pressure sample chamber (Deguchi and Tsujii 2002; Mukai et al. 2006). The instrument allows in situ observation of a specimen in water at temperatures and pressures up to 400°C and 40 MPa with an optical resolution of 2 μm. Rather surprisingly, the highly porous material made of a biopolymer remained unchanged in water up to 280°C at 25 MPa, above which it dissolved in water. Considering the known upper temperature limit for life (122°C) (Takai et al. 2008), it seems safe to conclude that the operable temperature range of the nanofibrous cellulose plate is wide enough for solid culture of any microorganisms.

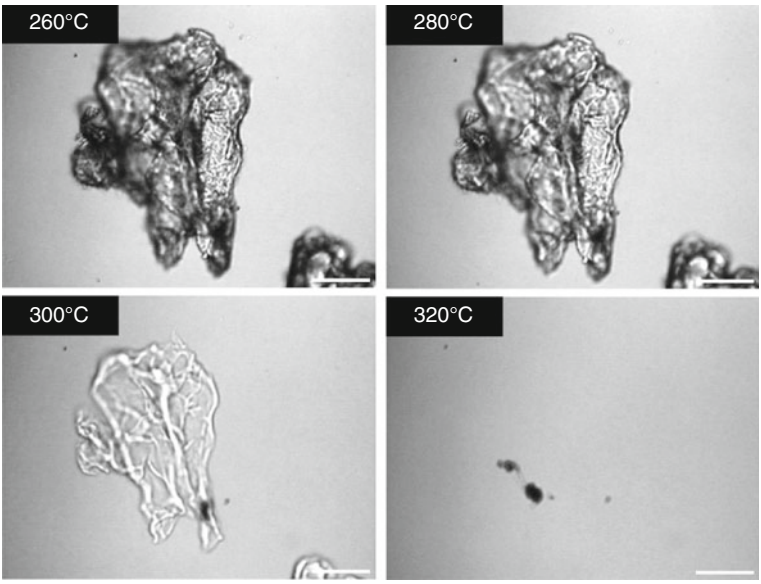

Fig. 5.8.4

A sequence of in situ optical microscopic images showing dissolution of a piece of a nanofibrous cellulose plate in water between 260°C and 320°C and at a constant pressure of 25 MPa. Scale bar represents 100 μm (Taken from Deguchi et al. 2007. Reproduced by permission of The Royal Society of Chemistry [RSC])

Permeation of Media

Unlike conventional agar-supported media, the nanofibrous cellulose plate contains pure water in the pores just after the salt removal is completed. For solid culture of microorganisms, the pores have to be filled with an appropriate nutrient fluid. The solvent exchange can be accomplished by overlaying an equal volume of a double strength medium on top of the plate that had been made sterile by autoclaving at 121°C for 20 min (Deguchi et al. 2007). The nutrient components are allowed to diffuse into the pores with the aid of gentle agitation on a shaker (*Fig. 5.8.5*).

Figure 5.8.6 shows kinetics of the solvent exchange, which was followed by measuring the absorbance of the supernatant (*Fig. 5.8.6*). On reaching equilibrium, the double strength medium is mixed with an equal amount of water and is diluted to half the original concentration. Accordingly, the absorbance of the supernatant should decrease with time as the solvent exchange proceeds and become half of the initial value when the equilibrium is reached. As is seen in *Fig. 5.8.6*, the solvent exchange process is rapid and seems to be almost completed in 1.5 h. The supernatant is discarded once the solvent exchange is completed.

Culture of Mesophilic Microorganisms

In our test using three representative mesophilic microorganisms, *E. coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*, no significant difference was seen in their growth on the agar and the cellulose plate (*Table 5.8.2*) (Deguchi et al. 2007). The number of colonies formed on the

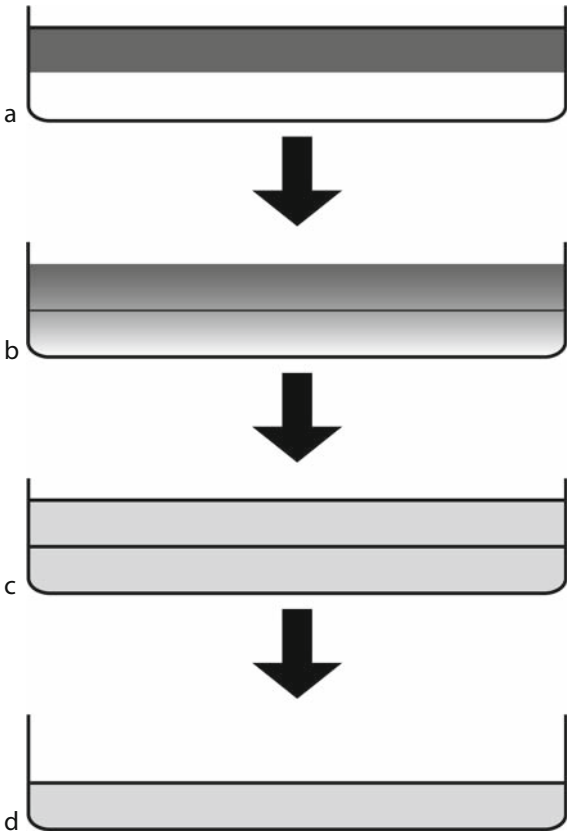

Fig. 5.8.5

Schematic diagram showing solvent exchange process: (a) double strength medium (*shaded area*) is overlaid on top of a cellulose plate (*white area*); (b) nutrient components are allowed to diffuse into pores of nanofibrous cellulose plate; (c) solvent exchange completes within a few hours; and (d) supernatant is discarded, and the plate is ready for use

agar plate and the cellulose plate agreed within experimental error, demonstrating that the nanofibrous cellulose plate can be used as a solid support for microbial cultures. The results also show that removal of $Ca(SCN)_2$ is complete enough that the residual salt if any does not affect the growth of the microorganisms.

Culture of Extremophiles

Wide variety of extremophiles were cultured successfully by using nanofibrous cellulose plates. Extremophiles tested include acidophilic *Acidiphilium acidophilum* ATCC 27807^T, alkaliphilic *B. clarkii* DSM8720^T, thermophilic *Geobacillus stearothermophilus* ATCC 12016 and *Thermus thermophilus* JCM10941^T, acidothermophilic *Sulfolobus acidocaldarius* DSM639^T, and alkalithermophilic *Bacillus* sp. strain TX-3 (JCM9162) (Kitada et al. 1989). Culture media

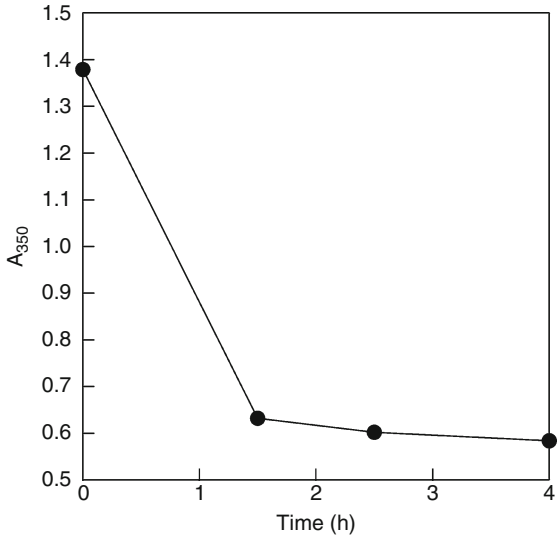

Fig. 5.8.6

Change of A_{350} of the supernatant of LB broth as a function of time. The absorbance decreased with time, and reached a constant value within 2 h. Taken from Deguchi et al. 2007 (Reproduced by permission of The Royal Society of Chemistry [RSC])

Table 5.8.2

Comparison of the number of colonies formed on agar and cellulose plates. Taken from Deguchi et al. 2007 (Reproduced by permission of The Royal Society of Chemistry [RSC])

	Agar	Cellulose
Escherichia coli W3110[a]	91 ± 10	80 ± 5
Bacillus subtilis 168[a]	51 ± 8	46 ± 9
Saccharomyces cerevisiae YPH499[b]	184 ± 10	171 ± 21

[a]Cultured on agar or cellulose plate containing LB broth at 37°C for 16–18 h.
[b]Cultured on agar or cellulose plate containing YPD broth at 25°C for 2 days.

and culture conditions are given in *Table 5.8.3*. The plates were inoculated from liquid cultures and incubated for 1–10 days (see *Table 5.8.3* for details). Conventional agar plates and/or gellan gum plates containing the same nutrients were used for control experiments.

In culturing thermophilic microorganisms, care was taken to prevent water evaporation. The lid and body of the culture dish were sealed with adhesive tape, wrapped with a wet paper towel, and sealed in a zip-lock bag during incubation at high temperature.

Culture of an Acidophile and an Alkaliphile

Hydrolysis of agar in acidic media and the toxicity of hydrolysates prevent growth of *A. acidophilum* on the agar-solidified medium (Kelly and Harrison 1989; Johnson 1995).

Table 5.8.3

Growth of extremophiles on media solidified with cellulose, agar, and gellan gum[a] (Taken from Tsudome et al. 2009. Copyright © American Society for Microbiology)

Organism	Basal medium	pH	Incubation		No. of CFU (mean ± SD) on medium with indicated solidifying agent[b]		
			Temp (°C)	Time	Cellulose	Agar	Gellan gum
Acidiphilium acidophilum	9-K glucose medium[c]	3.5	25	10 days	285 ± 21 (3)	0 (1.5)	240 ± 27 (0.7)
Bacillus clarkii	Horikoshi-II medium[d]	10.5	30	6 days	65 ± 23 (3)	63 ± 18 (1.5)	ND
Geobacillus stearothermophilus	NB	6.8	55	24 h	215 ± 32 (3)	259 ± 31 (2)	— (0.8)
Thermus thermophilus	TM medium[e]	7.2	80	2 days	379 ± 30 (3)	— (3[d])	— (1)
	TM medium + 0.1% (by wt) $MgCl_2 \cdot 6H_2O$	7.2	80	2 days	335 ± 66 (3)	ND	430 ± 65 (0.7)
Sulfolobus acidocaldarius	*Sulfolobus* medium[f]	2.0	80	7 days	100 <[h] (3)	ND	100 <[h] (0.7)
Bacillus sp. strain TX-3	Alkaline NB[g]	10.0	55	24 h	153 ± 39 (3)	115 ± 24 (1.5)	ND

[a]All cultures were incubated aerobically. The experiments were done at least three times.
[b]The numbers in parentheses show the concentration (%) of the solidifying agent. ND, not determined; —, medium did not remain solid.
[c]ATCC medium 738.
[d]JCM medium 181 with soluble starch added instead of glucose.
[e]JCM medium 273.
[f]DSM medium 88.
[g]DSM medium 31.
[h]Colonies were small and could not be enumerated reliably.

In contrast, visible colonies of *A. acidophilum* were formed on the cellulose and gellan gum plates after incubation for 8 days, showing their chemical inertness in the acidic environment (Tsudome et al. 2009). In the case of alkaliphilic *B. clarkii*, no difference was observed in the number of colonies formed on the cellulose and agar plates (Tsudome et al. 2009).

Culture of Thermophiles

G. stearothermophilus grew as well on nutrient cellulose at pH 6.8 and 55°C as it did on nutrient agar (Tsudome et al. 2009).

In the case of *T. thermophilus*, the conventional agar plate containing TM broth did not remain solid at 80°C, and colony formation was not observed (*Fig. 5.8.7a*). On the other hand, heating to 80°C did not affect the cellulose plate at all, and *T. thermophilus* formed yellow colonies on the cellulose plate containing TM broth at 80°C (*Fig. 5.8.7b*) (Deguchi et al. 2007).

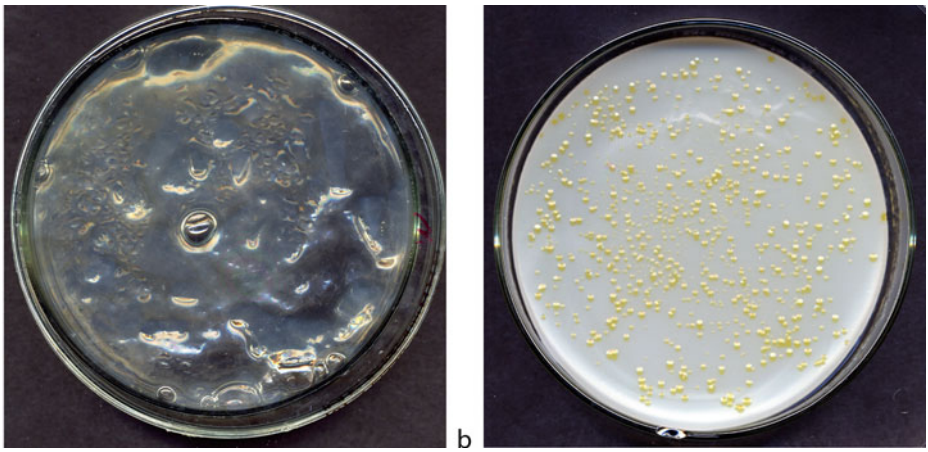

Fig. 5.8.7

Photographs of solid culture of *Thermus thermophilus* on agar plate (a) and cellulose plate (b) after incubation at 80°C for 3 days. The agar plate (a) was not solid at 80°C, but re-solidified upon cooling to room temperature when taking the image. Formation of colonies is seen only on the cellulose plate. Taken from Deguchi et al. 2007 (Reproduced by permission of The Royal Society of Chemistry [RSC])

Preparation of the gellan gum-supported media that can be used for solid culture of *T. thermophilus* at 80°C requires the use of a solidifying aid (Lin and Casida 1984). Without the solidifying aid, the plate containing 0.7 wt% gellan gum and TM broth did not solidify even at room temperature, and the plate with 1 wt% gellan gum solidified at room temperature, but did not remain solid at 80°C. Still, the effect of syneresis was evident for colonies of *T. thermophilus* formed on a TM medium solidified by gellan gum and a magnesium gelling aid, leading to irregular colony shapes and merger of neighboring colonies (*Fig. 5.8.8a*). No such effect was seen for colonies of *T. thermophilus* on the cellulose plate (*Fig. 5.8.8b*).

The addition of a gelling aid negatively affects microbial growth in some cases (Lin and Casida 1984). As cellulose-supported media does not require any solidifying aids, it allows examining the effects of magnesium and calcium gelling aids on the growth of *T. thermophilus*. *T. thermophilus* was cultured at 80°C for 4 days on cellulose plates that contained TM broth, TM broth plus 0.1% $MgCl_2 \cdot 6H_2O$, or TM broth plus 0.1% $CaCl_2 \cdot 2H_2O$. Culturing *T. thermophilus* on solidified TM medium that was free from a gelling aid was made possible only by using cellulose.

T. thermophilus formed yellow circular colonies on a TM cellulose plate (*Fig. 5.8.8c*). The pigmentation was so intense that the entire plate became yellowish after incubation for 4 days. The pigmentation is due to the formation of carotenoids and is one of the important phenotypic characteristics of *Thermus* strains (Williams et al. 1995). Interestingly, the pigmentation became less intense when the cellulose plate was supplemented with 0.1% $MgCl_2 \cdot 6H_2O$, although the colony shape remained unaffected. *T. thermophilus* formed nonpigmented umbonate colonies (*Fig. 5.8.8d*) on the cellulose plate that contained 0.1% $CaCl_2 \cdot 2H_2O$. The results show that the color and shape of *T. thermophilus* colonies are significantly affected by the type of gelling aid.

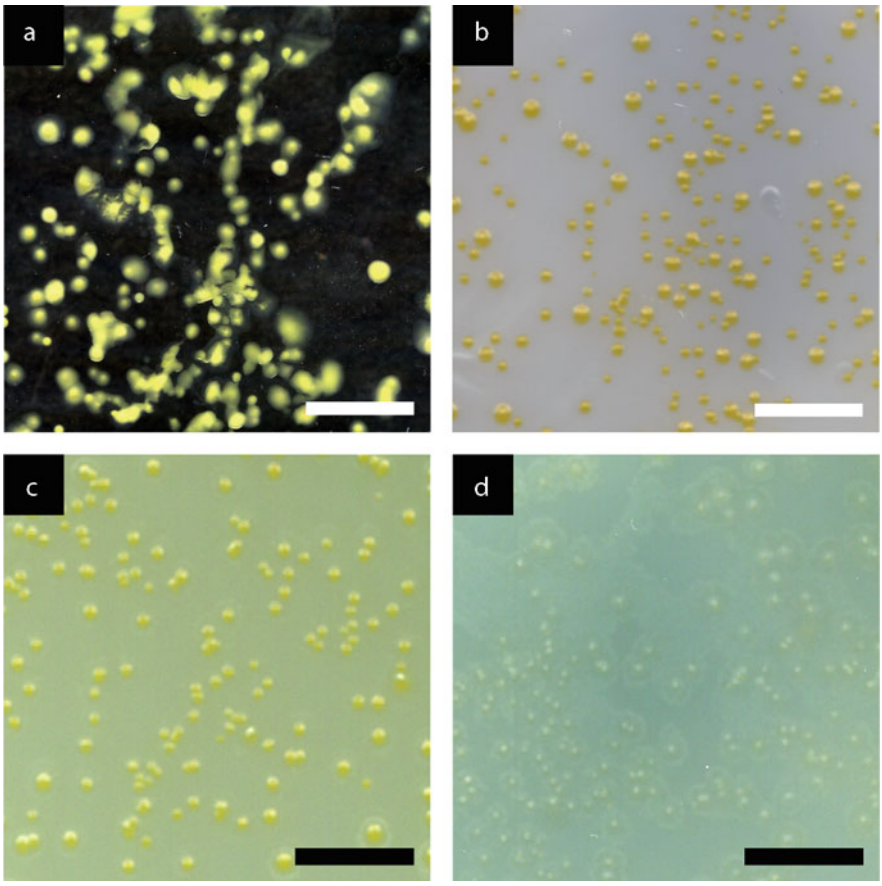

Fig. 5.8.8

(a and b) Colonies of *Thermus thermophilus* formed on a gellan gum plate containing TM broth plus 0.1% $MgCl_2 \cdot 6H_2O$ (a) and a cellulose plate containing TM broth (b). Images were taken after incubation at 80°C for 2 days. Syneresis prevented formation of well-isolated colonies on the gellan gum plate. Scale bar represents 1 cm. (c and d) Colonies of *T. thermophilus* formed on a cellulose plate containing TM broth (c) and TM broth plus 0.1% $CaCl_2 \cdot 2H_2O$ (d). Images were taken after incubation at 80°C for 4 days. Scale bar represents 5 mm (Taken with modification from Tsudome et al. 2009. Copyright © American Society for Microbiology)

Culture of an Acidothermophile and an Alkalithermophile

Acidothermophilic *S. acidocaldarius* formed colonies on both cellulose and gellan gum plates at pH 2 and 80°C, although the colonies were smaller than 300 μm in diameter and difficult to enumerate accurately (*Fig. 5.8.9a, b*) (Tsudome et al. 2009). Close examination revealed significant effects of syneresis only on the colonies on the gellan gum plate. Alkalithermophilic *Bacillus* sp. strain TX-3 was cultured successfully on the cellulose plate at pH 10 and 55°C (Tsudome et al. 2009). Colony formation was also confirmed at 65°C with cellulose, whereas culture on the agar plate met with sporadic success due to syneresis. Gellan gum was unusable because the addition of a calcium or magnesium gelling aid to alkaline NB resulted in precipitation.

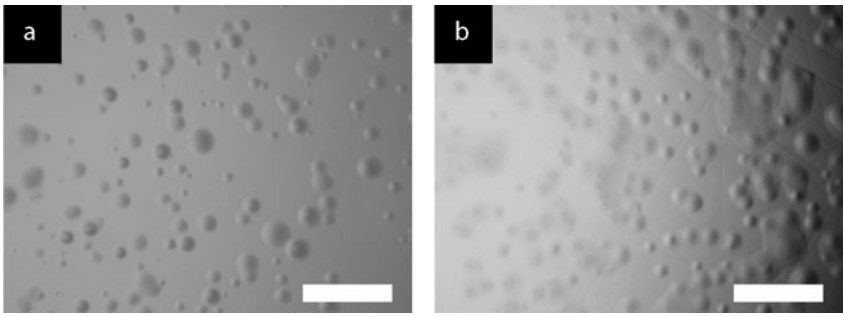

Fig. 5.8.9

Optical micrographs showing colonies of *Sulfolobus acidocaldarius* formed on the cellulose plate (a) and the gellan gum plate (b) after incubation for 7 days at pH 2 and 80°C. Scale bar represents 1 μm (Taken with modification from Tsudome et al. 2009. Copyright © American Society for Microbiology)

Summary

Due to unsurpassed stability of porous network structures, solidified media using nanofibrous cellulose plates can be used for culturing various extremophiles. The cellulose-supported media have several advantages over conventional solidified media using agar or gellan gum (Lin and Casida 1984; Deming and Baross 1986) for culture of extremophiles.

1. Essentially any medium can be solidified with the cellulose plate, regardless of the culture conditions, while the solidification of conventional solidified media depends strongly on the composition of the medium, such as the concentrations of solidifying agent and gelling aids (Moorhouse et al. 1981; Johnson 1995; Miyoshi and Nishinari 1999). The cellulose plate, for example, allows the preparation of solidified media for extremophiles that contain very dilute and low-nutrient fluids. Various previously uncultured microorganisms have been propagated successfully by using such dilute, low-nutrient media (Connon and Giovannoni 2002; Rappé et al. 2002; Zengler et al. 2002). However, solidifying such media with agar or gellan gum for use under extreme culture conditions is difficult.
2. The stability of the cellulose plate is unparalleled. It remains unchanged even at temperatures much higher than the known upper temperature limit for life (122°C) (Takai et al. 2008). Because oxygen does not affect the stability of the cellulose plate, it may be possible to use the plates to culture anaerobic hyperthermophiles at temperatures at or above 100°C, although this has not been tested.
3. Syneresis of the cellulose plate is negligible.
4. Preparation of the cellulose medium obviates such hassles as handling very hot solutions, which is often necessary for preparing gellan gum medium (Baross 1995).

A major drawback of the cellulose plate is its preparation, which is difficult and time-consuming compared with agar- or gellan gum-supported ones. To address the issue, we have partnered with a company and considered the possibility of commercial production of the cellulose plate. The collaboration has proceeded well, and we expect product launch sometime in 2010.

Cross-References

- 1.1 Prologue: Definition, Categories, Distribution, Origin and Evolution, Pioneering Studies, and Emerging Fields of Extremophiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 4.10 Physiology, Metabolism and Enzymology of Thermoacidophiles
- 7.1 Microbiology of Volcanic Environments
- 11.1 Ecology and Cultivation of Marine Oligotrophic Bacteria

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Part 6

Extremophiles: Psychrophiles

6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_6.1,

Introduction

A significant portion of the Earth's biosphere is found in perennially cold environments (less than 5°C) including the deep ocean, frozen soils, terrestrial glaciers, perennially ice-covered lakes, and polar sea ice and ice sheets (Priscu and Christner 2004). Approximately 20% of Earth's surface is currently ice-covered with the Greenland and Antarctic ice sheets covering ~10% of the terrestrial land surface. This ice stores ~75% of the Earth's freshwater supply (Paterson 1998). The Antarctic ice sheets, which contain ~90% of the planet's ice, would result in a sea level rise of ~70 m if it melted (http://www.nsidc.org). Approximately 20% of world's soil ecosystems are frozen ground (permafrost). Ice is a dynamic feature on our planet and a key regulator of the global climate system; how cold systems will respond to a warming world is of fundamental concern. The awareness that icy systems harbor cold-loving microorganisms raises new questions about climatic feedbacks between microbial activity and ice extent. Despite the prevalence and importance of cold ecosystems, little is known about the diversity and function of icy microbial communities.

Cold adapted organisms are known as psychrophiles. Richard Morita (1975) first defined the term in his now classic 1975 publication where he differentiated between psychrophiles (or "cold-loving") organisms that grow optimally at temperatures <15°C and cannot grow above 20°C and psychrotolerant (or "cold-tolerant") organisms that are capable of growth at temperatures <15°C, but grow faster at temperatures above 25°C. Terms from animal physiology have also been used (Helmke and Weyland 1995; Laucks et al. 2005) including Stenopsychrophile and Eurypsychrophile (Rothschild and Mancinelli 2001; Cavicchioli 2006). Stenopsychrophiles (i.e., true psychrophiles *sensu* Morita) are restricted to growth within a narrow range and do not tolerate high temperatures (above 25°C). Eurypsychrophiles (i.e., psychrotolerant *sensu* Morita) can grow over a broad range of temperatures, −10°C or lower, but can also tolerate growth at higher temperatures (up to 30°C). Here, we use the term psychrophile to indicate any organism that is active in a low-temperature environment (i.e., temperatures below 5°C).

Until recently, environments covered with ice such as glaciers, ice sheets and some perennially ice-covered lakes, were thought to be devoid of life. This was based largely on our limited understanding of the ability of life to tolerate environmental extremes and the notion that if sunlight was absent, life could not be present. Discoveries of life in extremely cold, isolated environments have challenged our notion of the habitable zone on Earth. In the late 1990s, life was found locked in deep (~3,590 m) glacial ice (Priscu et al. 1999; Karl et al. 1999) and sediments below glaciers (Sharp et al. 1999). Microbial cells have also been found growing in permafrost sediments (i.e., Rivkina et al. 2000) and sea ice (Junge et al. 2004). Despite the excitement around these discoveries, questions remained about the role of these organisms in the environment: were they metabolically active at freezing in situ temperatures or were these organisms simply preserved in the ice?

Microbial cells in frozen environments, regardless of their in situ activity, represent a previously unaccounted for component of the global carbon budget. Priscu et al. (2008) estimated that within and beneath the Antarctic and Greenland ice sheets there may be as many as 4.0×10^{29} prokaryotic cells which translates into ~4.4 Pg of cellular carbon, rivaling the number of cells found in all of the freshwater environments on Earth (Whitman et al. 1998). Icy environments are difficult to access and these numbers were based on only a few samples from within and beneath the ice sheets. Polar regions, in general, are remote and lack convenient infrastructure, while subglacial environments are covered by 100s to 1,000s of meters of ice and

collecting clean samples for microbiological analyses presents significant technical challenges (i.e., Christner et al. 2005). Still, these preliminary estimates serve to illustrate the point that icy environments are a vast, unexplored repository of microbial life whose function in global processes remains unclear.

Knowing if life is metabolically active at sub-freezing temperatures is fundamental to defining the ecological role of microorganisms in icy systems. Dormant cells in frozen systems are a sink for carbon, nitrogen, phosphorous and other vital nutrients, while metabolically active cells are dynamic participants in biogeochemical cycles. Measuring metabolic activity below the freezing point of water is not without difficulty; however a variety of methods have been developed in attempts to measure rates of sub-freezing microbially mediated processes. Despite inherent biases for each method, the case for in situ low temperature metabolism is gaining support. Christner (2002) demonstrated that bacterial cells can maintain their macromolecular components by incorporation of radiolabeled DNA and protein precursor molecules during extended periods (100 days) at freezing temperatures (−15°C). Metabolic activity in Arctic sea ice was measured at temperatures between −2°C and −20°C using a fluorescent dye (5-cyano-2,3-ditoyl tetrazolium chloride or CTC) which identifies bacterial cells that are actively respiring via oxidative metabolism (Junge et al. 2004). Bakermans et al. (2003) documented both reproduction (by microscopic visualization of dividing cells) and metabolism (based on the rate of resazurin reduction) at −10°C in isolates from Siberian permafrost. Rivkina and colleagues (2000) measured activity in permafrost sediments by monitoring the incorporation of radiolabeled acetate over a 550-day period, sigmoidal growth was found down to −10°C and evidence for incorporation to −20°C. Price and Sowers (2004) summarized these and other reports of metabolic activity from low temperature environments and concluded that metabolic rates in cold systems can be characterized by three categories: (1) capable of supporting growth, (2) maintenance metabolism or (3) sufficient only for dormancy. Metabolic status of resident microorganisms is an important yet poorly understood factor in cold environment microbial ecology.

Physchrophilic representatives can be found in all three domains of life (i.e., Bacteria, Archaea, and Eukarya) and exhibit a wide variety of metabolic lifestyles, including sulfate reduction and oxidation, iron reduction and oxidation, methanotrophy and methanogenesis. Archaea are often thought to be analogous with "extremophiles" and in many perennially cold environments such as the deep ocean or polar marine waters, Archaea become more abundant relative to Bacteria (Murray et al. 1998; Massana et al. 1997). Methanogenic archaea are common and metabolically active in permafrost systems (Steven et al. 2009) and methanogenesis may be an important process in some subglacial environments that contain high concentrations of organic carbon from overrun soils and boreal forests (Skidmore et al. 2000; Wadham et al. 2008). However, in frozen environments such as glacial ice, subglacial systems, lake ice, and sea ice, Archaea appear to be rare or absent. It is not clear why this should be the case; however, it may be correlated to the highly oligotrophic nature of these communities.

Niches in Icy Systems

A broad range of cold and icy environments are found on Earth. These environments can be distinguished according to the portion of rock or sediment to liquid water to ice they are comprised of (*Fig. 6.1.1*). Cold rock and sediment environments (*Fig. 6.1.1a*) include

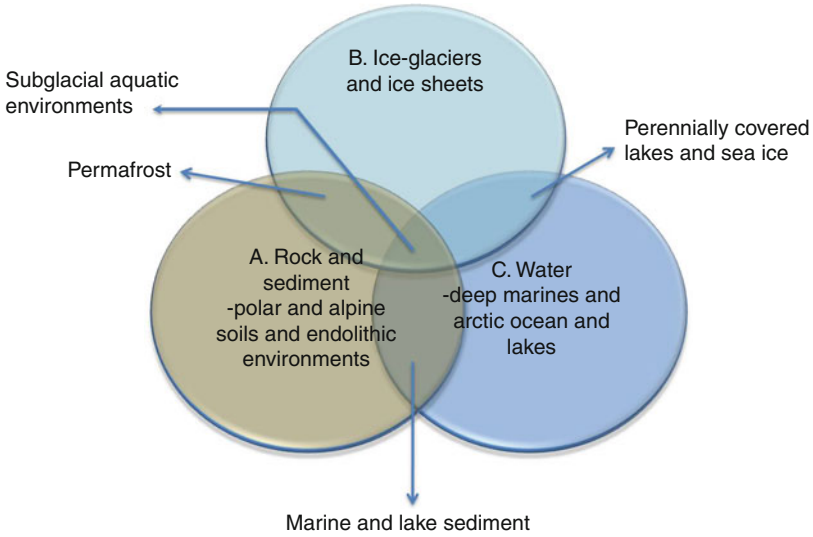

Fig. 6.1.1

Characterization of icy environments based on the influence of rock or sediment, liquid water and ice

alpine soils and lithic environments (i.e., environments within or beneath the surface of rocks). Environments that are exclusively "cold water" (*Fig. 6.1.1c*) include deep and polar oceans and polar lakes. Ice-dominated environments (*Fig. 6.1.1b*) are found within glaciers and ice sheets. Saturated marine and lake sediment systems are found at the interface of cold rock and sediment environments and cold water systems. Permafrost is the primary cold environment with both rock/sediment and ice components. Sea ice and perennially ice-covered lakes have both an ice and water component. Subglacial environments are found at the intersection of all three components; ice from the glacier, bedrock or sediment which underlie the glacier, and water generated at the ice/rock interface. The varying composition of these abiotic parameters and the interactions between them help constrain the structure and function of the microbial communities they host. In this chapter, we focus on two specific environments that include ice and rock as major factors regulating the environment: subglacial systems (which have aspects of rock, ice, and water) and permafrost (rock and ice). Other cold environments are covered elsewhere in this volume, including soils and lithic environments (Kirby et al.), glacial, ice sheet environments and sea ice (Junge et al. 2004). Here we describe what is known about microbial processes in the subglacial and permafrost environment and discuss where potential feedbacks between microbial metabolism and global climate occur.

The Subglacial Niche

Ice covers extensive portions of the Earth's terrestrial surface (~10%) making subglacial environments the most widespread habitat for psychrophiles after the deep cold ocean (*Fig. 6.1.2*). It has been estimated that subglacial sediments beneath Antarctica ice range from 10^4 to 10^6 km^3 (Priscu et al. 2008). Glaciers have highly variable rates of advance, retreat,

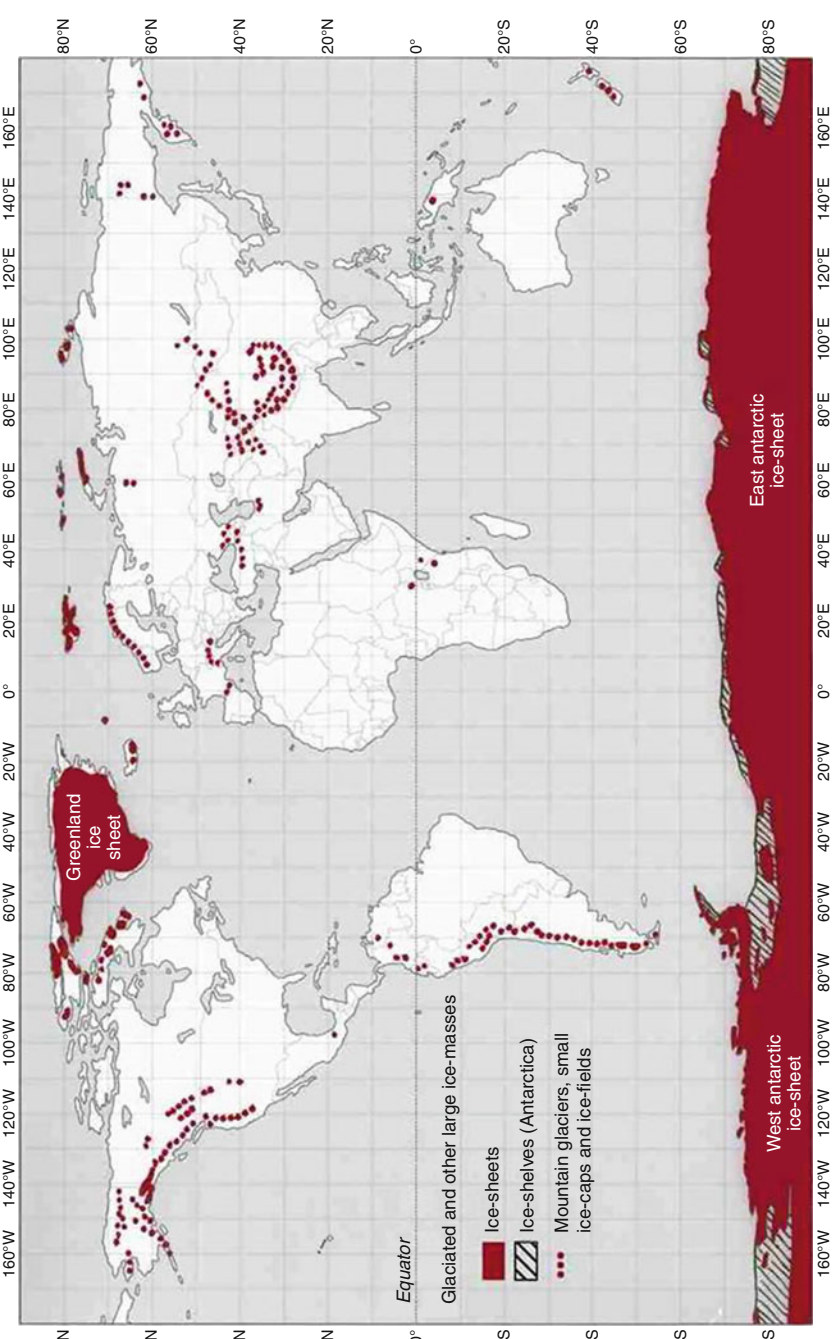

Fig. 6.1.2
The global distribution of glaciers. Figure generated from the World Glacier Inventory, Snow and Ice Data Center. (http://map.ngdc.noaa.gov/website/nsidc/glacier/viewer.htm)

and dynamic thinning (i.e., ice loss due to acceleration). While the mechanisms behind these differences are complex, they are primarily the result of the presence, availability, and pressure of liquid water in contact with deformable sediments at the ice/rock interface. Two major glacial types exist: (1) those that are unconstrained and blanket the landscape, including ice caps and ice sheets and (2) those that are constrained by the surrounding topography (i.e., valley glaciers, ice fields, and cirques) (reviewed in Tranter 2003). The latter can be grouped along a subglacial thermal continuum between warm-based and cold-based glaciers. Two factors exert strong influence on the ecosystem function in these systems: water residence time and the rock/water ratio; both influence the rates of chemical weathering and biological processes.

The ice at the base of warm-based glaciers, by definition, is at the pressure-depressed freezing point; thus, liquid water is present throughout the subglacial environment (Tranter 2003). Consequently they have a shorter subglacial residence time. Most valley glaciers in temperate regions fall into this category. During winter, water pressure in the subglacial environment is low enough that the glacier rests directly on the bed in many places and water flow is essentially non-existent. This water is stored for many months and tends to have high(er) solute concentrations than late season waters (i.e., Tranter et al. 1993; Skidmore and Sharp 1999). As the season progresses and surface melt water reaches the base of the glacier, hydrologic pressure begins to rise, lifting the ice off the bed and allowing glacial motion. When water content (and thus hydrologic pressure) is relatively low, water is found distributed throughout the basal environment in the so-called "distributed flow" model (Fountain and Walder 1998). Later in the season as more water is injected into the subglacial environment and hydrologic pressure increases, water converges into larger subglacial conduits or streams that flow through channels in the so-called "channelized flow" model (Fountain and Walder 1998). Water in these two scenarios (distributed vs. channelized) represents distinct physiochemical systems. Because the water in channelized flow is not fully connected to the greater subglacial hydrological system, stored water may remain isolated for multiple seasons and (if sufficient biological activity occurs) anoxic conditions may exist in some regions (Tranter 2003). Water flow remains in this mode until decreasing temperatures and decreasing water input cause water pressure to drop and the water to convert back to a distributed flow and eventually to stored water. These processes create complex and varied hydrological conditions, which influence the chemical and physical environment. Furthermore, seasonal shifts in temperate settings, from relatively warm to relatively cold conditions, lead to the development of basal ice formed by the "freeze-on" of melted water from the subglacial environment that is highly enriched in sediments entrainment from direct interaction of the ice with the glacial bed (Sharp et al. 1999).

Polythermal glaciers are found primarily in the higher latitudes and exhibit greater complexity in their hydrologic regimes often storing subglacial water on seasonal time scales. The ice at the exterior of a polythermal glacier is extremely cold with the basal ice frozen directly to the glacier bed, whereas the core of the glacier remains warm and the basal ice is at the pressure-depressed freezing point (Wadham et al. 2000). Water still enters the subglacial system via surface melt inputs, but cannot flow through a distributed mechanism. Instead, it is stored either as refrozen ice at the perimeter or as liquid water toward the center of the glacier. Once hydrologic pressure rises sufficiently, the ice is lifted off the bed and water flows out, likely in a channelized mechanism, from one or a few outflow points (Tranter 2003). Similar to temperate glaciers, basal ice is the result of freeze-on of liquid water and contains high concentrations of sediment. Thin valley glaciers at high latitudes can have ice below the pressure-depressed melting point throughout the glacier. In these glaciers, minimal water is

present in the subglacial environment and thus chemical weathering and biological activity is minimal (Hodgkins et al. 1997).

Ice caps and ice sheets cover regions ranging from $<$50,000 and $>$50,000 km^2 area respectively (Bennet and Glasser 1996) and may contain hydrologic elements of each of the above glacier types (Tranter 2003) resulting in varying and possibly extended residence times. When ice flows over regions with relatively high water content and soft deformable sediments it tends to move faster than the surrounding ice, generating ice streams. Ice streams are thought to be the major mechanism by which the Antarctic and Greenland ice sheets are drained (Bentley 1987). It has been argued that many ice streams have subglacial lakes at their heads, and that these lakes may act as a source of water to the downstream environment (Bell et al. 2007; Fricker et al. 2007). In fact a diversity of hydrologic systems may exist below ice caps and ice sheets including "wetlands" (i.e., regions with low or non-existent standing water but with water-saturated sediments), "streams" (i.e., regions with detectable liquid water moving through a hydrologic gradient), and lakes (i.e., relatively deep regions of standing water that may or may not be hydrologically connected to other water systems) (Fricker et al. 2007; Priscu et al. 2008; Smith et al. 2009).

Life in Subglacial Environments: The Geochemical Implications

The subglacial environment was long thought to be devoid of life (Raiswell 1984; Chillrud et al. 1994). Then, in the mid-1990s, researchers focusing on the geochemistry of subglacial environments noticed that subglacial waters that had been stored over the winter tended to be anoxic and depleted in nitrate. This chemical alteration from what would be predicted for abiotic subglacial waters suggested that biological activity might be involved in subglacial chemical transformations (Fairchild et al. 1993; Tranter et al.1993; Tranter et al. 1994; Tranter et al. 1998). In 1999, Sharp et al. (1999) discovered microorganisms in sediment-rich basal ice and subglacial waters from the Haut Glacier d'Arolla in the Alps. These authors hypothesized that the microorganisms may be playing a significant role in weathering processes at the glacier bed by mediating redox chemistry, generating acid equivalents and oxidizing sulfide. Since these first discoveries, subsequent research on glaciers in Svalbard, the Canadian Arctic and Antarctica have confirmed a biological contribution to subglacial chemical weathering processes (Wadham et al. 2004; Hodson et al. 2008; Skidmore et al. 2009; Mikucki et al. 2009); however, the magnitude of these processes and their contribution to global biogeochemical cycles remains unclear.

Subglacial processes may provide an important negative feedback on global climate change primarily through increased weathering of silicate minerals, which can lead to increased sequestration of atmospheric carbon dioxide (Sharp et al. 1995), although this hypothesis is somewhat controversial (Anderson et al. 2000). Chemical weathering rates in glaciated environments are comparable to non-glaciated environments with similar water flow rates, and this is likely due to the high levels of freshly exposed mineral surfaces from glacial grinding and low levels of organic carbon (which can protect surfaces from weathering) in glaciated systems (Anderson et al. 1996). Global carbon models generally assume that once a region is glaciated, organic carbon mineralization ceases and calculations have shown a negligible impact of glacial nutrient pools on global carbon budgets (Ludwig et al. 1998; Tranter et al. 2002); however, it is possible that this is a result of an incomplete understanding of the relationship between terrestrial and marine carbon sequestration processes (Tranter 2003). Changes in microbial

activity may accelerate under warming conditions generating more uncertainty in the role of glacial systems in climatic feedbacks.

Methanogenesis however, presents a clear link between subglacial microbial activity and the global carbon cycle. If methane is produced in subglacial environments during glacial periods, this stored methane could be released during glacial retreat under warming conditions providing a significant positive feedback during the glacial-interglacial transition (Delmotte et al. 2004; Wadham et al. 2008). Wadham et al. (2008) estimated that as much as 63 Pg of soil organic carbon could be converted to methane during a glacial cycle. As mentioned above, it is rare to find Archaea in icy environments; however, methanogenic activity was detected in enrichment cultures from a subglacial environment (Skidmore et al. 2000) and genetic, biochemical and geochemical indicators of in situ methanogenic activity have been detected in a subglacial environment in the Canadian Rockies (Boyd et al. 2010). What regulates rates of methanogenesis in subglacial environments is a key area for future research (Tranter 2003).

Bacterial Diversity in Subglacial Environments

A diversity of microorganisms has been detected in subglacial materials using molecular, geochemical and culture techniques. Evidence for aerobic and anaerobic lifestyles and autotrophic and heterotrophic modes of carbon fixation have been found; perhaps photosynthesis is the only mode of metabolism not possible in the subglacial environment. Molecular studies indicate that the energy requirements of subglacial microbes can be met by all forms of redox chemistry and denitrification, sulfate reduction and methanogenesis have been demonstrated with enrichments or isolates (Skidmore et al. 2000; Foght et al. 2004; Cheng and Foght 2007). Still, the vast majority of Bacteria and Archaea are resistant to cultivation (Amann et al. 1995). Molecular studies based on 16S rRNA gene sequence similarity reveal assemblages from subglacial environments around the world with high similarity in composition, possibly indicating a biogeographical pattern to the distribution found in subglacial microbial communities. Below we speculate about several factors that may determine the composition of subglacial communities and how these factors may shape the net function of the assemblage.

Subglacial microbial assemblages appear to be distinct from those found in supraglacial waters, lateral moraines, and supraglacial snow (i.e., Bhatia et al. 2006; Skidmore et al. 2005). Important controls on subglacial microbial communities are structured by three key factors: (1) glacier hydrology (2) mineralogy or bedrock lithology and (3) preglacial ecosystem (Skidmore et al. 2005; Tranter et al. 2005; Mikucki and Priscu 2007). An additional factor that likely contributes to the composition of subglacial communities is the ability of invading organisms to survive transit from the glacial surface to the subglacial environment. In temperate glaciers, surface organisms and materials are readily transported to the subsurface via englacial conduits such as moulins; such direct transport does not occur in most ice caps and ice sheets, with the exception of the Greenland ice sheet (Das et al. 2008). However, microbes deposited on the surface of ice caps and ice sheets become entrained in the ice matrix for possibly hundreds of thousands of years. The physical processes of firnification and subsequent ice crystal formation over extended periods of time is a strong selective filter on microbial cells. Still, cells can survive this process (Christner et al. 2000, 2001) and may eventually be deposited in the subglacial setting. Thus microorganisms that can withstand freezing, constant low temperatures, low nutrients and organic carbon content should, *a priori*, be found in most glacier ice, since glacial ice is formed in a similar fashion globally. On the other hand,

components of the assemblage that are selected for by geochemical factors in the subglacial environment itself, such as mineralogy of the bedrock, redox state, or hydrological parameters, should vary from site to site.

Bedrock lithology thus plays a strong role in shaping subglacial microbial communities. Skidmore et al. (2005) compared two subglacial systems that were dominated by distinct weathering processes: Bench Glacier, Alaska a warm-based glacier that was similar to the majority of valley glaciers with carbonate dissolution and sulfide oxidation (Tranter 2003) and John Evans Glacier, Nunavut, Canada a polythermal glacier where subglacial geochemistry was controlled by gypsum and carbonate dissolution. In both systems sulfate was the dominant anionic solute but the source of sulfur was distinct. Otherwise, these two glacial systems had a similar geochemical profile. Molecular surveys revealed that both systems were dominated by members of the *Betaproteobacteria* that were related to the *Comomonadaceae*. The *Betaproteobacteria* are commonly isolated from soil and water samples and consist of several groups of aerobic or facultative Bacteria which are highly versatile in their metabolic capacities; of note, chemolithotrophic genera are present in this group. Foght et al. (2004) also found a predominance of *Comomonadaceae* isolates in subglacial samples from the Fox and Franz Josef Glaciers in New Zealand and Nemergut et al. (2007) also isolated the *Comomonadaceae* in samples of recently deglaciated glacial foreland soils; both aerobic environments. The *Epsilonproteobacteria* and relatives of *Gallionella ferruginea* were more prevalent in the Bench Glacier assemblage. Members of the *Epsilonproteobacteria* are often found in sulfide rich environments and most cultivated members are chemolithoautotrophic sulfide oxidizers (Takai et al. 2005). *Gallionella ferruginea* is a neutrophilic iron-oxidizing chemolithoautotrophic organism (Lütters-Czekalla 1990). Sequences related to the primarily heterotrophic *Bacteroidetes*, *Planctomycetales*, and *Verrucomicrobia* were found more frequently below the John Evans Glacier. Since the primary source of sulfur below the Bench Glacier is in the form of pyrite (FeS_2), the likely presence of relatives of iron- or sulfide oxidizing organisms and their absence beneath John Evans where sulfur exists as gypsum, supports the notion that mineralogical substrate is a key factor driving the selection of microbial species in the subglacial environment.

Iron and sulfur are common elements in bedrock materials and, in the subglacial environment bedrock minerals present a likely source of chemotrophic energy for microbial metabolism. In sediment samples collected below the Kamb Ice Stream in West Antarctica, Lanoil et al. (2009) found a less diverse assemblage containing only *Comomonadaceae*, relatives of *Gallionella ferruginea*, and relatives of *Thiobacillus denitrificans*, an obligately chemolithoautotrophic *Betaproteobacteria* known for coupling sulfide- or iron-oxidation to denitrification. These results were similar to the analyses of Bench and John Evans Glaciers; relatives of *T. denitrificans* were found at low levels in both studies. In subglacial samples from an outlet glacier in the McMurdo Dry Valleys, Antarctica, Mikucki and Priscu (2007) also found that the most abundant 16S rRNA gene sequences were related to organisms known to cycle iron and sulfur compounds. At this site, clones detected showed a high sequence similarity to chemolithoautotrophic sulfide-oxidizers and metal reducers (Mikucki and Priscu 2007). Although these organisms are phylogenetically unrelated to either *G. ferruginea* or the members of the *Epsilonproteobacteria* detected in other glaciers, they may be filling a similar niche to these organisms as they has the same central metabolism.

The link between potential energy metabolisms and the phylogenetic similarity among distinct subglacial microbial assemblages makes it tempting to develop a theoretical model of subglacial energetics. In these environments, there is little to no input from photosynthesis –

the subglacial environment itself is completely dark, and, in general, there are no surrounding plants to provide photosynthetic carbon from the surface, with the possible exception of cryoconite holes which contain cyanobacteria and algae and snow algae on temperate glaciers (Hodson et al. 2008). However, many glaciers have overrun carbon-rich soils or sediments during their expansion; recalcitrant organic matter from these soils or sediments may remain as a significant source of organic carbon. The abundance of sequences related to chemoorganotrophic organisms in the subglacial system is consistent with such a carbon source. However, autotrophic carbon fixation might also be an important process. Carbon dioxide fixation has been measured using labeled substrate incorporation experiments from subglacial samples (Gaidos et al. 2004; Mikucki and Priscu 2007) and gene sequences for obligate autotrophs have been identified in numerous subglacial environments. The prevalence of sequences related to sulfide- and/or iron-oxidizing organisms in subglacial environments seems to indicate that bedrock minerals provide key energetic substrates such as pyrite. The product of pyrite oxidation is sulfate, the predominant anion in most subglacial systems; furthermore, both chemoheterotrophic and chemolithoautotrophic processes produce acid equivalents that lead to carbonate dissolution. Subglacial chemosynthesis may fix enough carbon to support heterotrophic growth. This model of subglacial metabolism is consistent with other models (Tranter et al. 2005; Christner et al. 2006) and is similar that proposed by Mikucki et al. (2009) for Blood Falls (see details below); however, it has yet to be confirmed in other subglacial systems.

Blood Falls: A Unique Ecosystem or a Subglacial Paradigm?

The Taylor Glacier in the McMurdo Dry Valleys of Antarctica releases subglacial brine at Blood Falls. The source of the brine is believed to be a remnant sea trapped under the Taylor glacier approximately 4 km from the ~400 m thick snout (Hubbard, et al. 2004). Outflow at Blood Falls is circumneutral (pH 6.5), cold (~5–6°C) hypersaline (~2.5 times seawater salinity), anoxic (E_h = 90 mV) and ferrous (3.35 mM Fe(II)). When brine is released at Blood Falls, iron and salts rapidly precipitate staining the glacier surface a deep red (*Fig. 6.1.3a*). The brine that emanates at Blood Falls is host to a viable, metabolically active microbial assemblage (Mikucki et al. 2004; Mikucki and Priscu 2007). This marine system is thought to have been isolated from phototrophic production for at least ~1.5 million years however biogeochemical

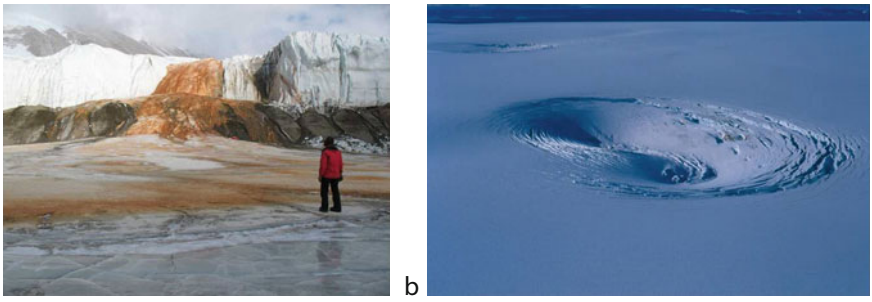

Fig. 6.1.3

Blood Falls (a) at the snout of Taylor Glacier, McMurdo Dry Valleys, Antarctica. Skaftar Cauldron system (b) forms beneath the Vatnajokull ice-cap in Iceland

measurements suggest that the subglacial community has remained active. Culture and molecular diversity of the Blood Falls brine showed the presence of both heterotrophic and autotrophic phylotypes, the majority of which share gene sequence similarity with marine organisms. The most dominant clones were related to microorganisms known to cycle iron and sulfur compounds including *Thiomicrospria* sp., a chemolithoautotrophic sulfur-oxidizer; *Geopsychrobacter* sp. an iron reducer and *Desulfocapsa* sp. a sulfur disproportionator. The detection of putatively chemoautotrophic phylotypes suggests that subglacial systems can be sustained independent of new carbon fluxes by in situ carbon dioxide fixation (Mikucki and Priscu 2007).

Like other subglacial environments it is thought that iron and sulfur are cycled to maintain growth below the Taylor Glacier. Dissolved organic carbon is low (450 μM) but bacterial heterotrophic activity has been measured based on radiolabeled thymidine incorporation demonstrating active consumption of organic matter (Mikucki et al. 2004 and Mikucki et al. 2009). Isotopic measurements of sulfur and oxygen in sulfate and water ($\delta^{34}S_{sulfate}$, $\delta^{18}O_{sulfate}$, $\delta^{18}O_{water}$) coupled with functional gene analyses of Adenosine 5′-Phosphosulfate (APS)-reductase, indicate that in the brine, sulfate is reduced, and appears to be acting as an electron shuttle to facilitate the reduction of iron oxides. The reduction of sulfate to sulfite is a biological process mediated by the APS reductase enzyme and typically proceeds sequentially to sulfide (H_2S). However, below the Taylor Glacier, it appears that sulfide is not produced, rather the reduction of sulfate stops at an intermediate oxidation state. Interestingly, to maintain the mass balance measured in the $\delta^{34}S_{sulfate}$, any intermediates produced must be quantitatively reoxidized to sulfate. This is because the $\delta^{34}S_{sulfate}$ in the brine is unchanged from that of seawater values. The Blood Falls subglacial system is net iron reducing but it is unclear whether this is a result of dissimilatory iron reduction via direct transfer of electrons from organic matter to the iron oxides from the bedrock, or if the resident microbes employ the electron shuttle system described above. Given the diversity of microorganisms detected in the brine, syntrophic relationships may support this catalytic cycle to maximize energy yields for slow, subglacial growth.

Antarctic Subglacial Lakes

Perhaps the most biologically intriguing subglacial systems are the Antarctic subglacial lakes. To date, more than 145 subglacial water bodies have been detected in Antarctica (Siegert 2005). At least some of these subglacial lakes are hydrologically connected to each other and periodically drain down a hydrological gradient (Fricker et al. 2007; Wingham et al. 2006). Such lakes are considered "active." The largest and deepest of these lakes is Lake Vostok (Kapitsa et al. 1996). Lake Vostok is covered by more than 3,800 m of ice and has been isolated from direct surface input for more than 15 million years. The ice directly over the lake, at the base of the ice sheet may be as much as 1.5 million years old. Lake Vostok is cold, dark, and ultraoligotrophic (due to the lack of photosynthetic input) for geologically and evolutionarily significant periods of time. Due to concerns about contamination of this pristine environment, the ice cover has not been fully penetrated, however, some of the nearly 200 m of ice accreted on to the bottom of the ice sheet, which represents refrozen lake water, has been sampled by coring (Jouzel et al. 1999). Several studies have provided insight into the geochemistry and microbiology of the lake itself (Priscu et al. 1999; Karl et al. 1999; Christner et al. 2001; Poglazove et al. 2001; Bulat et al. 2004; Christner et al. 2006). The extent of analysis on

these samples has been limited due to the small volume of core and extremely low numbers of cells contained within the cores (1×10^3 or less; Christner et al. 2006). Still the analyses to date have been impressive despite sample constraints and include broad scale activity measurements, cultivation of isolates, abundance measurements, and some community composition surveys using 16S rRNA gene sequencing. These reports have disparate findings and may indicate either highly variable conditions in accreted basal ice, contamination of samples, or methodological differences. To date, there is no consensus on the composition, abundance, or even existence of microorganisms in Lake Vostok. These subjects have been reviewed extensively elsewhere: for more information see (Siegert et al. 2001; Siegert et al. 2005; Christner et al. 2006; Priscu et al. 2008) and references therein.

Subglacial Caldera Lakes in Iceland

The Vatnajokull ice-cap in Iceland covers approximately 20% of the country's total land area and overlies several active volcanoes. This creates a situation where geothermal heat melts the bottom of the ice creating a volcanic caldera lake beneath the ice (*Fig. 6.1.3b*; Agustsdottir and Brantley 1994). These lakes are ice-dammed, and do not drain until the water pressure in the lake is high enough to lift the ice cap to allow drainage downstream. Occasionally, volcanic eruptions will break the ice dam and lead to catastrophic draining, called a jökulhlaup (Roberts 2005). Due to the influence of the geothermal fluids, there is a continuous flux of volatile and reduced compounds into these lakes providing potential sources of energy for microorganisms in the system (Agustsdottir and Brantley 1994). Two such subglacial volcanic caldera systems have been investigated (Gaidos et al. 2004; Gaidos et al. 2009) and both contained 16S rRNA gene sequences related primarily to mesophilic or psychrophilic organisms rather than thermophilic organisms suggesting that these organisms originate from the glacial environment. These initial studies reveal a low diversity assemblage; notably, no Archaeal sequences were found in either system. In the Skaftar Cauldron system, a high number of sequences related to the genus *Acetobacterium* were present and ~10% of the total cells were related to *Acetobacterium* based on fluorescent in situ hybridization or FISH probes; cultivated members of this genus are obligately anaerobic homoacetogens. It is unclear why only homoacetogens were detected in this system as estimates of thermodynamic favorability suggest methanogenesis should be present (Gaidos et al. 2009). It is possible that the caldera system is not reducing enough for methanogens as oxygen might be introduced with glacial melt, acetogens can also out-compete methanogens for H_2 substrate under certain conditions (Kotsyurbenko et al. 2001). *Sulfuricurvum* and *Sulfurospirillum* were the most abundant cells based on FISH counts (39% and 25% respectively); cultured representatives from these genera utilize sulfite, thiosulfate and elemental sulfur as terminal electron acceptors and H_2 and organic compounds as electron donors. Thus, it appears that two major chemoautotrophic pathways may serve as the base of the food chain in these systems: homoacetogenesis and sulfur oxidation. Several hundred other sequences were found in the Skaftar Cauldrons; however, in total they comprised no more than 10–20% of the total assemblage. *Acinetobacter* sp. was also present in the Grímsvötn caldera lake system; the methods used in this study did not allow for quantification of members in each group (Gaidos et al. 2004). Subglacial calderas are unusual and may not be representative of other subglacial systems on Earth; however, it has been argued that these systems may be good proxies for cryovolcanism-based meltwater on Mars and elsewhere in the solar system (Gaidos et al. 2004).

Permafrost

Permafrost is defined as frozen ground that remains at or below 0°C for at least two consecutive years (van Everdingen 1998). Much of the permafrost regions on Earth have been frozen for thousands of years while some areas have formed more recently (Steven et al. 2009). The majority of permafrost is located in high latitudes but can also be found at high altitudes in the lower latitudes. Permafrost exists in 24% of exposed land in the Northern Hemisphere (Zhang et al. 1999) and underlies 20–25% of Earth's land area, including about 99% of Greenland, 80% of Alaska, 50% of Russia, 40–50% of Canada and 20% of China. Antarctica is covered by two large sheets of ice, so there is only a relatively small ice-free region where permafrost soils occur on ~0.35% of the continent.

The active layer of the permafrost system is the dynamic surface zone that freezes and thaws annually and is typically 1–2 m thick. In colder regions, (i.e., higher latitudes and altitudes) where the average annual temperatures are cooler, the active layer thins and the permafrost layer thickens and can extend hundreds of meters deep. Siberia harbors the deepest known permafrost layer which is 1,450 m deep (Steven et al. 2009). In Antarctica the active layer can be as shallow as (<2 cm) and large masses of ice are often found immediately below the permafrost layer.

Permafrost is one of the largest carbon reservoirs on Earth and is estimated to contain ~190–900 gigatons carbon; the variation is due to the dynamic nature of soil types and depths (Post et al. 1982; Anisimov and Reneva 2006; Zimov et al. 2006). Research has shown that under warming conditions, organic matter in permafrost rapidly decomposes releasing carbon to the atmosphere as methane and carbon dioxide (i.e., Zimov et al. 2006). There are an estimated 7.5×10^9 tons of methane trapped in frozen peat bogs in West Siberia which constitute 25% of the estimated methane trapped in soil and ice-age permafrost worldwide. Methane has even been shown to bubble dramatically from thermokarst or thaw lakes that form in Siberia (i.e., Walter et al. 2006, 2007); the dynamics of permafrost carbon release may represent a significant unknown in global climate models. Although there is still much to learn about the mechanism of carbon release and the role of microorganisms in the decomposition of organic matter in permafrost, the positive feedback between permafrost and climate warming suggests that continued increase in global temperatures will result in rapid carbon loss to the atmosphere and overall decrease in permafrost extent (Eugster et al. 2000; Zimov et al. 2006).

Large masses of ice called ice wedges are common in permafrost sediments whereas smaller structures form in the freezing processes such as concentrated brines called cryopegs (Gilichinsky et al. 2003, 2005). Importantly, some liquid water remains in permafrost soils, even below freezing (Yershov 1998). Anderson (1967) calculated a thickness of ~50 nm of external unfrozen water which formed a shell around bacterial cells in the soil. Within the permafrost matrix, nano-scale amounts of liquid water within sediment and ice structures mix with air bubbles, organic matter, vegetative debris and clastic material (Black 1954) forming potential refugia for microbial life. Several studies have reported on the abundance and diversity of microorganisms in permafrost sediments. Total cell numbers vary from moderate, up to 10^6 cells gram dry weight^{-1} in Antarctica (Gilichinsky et al. 2007) to high with approximately 10^9 cells gram dry weight^{-1} in Arctic islands (Hansen et al. 2007). These microscopic investigations revealed dead or compromised cells in permafrost samples as well and such cells may contribute to previously published cell count numbers (Steven et al. 2009). Low temperatures and dry conditions slow the rate DNA degradation- therefore frozen environments can serve as reservoirs for ancient DNA molecules. Ancient DNA and viable

cells were isolated from frozen sediments dated to be eight million years old in Antarctica (Bidle et al. 2007) and DNA was extracted from an ice core sample that was dated at 450,000–800,000 years old in Greenland (Willerslev et al. 2007). However, not all DNA and cells obtained from frozen samples are in a state of cryopreservation.

Many microorganisms in frozen soil are likely in the dormant stage (such as spores or anabiotic cells) (i.e., Price and Sowers 2004; Bakermans and Nealson 2004). Studies show that some portion of cells are viable and may be metabolizing strictly for cellular maintenance and a growing number of reports suggest that a portion of the permafrost community is actively growing in situ. Gilichinsky and colleagues (1995), in a review of numerous microbiological reports of diverse permafrost samples from Siberia, the Canadian Arctic, Alaska and Antarctica, reported that microbial cells were readily cultivated at 22°C on nutrient rich agar plates; but concluded that it was often difficult to rule out possible contamination from drilling and handling in these early studies. It is estimated that only 0.1–10% of microorganisms in Arctic permafrost samples can be cultured by standard plating techniques and recovery is significant lower in Antarctic permafrost samples, ~0.001–0.01% (Vorobyova et al. 1997). Still, aerobic and anaerobic heterotrophs, methanogens, denitrifying bacteria and sulfate reducers have all been detected in various permafrost environments (Steven et al. 2009). *Crenarchaeota* and *Euyarcheaota* members of the Archaea and Bacterial representatives from the *Proteobacteria*, *Firmicutes* and *Actinobacteria* have been found using both culture-dependent and molecular approaches (Gilichinsky et al. 2007; Shi et al. 1997; Spirina et al. 2003; Vorobyova et al. 1997; Vishnivetskaya et al. 2000). In addition to viability measured through cultivation, in situ metabolic activity has also been measured (Rivkina et al. 2000). Winter tundra boreal soils emit various microbially generated gases including methane, carbon dioxide and nitrous oxide; rates of metabolic activity at subzero temperatures may slow considerably, but the evidence suggests it does not stop completely. Total winter carbon fluxes from permafrost may account for as much as ~2–20% of the annual methane emission and 60% of carbon dioxide efflux (Panikov 2009).

Subsea Permafrost and Hydrates

A particular variety of Arctic permafrost is the subsea permafrost, which underlies the shallow shelves of the Arctic coastal areas such as the Laptev Sea in north-east Siberia. This permafrost was formed by flooding of terrestrial permafrost with ocean water during the Holocene sea level rise (Rachold et al. 2007). Flooding of the cold terrestrial permafrost (−5 to −15°C) with relatively warm (−0.5 to −2°C) saline sea water changed the system profoundly and resulted in a warming of the frozen sediments (Overduin 2007), which might enable and enhance microbial carbon turnover. In permafrost environments it is thought that three main pathways of energy metabolism dominate: (1) the reduction of carbon dioxide to methane using hydrogen as an electron donor and (2) the disproportionation of acetate to methane and carbon dioxide and (3) methane oxidation. For example, microbial community and carbon dynamics were studied in a subsea permafrost core of the Siberian Laptev Sea Shelf (Koch et al. 2009). At the depth of maximum methane concentration, the Archaeal community structure was dominated by the methylotrophic genera *Methanosarcina* and *Methanococcoides* as well as two uncultured Crenarchaeota.

Gas hydrates are ice-like structures that crystallize under conditions of high pressure, low temperature, and high gas concentration (Kvenvolden 1988; Buffett 2000). They are composed

of hydrocarbon and non-hydrocarbon gases held in cages of water molecules. The majority of hydrate methane is biogenic, a result of microbial methanogenesis (Blunier 2000). Although the vast majority of gas hydrates are found in marine systems, permafrost-associated gas hydrates are also widespread throughout the Arctic and Antarctic (Kvenvolden 1988). Hydrate stability depends on maintaining pressure and temperature equilibrium, and drilling in sediments containing hydrates can compromise structural stability and pose explosion hazards (Kvenvolden 1999).

In marine sediment gas hydrate deposits, sulfate-reducing Bacteria and methanogenic Archaea associated with the anaerobic oxidation of methane are commonly found (Bidle et al. 1999; Cragg et al. 1996; Lanoil et al. 2001; Sassen et al. 1998). Methanogens are associated with the formation of gas hydrates (Waseda 1998) and within the hydrate-containing sediments (Mikucki et al. 2003), while sulfate reducing and anaerobic methane oxidizers are associated with their dissolution (Sassen et al. 1998; Joye et al. 2004; Elvert et al. 1999; Nauhaus et al. 2002). Microbial communities physically associate with intact gas hydrates (Lanoil et al. 2001) and aerobically and anaerobically consume hydrocarbons, including methane, from within the gas hydrate structure (Elvert et al. 1999; Sassen et al. 1998). Thus, it appears that gas hydrates could be an important source of organic carbon for psychrophiles inhabiting permafrost or coastal marine sediments.

It is estimated that gas contained in hydrates is equivalent to twice the carbon in all other coal, oil and gas deposits (Appenzeller 1991) making methane hydrate deposits an attractive an energy source. However, harvesting gas hydrates is a considerable challenge and presents a significant geohazard. Methane hydrates act to cement sediments together where they form in oceanic continental shelves and slopes. Drilling into a hydrate deposit hydrates involves dissociation of the clathrate cage to release the gas. Destabilizing the "cement" of oceanic sediments can cause large-scale slumping; releasing large volumes of greenhouse gas and presenting a potential explosion risk near the drilling operation. Although methane only contributes about a half of a percent of the carbon that carbon dioxide delivers to the atmosphere, each molecule of methane has the potential to contribute about 25 times as much greenhouse warming as each molecule of carbon dioxide (Schlesinger 1997). And since carbon dioxide absorption bands are now nearly opaque, or at the point of saturation and atmospheric methane will play an increasingly important role in global climate change in the future. It has been hypothesized that hydrate slumping events have been associated with past global-scale climatic change (Kennett et al. 2000) including transient greenhouse warming events at the Paleocene/Eocene transition (Katz et al. 1999; Norris and Röhl 1999; Bowen et al. 2004; Dickens et al. 1997) and in a Jurassic oceanic anoxic event (Hesselbo et al. 2000). This association between rapid climate change and methane release from gas hydrates has also been extended into the Quaternary period, in a model known as "the clathrate gun hypothesis" but is considered controversial (Kennett et al. 2000; Sloan 2003).

Conclusions

Ice-bound environments such as subglacial sediments, deep subglacial lakes and permafrost described here are relatively unexplored portions of the biosphere and are significant, yet relatively unknown variables in the global system. Polar ecosystems are more sensitive to warming and are likely to experience greater temperature increases as compared to the earth as a whole (IPCC 2007; Chapin et al. 2005). In global circulation models, polar regions respond

more strongly to global warming than temperate regions, primarily due to various positive feedback mechanisms such as decreased albedo as ice melts, increased greenhouse gas flux as permafrost melts, and increased heat capacity as sea-ice is converted into open water (Moritz et al. 2002). Predicting polar ecosystem response to change will also require an understanding of cold environment microbial systematics. Of particular importance is describing the diversity present and estimating rates of metabolic processes. Microbial metabolism is responsible for substantial weathering, particularly in subglacial environments and their metabolism contributes to the flux of greenhouse gases to the atmosphere. Understanding how the rates of these activities may change under future warming scenarios will improve our ability to model and manage global change.

Cross-References

- 6.3 Diversity of Psychrophilic Bacteria from Sea Ice- and Glacial Ice Communities
- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats

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6.2 Taxonomy of Psychrophiles

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Introduction

Psychrophiles are extremophiles that are capable of growth and reproduction at low temperatures. The environments they inhabit are ubiquitous on Earth, as a large fraction of our planet's surface is at temperatures lower than 15°C. They are present in the Arctic and Antarctica, glaciers, alpine regions, and deep sea. Psychrophiles utilize a wide variety of metabolic pathways, including photosynthesis, chemoautotrophy, and heterotrophy, and form robust, diverse communities. Psychrophiles are characterized by lipid cell membranes chemically resistant to hardening in response to the cold. In some cases, they create antifreeze protein to maintain their internal space in liquid form even at temperatures below the freezing point of water. Additionally, an understanding of their biology will lead to insights into astrobiology, the possibility of extraterrestrial life, and geomicrobiology, the study of microbes active in geochemical processes. Most psychrophiles are Bacteria or Archaea, and psychrophily is present in widely diverse microbial lineages within those broad groups. Recent research has discovered novel groups of psychrophilic cyanobacteria, fungi, and viruses (Margesin et al. 2008). However, this chapter discusses only bacteria.

Characteristics of Psychrophilic Bacteria

Definition of Psychrophilic Bacteria

The ability of bacteria to reproduce at 0°C was first reported by Forster 1887. In 1902, Schmidt-Nielsen used the term "psychrophilic" for the first time to define this bacterial type, but only the minimum growth temperature was considered in this early definition. Without adequate data in the literature on the existence of psychrophiles, the *Dictionary for Microbiology* (Jacobs et al. 1957) defined psychrophiles as those bacteria with an optimum growth temperature of 15°C or lower. Organisms that grew at 0°C were separated into two categories, obligate and facultative. The former were capable of growth at 0°C but not at 30°C, whereas the latter could grow at 0°C and 30°C. Many microbiologists at that time considered psychrophiles to be organisms that more or less fit the facultative category. However, with the isolation of true psychrophiles in the 1960s, cold-adapted bacteria were consequently subdivided into the stenothermic and eurythermic categories, and Morita (1975) ended the confusion with a new definition: "Psychrophiles are defined as organisms having an optimal temperature for growth at about 15°C or lower, a maximal temperature for growth at about 20°C, and a minimal temperature for growth at 0°C or below." This definition is still in common use, although the term "psychrotrophic" is today replaced with "psychrotolerant." Morita's definition has in principle been useful, but the temperature ranges should be reconsidered on the basis of increased numbers of cold-adapted bacteria isolated from various habitats and in the light of new ecological data (Helmke and Weyland 2004).

Lipid Composition

One of the characteristics believed critical for life at low temperatures is the maintenance of appropriate membrane fluidity or phase. Low temperature exerts profound physical effects on

biological membranes, typically leading to a reduction in membrane fluidity, the onset of a gel-phase transition and, ultimately, the loss of function. The lipid composition controls the physical properties of membranes. In general, lower growth temperatures produce a higher content of unsaturated, polyunsaturated, and methyl-branched fatty acids, and/or a shorter acyl-chain length (Russell 1997). This altered composition is thought to have a key role in increasing membrane fluidity by introducing steric constraints that change the packing order or reduce the number of interactions in the membrane (D'Amico et al. 2004).

Distribution of Psychrophilic Bacteria

The surface area of the Earth totals 5.1×10^8 km^2. The seas occupy just over 70%, and land areas just under 30%. The amount of land above sea level is very much less than the volume of sea. If the land were spread into the sea, the oceans would still be 3,000 m deep. The highest point on land is Mount Everest (8,848 m). The deepest point in the sea is in the Mariana Trench (11,034 m). The seas have an average depth of about 3,800 m, with average salinity of 3.5%. The warmest temperatures are near the equator and change gradually with latitude, so that polar water is relatively cold. The north-south thermal structures of the Atlantic and Pacific oceans are shown in the upper panel of *Fig. 6.2.1*. Temperature tends to be the highest at the surface, but deeper water is colder and maintains a constant temperature with depth. In some places, the surface temperature of the sea exceeds 25°C, although in most the temperature is less than 10°C at a depth of 500 m (*Fig. 6.2.1*, lower panel). In addition, in most locations the seawater temperature is lower than 4°C at depths greater than 1,000 m. In short, in most places the sea temperature is below 4°C. Most of the glacier ice on Earth is represented by the ice sheets of Antarctica and Greenland, corresponding to about 10% of Earth's terrestrial surface and contain ~75% of the fresh water on the planet (Paterson 1994). Together, polar plus temperate glaciers on other continents cover an area of 1.5×10^7 km^2. Despite the fact that >80% of the biosphere (by volume) has a permanent temperature lower than 4°C and most of the biomass is microbial (Priscu et al. 2008), very little is known about the biology of microorganisms inhabiting permanently cold environments. Therefore, the greater part of the earth is an environment appropriate for the growth of psychrophilic bacteria, and it will be necessary to conduct more research on them in the future.

Biodiversity

Numerous psychrophilic bacterial strains have been isolated and characterized in an effort to understand the interaction between the permanently cold environment and its microbial inhabitants. Thus far, psychrophilic bacterial isolates fall into diverse bacterial genera according to the phylogenetic classifications based on 16S ribosomal RNA sequence information. However, the only genus composed solely of psychrophilic bacteria is *Moritella*. Other bacterial genera include both mesophilic and psychrophilic species. The most commonly reported microorganisms in deep-sea or polar areas are the Gram-negative Alpha-, Beta-, Delta-, and Gammaproteobacteria (*Shewanella* sp. and *Moritella* sp.) and the *Cytophaga–Flavobacterium–Bacteriodes* phylum. *Firmicutes* and *Deinococcus* are frequently

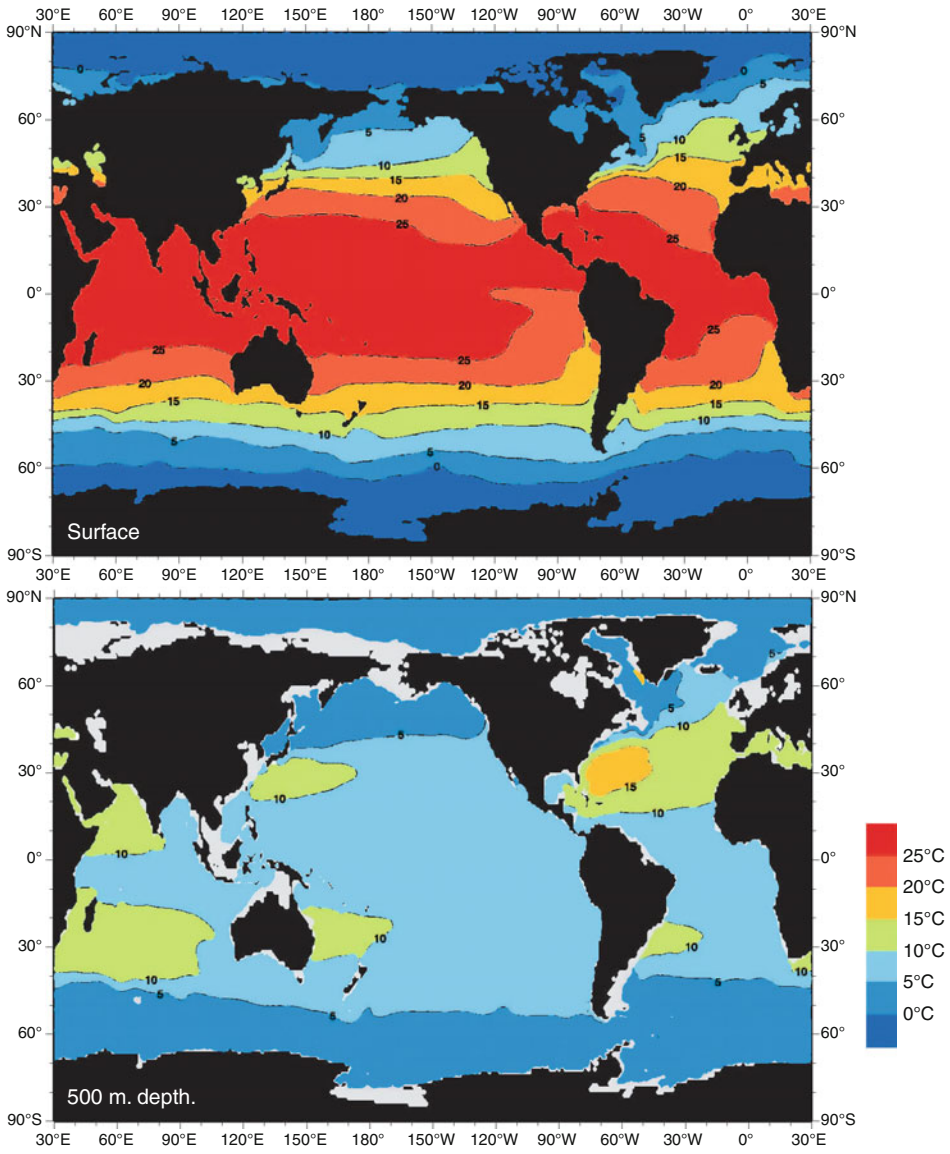

Fig. 6.2.1

Annual temperature observations at the surface and at 500-m depth. The map is based on data from the *World Ocean Atlas 2005* of the US National Oceanic and Atmospheric Administration

found in Antarctic and alpine environments. In permanently cold environments, the Bacteria generally dominate in number and diversity over the Archaea. In some areas such as the deep sea, the Archaea such as the Methanomicrobiaceae and Methanococcaceae families are found in equivalent numbers and are the most common organisms. The species of typical psychrophilic bacteria are described below (*Fig. 6.2.2*).

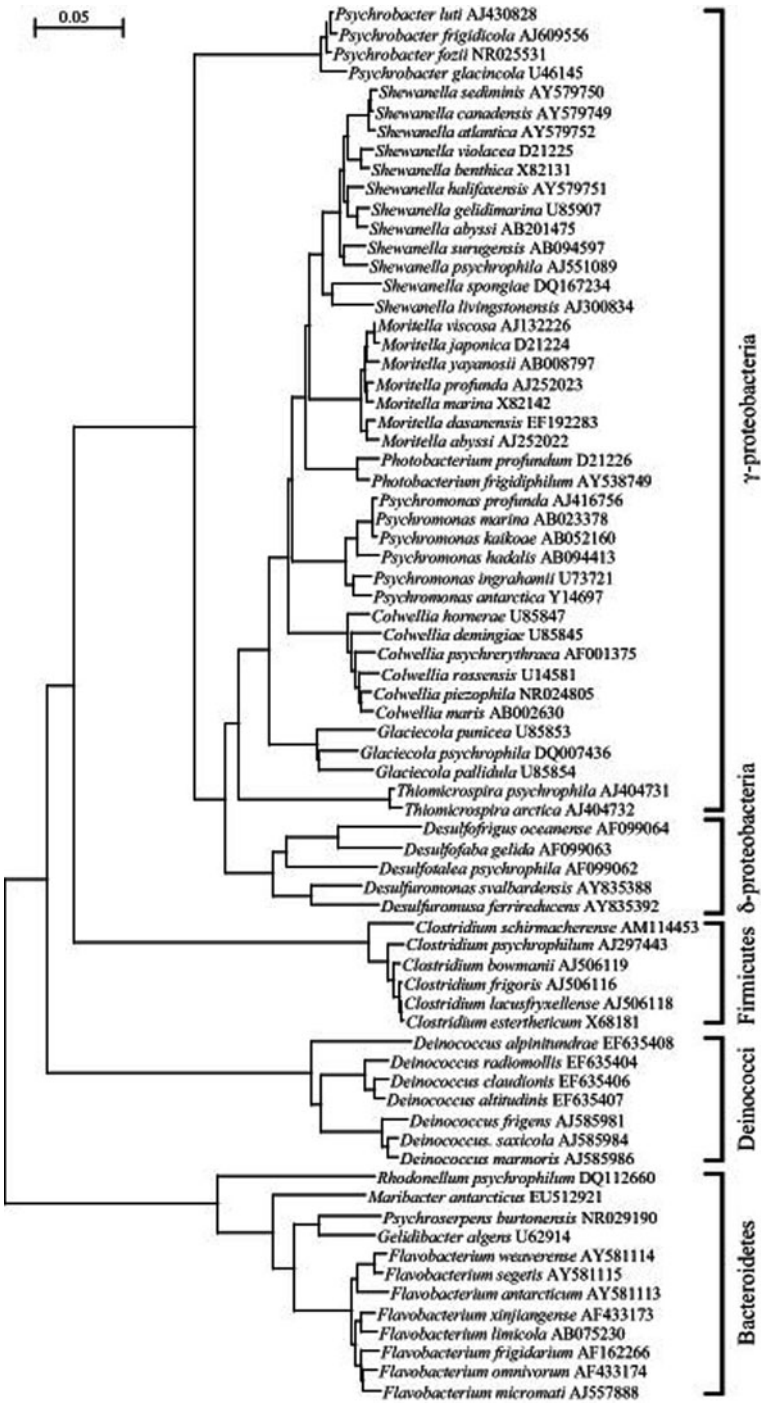

Fig. 6.2.2

Phylogenetic relationships of psychrophilic bacteria isolated from several environments. The tree was produced by comparing 16S rDNA sequences using the neighbor-joining method

Gammaproteobacteria

The Genus *Colwellia*

The family Colwelliaceae, which belongs to the Gammaproteobacteria (Ivanova et al. 2004), includes the genera *Colwellia* and *Thalassomonas*. The genus *Colwellia* was originally proposed by Deming et al. (1988) with the description of two facultatively anaerobic bacteria, *Colwellia psychrerythraea* and *C. hadaliensis*. Species of the genus *Colwellia* are characterized as Gram-negative and motile (except for *C. rossensis*), chemoorganotrophic, curved or straight rods, psychrophilic, halophilic, containing ubiquinone-8 (Q-8) as the predominant isoprenoid quinone, and with a DNA G+C content in the range of 35–42 mol% (Nogi et al. 2004; Ivanova et al. 2004; Zhang et al. 2008). Most species produce polyunsaturated fatty acids (PUFAs) such as 16:1 and docosahexaenoic acid (DHA; 22:6ω3) (Bowman et al. 1998a; Nogi et al. 2004). At the time of writing, the genus *Colwellia* comprised 10 species isolated from marine environments. Two psychropiezophilic species, *C. hadaliensis* (Deming et al. 1988) and *C. piezophila* (Nogi et al. 2004) were isolated from deep-sea sediment. Five psychrophilic species, *C. psychrerythraea*, *C. demingiae*, *C. hornerae*, *C. rossensis*, and *C. maris*, were isolated from fish eggs, the Antarctic, and Japanese coastal waters (Deming et al. 1988; Bowman et al. 1998a; Yumoto et al. 1998). Three nonpsychrophilic species, *C. psychrotropica*, *C. aestuarii*, and *C. polaris*, were isolated from the Antarctic, tidal flats, and Arctic sea ice, respectively (Bowman et al. 1998a; Jung et al. 2006; Zhang et al. 2008).

The Genus *Glaciecola*

The genus *Glaciecola* was originally described as Gram-negative, aerobic, psychrophilic, slightly halophilic bacteria and initially comprised two species, *Glaciecola punicea* and *G. pallidula* (Bowman et al. 1998b). Both species were isolated from sea-ice diatom assemblage samples collected from coastal areas of eastern Antarctica. Since then, further species of the genus *Glaciecola* have been isolated from marine environments: marine invertebrates, Arctic Ocean seawater, coastal surface seawater, deep-sea sediment, and East Sea sediment. They show diverse biochemical properties, such as agar digestion, nitrate reduction, and polysaccharide formation (Romanenko et al. 2003; Van Trappen et al. 2004a; Baik et al. 2006; Matsuyama et al. 2006; Zhang et al. 2006b; Chen et al. 2009). At the time of writing, the genus *Glaciecola* comprised nine recognized species. However, the range of growth temperature for *G. nitratireducens* (Baik et al. 2006) is 15–30°C (optimum temperature 25°C), and thus it is not a psychrophile. All other species grow at temperatures below 7°C. There are three species of true psychrophiles with growth at temperatures lower than 15°C: *G. punicea*, *G. pallidula*, and *G. psychrophila* (Zhang et al. 2006b).

The Genus *Moritella*

The genus *Moritella*, currently the only known member of the family Moritellaceae (Ivanova et al. 2004), belongs to the order Alteromonadales (Bowman and McMeekin 2005) within the class Gammaproteobacteria. Species of the genus *Moritella* are characterized as Gram-negative curved or straight rods, motile, chemoorganotrophic, psychrophilic, halophilic, and aerobic or

facultatively anaerobic. They do not form endospores or microcysts. Most species produce PUFAs such as C22:6 (DHA) and 16:1 (palmitoleic acid) (Urakawa et al. 1998; Nogi and Kato 1999; Xu et al. 2003a). At the time of writing, the genus *Moritella* consisted of seven species. All members of this genus are true psychrophilic bacteria isolated from marine environments. Nonpiezophilic *Moritella marina* and *M. dasanensis* were isolated from seawater of the North Pacific Ocean and near a glacier in Norway, respectively (Urakawa et al. 1998; Kim et al. 2008). *M. viscosa* was isolated from fish farms and is pathogenic for salmon species (Lunder et al. 2000; Benediktsdottir et al. 2000). Four psychropiezophilic species, *M. japonica*, *M. yayanosii*, *M. profunda*, and *M. abyssi*, were isolated from the Japan Trench, Mariana Trench, and the deep eastern tropical Atlantic Ocean (Nogi et al. 1998a; Nogi and Kato 1999; Xu et al. 2003a).

The Genus *Psychrobacter*

The family Moraxellaceae, which belongs to the Gammaproteobacteria (Rossau et al. 1991), includes the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter*. The genus *Psychrobacter* was first described by Juni and Heym (1986) as comprising psychrophilic or psychrotolerant and halotolerant, Gram-negative coccobacilli that are aerobic, nonmotile, nonpigmented, and oxidase positive. It was first described as a genus separate from *Acinetobacter* in 1986 (Juni 2005; Juni and Heym 1986). Members of the genus *Psychrobacter* are capable of growing at temperatures ranging from −10°C to 37°C and they vary from stenothermal psychrophiles, for example, *Psychrobacter frigidicola* (0–22°C), to eurythermal psychrophiles, for example, *P. fulvigenes* (−5°C to 37°C). At the time of writing, the genus *Psychrobacter* comprised 31 recognized species. Fourteen species of this genus were isolated from a variety of low-temperature marine environments including Antarctic sea ice, ornithogenic soil and sediments, the stomach contents of the Antarctic krill *Euphausia*, seawater (northwestern Pacific Ocean, 300 m in depth), the deep sea, and the internal tissues of a marine ascidian (Bowman et al. 1997a; Maruyama et al. 2000; Romanenko et al. 2002, 2009). However, the optimum growth temperature of most species is higher than 20°C, and only *P. glacincola* isolated from Antarctic sea ice has an optimum growth temperature lower than 15°C (Bowman et al. 1997a). Other sources of *Psychrobacter* include pigeon and seal feces, fish, poultry, dairy products, clinical sources, and an infected lamb (Yassin and Busse 2009; Juni and Heym 1986); fermented seafood (Yoon et al. 2003), Antarctic ornithogenic soil, ponds, and cyanobacterial mat samples (Bowman et al. 1996; Bozal et al. 2003); and Arctic permafrost (Bakermans et al. 2006). The majority of the other 17 species have optimum growth temperatures above 20°C, and only *P. fozii*, *P. frigidicola*, and *P. luti* (Bozal et al. 2003; Bowman et al. 1996) have optimum growth temperatures below 15°C. The genus *Psychrobacter* therefore includes only four species of true psychrophiles, although all species were isolated from low-temperature environments and most grow at 4°C.

The Genus *Psychromonas*

The genus *Psychromonas*, currently the only known member of the family Psychromonadaceae (Ivanova et al. 2004), belongs to the order Alteromonadales (Bowman and McMeekin 2005) within the class Gammaproteobacteria. Since the description of *Psychromonas antarctica*

(Mountfort et al. 1998), additional members of the genus *Psychromonas* have been isolated and described. Species of this genus are Gram-negative, curved or straight rod-shaped bacteria, motile or nonmotile, chemoorganotrophic, psychrophilic, aerobic or facultatively anaerobic, and halophilic but some species do not require Na^+ ions for growth. The DNA G+C content is 38–44 mol%, the major isoprenoid quinone is Q8, and the major fatty acids are 16:0 and 16:1ω7. Beyond these similarities, members of the genus *Psychromonas* described to date have a great deal of variety in their other physiological characteristics including cell size, temperature range for growth, piezophily, presence of gas vacuoles, and carbon source utilization (Mountfort et al. 1998; Bowman and McMeekin 2005; Nogi et al. 2002, 2007; Kawasaki et al. 2002; Xu et al. 2003b; Groudieva et al. 2003; Hosoya et al. 2008; Miyazaki et al. 2008; Auman et al. 2006, 2010). At the time of writing, the genus *Psychromonas* comprised 14 recognized species isolated from marine environments. Half of them are true psychrophiles. Three psychropiezophilic species, *P. kaikoae, P. hadalis* (Nogi et al. 2002, 2007), and *P. profunda* (Xu et al. 2003b) were isolated from deep-sea sediment. Four psychrophilic species, *P. antarctica* (Mountfort et al. 1998), *P. ingrahamii*, *P. boydii* (Auman et al. 2006, 2010), and *P. marina* (Kawasaki et al. 2002) were isolated from Antarctic, Arctic sea-ice, and Japanese coastal waters.

The Genus *Shewanella*

The family Shewanellaceae was established from the emended description of a group of marine *Alteromonas*-like bacteria because of their deep phylogenetic branching and lack of association with any other genus in the family Alteromonadaceae (Ivanova et al. 2004). At present, the family Shewanellaceae includes only one genus, *Shewanella* (MacDonell and Colwell 1985), which was created from the reclassification of two species previously assigned to the genus *Alteromonas*, [*Alteromonas*] *putrefaciens* and [*Alteromonas*] *hanedai*. *Shewanella* species comprise Gram-negative, motile, straight or curved rod-shaped, aerobic or facultatively anaerobic, oxidase-positive, nonfermentative bacteria with genomic DNA containing 38–54 mol% G+C (Bowman 2005). At the time of writing, there were 51 species of *Shewanella* with validly published names, mostly isolated from marine environments, with a few isolated from freshwater sediment, industrial wastewater, a clinical specimen, and spoiled food. In the past decade, the number of recognized species in this genus has increased; they have been studied extensively because of their capacity for dissimilatory reduction of manganese and iron oxides (Myers and Nealson 1988; Bowman et al. 1997b; Venkateswaran et al. 1998), reducing alternative electron acceptors such as trimethylamine *N*-oxide and nitrate (Bowman 2005), destructive souring of crude petroleum (Semple and Westlake 1987), and the production of tetrodotoxin (Simidu et al. 1990). Some species isolated from cold and deep-sea environments, such as *S. hanedai*, *S. gelidimarina*, *S. violacea*, and *S. benthica*, are known for the production of PUFAs (Kato and Nogi 2001; Russell and Nichols 1999; Bowman et al. 1997b). Species such as *S. oneidensis* and *S. putrefaciens* have been reported to be able to reduce heavy-metal oxides (Myers and Nealson 1988; Roh et al. 2006), cometabolize halogenated organic pollutants (Petrovskis et al. 1994), and have the potential to generate electricity (Ringeisen et al. 2006; Park and Zeikus 2002). The 12 psychrophilic *Shewanella* species isolated from the deep sea are *S. abyssi, S. surugensis* (Miyazaki et al. 2006), *S. sediminis*, *S. halifaxensis*, *S. atlantica*, *S. canadensis* (Zhao et al. 2005, 2006, 2007), *S. benthica* (MacDonell and Colwell 1985), *S. psychrophila* (Xiao et al. 2007), and *S. violacea* (Nogi et al. 1998b). Those isolated from

Antarctic sea ice or coastal waters and sponges are *S. gelidimarina* (Bowman et al. 1997b), *S. livingstonensis* (Bozal et al. 2002), and *S. spongiae* (Yang et al. 2006). They all require Na^+ and produce PUFAs such as 16:1, 18:1, and/or EPA (20:5ω3).

Deltaproteobacteria

Some psychrophilic species occur in the class Deltaproteobacteria. The order Desulfuromonadales, family Desulfuromonadaceae of the Deltaproteobacteria (Kuever et al. 2005), includes the genera *Desulfuromonas* and *Desulfuromusa* (Pfennig and Biebl 1976; Liesack and Finster 1994). These genera are Gram-negative, rod-shaped and curved cells, strictly anaerobic chemolithoheterotrophs or chemoorganotrophs with a respiratory or fermentative type of metabolism. Sulfur or ferric iron can be used as an electron acceptor and reduced to sulfide and ferrous iron. Two psychrophilic species, *Desulfuromonas svalbardensis* and *D. ferrireducens*, were isolated from Arctic sediments (Vandieken et al. 2006).

The order Desulfobacterales, families Desulfobacteraceae and Desulfobulbaceae, which belong to the Deltaproteobacteria (Kuever et al. 2005), includes the genera *Desulfofrigus*, *Desulfofaba*, and *Desulfotalea* (Knoblauch et al. 1999). These genera are Gram-negative, strictly anaerobic chemoorganotrophs, chemolithoheterotrophs, or chemolithoautotrophs with a respiratory metabolism. Sulfate is used as an electron acceptor and reduced to sulfide. Fermentative growth on pyruvate or other carbon substrates is possible. Simple organic compounds such as long-chain fatty acids and alcohols serve as electron donors and carbon sources. Three psychrophilic species, *Desulfofrigus oceanense*, *Desulfofaba gelida*, and *Desulfotalea psychrophila*, were isolated from Arctic marine sediments (Knoblauch et al. 1999).

Cytophaga–Flavobacterium–Bacteriodes Phylum

The Genus *Flavobacterium*

The genus *Flavobacterium* was proposed by Bergey et al. (1923) and belongs to the family Flavobacteriaceae in the phylum Bacteroidetes. With emendation of the description of the genus (Bernardet et al. 1996), several species previously belonging to the genus were reclassified and placed in new or different genera. Recently, Bernardet and Bowman (2006) have provided an excellent overview of the taxonomy and ecology of members of the genus *Flavobacterium*. It currently accommodates Gram-negative, non-spore-forming, yellow-pigmented, rod-shaped bacteria that are usually motile by gliding, contain menaquinone 6 (MK-6) as the sole respiratory quinone, and have a DNA G+C content in the range of 32–37 mol%. *Flavobacterium* species are physiologically diverse and found in a variety of environments such as marine and freshwater environments, soil, sediment, sea-ice biofilms, and diseased fish. Many species are psychrotolerant, psychrophilic, or mesophilic, and the genus includes species that are halotolerant, halophilic, or sensitive to salts, and others produce a variety of enzymes (Humphry et al. 2001; Tamaki et al. 2003; Zhang et al. 2006a). Most species of *Flavobacterium* are harmless, but some are opportunistic or true pathogens (Bernardet and Bowman 2006). In the past few years, many novel species have been added to the genus, which were isolated from freshwater sediments (Kim et al. 2009; Ali et al. 2009), glacier ice (Xin et al. 2009), soil (Liu et al. 2008), Antarctic habitats (Yi and Chun 2006), and wastewater (Yoon et al. 2009a). A number of

cold-adapted *Flavobacterium* species have also been isolated from glaciers, sea ice, and Antarctic lakes (Tamaki et al. 2003; Zhu et al. 2003; Van Trappen et al. 2004b; Yi et al. 2005; Nogi et al. 2005). The genus *Flavobacterium* is a diverse group of bacteria comprising more than 60 recognized species. There are eight species of true psychrophiles in this genus: *Flavobacterium frigidarium*, *F. limicola*, *F. omnivorum*, *F. xinjiangense*, *F. micromati*, *F. antarcticum*, *F. segetis*, and *F. weaverense* (Humphry et al. 2001; Tamaki et al. 2003; Zhu et al. 2003; Van Trappen et al. 2004b; Yi et al. 2005; Yi and Chun 2006).

The Genus *Psychroflexus*

The genus *Psychroflexus*, in the family Flavobacteriaceae, was first proposed by Bowman et al. (1998c) with the description of *Psychroflexus torquis* as well as the reclassification of *F. gondwanense* (Dobson et al. 1993) as *P. gondwanensis*. The genus was defined as Gram-negative. Cellular morphology ranges from rod-like cells to coiled filaments and filaments of indeterminate length, nonmotile or motile via gliding, catalase and oxidase positive, strictly aerobic, heterotrophic, slightly or moderately halophilic, psychrophilic or psychrotrophic, with the major respiratory quinone MK-6 (Bowman et al. 1998c). Members of the genus *Psychroflexus* have been isolated from a variety of salty or marine environments including Antarctic sea ice and lakes, salt lakes, and marine solar salterns (Bowman et al. 1998c; Donachie et al. 2004; Yoon et al. 2009b). At the time of writing, the genus *Psychrobacter* comprised five recognized species. However, most species grow at 4°C, and the optimum growth temperatures are higher than 20°C. Only *P. torquis*, isolated from Antarctic sea ice, is a true psychrophilic species.

Deinococci

The Genus *Deinococcus*

The genus *Deinococcus* was created in 1981 to accommodate radiation-resistant cocci exemplified by *Micrococcus radiodurans* (Brooks and Murray 1981). The 16S rRNA gene sequence analyses showed that the deinococci form a phylogenetically diverse group in a deeply branching lineage within the Bacteria. Although similarity may be considered sufficiently low to differentiate some species into other genera, the genus *Deinococcus* has not been divided because of the common characteristics of its members (Rainey et al. 1997, 2005). Since the first species, *Deinococcus radiodurans*, was reported, a further 39 species of this genus have been reported, isolated from a variety of environments such as laboratory contamination, radioactive sites, Antarctica, alpine treelines, deserts, hot springs, industrial waste, the plant rhizosphere, marine fish, and animal feces and tissues. Species of the genus *Deinococcus* are strictly aerobic, have high resistance to ionizing radiation, are Gram-positive, have L-ornithine in the peptidoglycan, and lack teichoic acids (Brooks and Murray 1981; Ferreira et al. 1997; Rainey et al. 1997, 2005; Hirsch et al. 2004; Callegan et al. 2008; Lai et al. 2006; Shashidhar and Bandekar 2009; Yang et al. 2009). The most studied characteristic of members of the genus *Deinococcus* is their ability to survive exposure to ionizing radiation and to repair their DNA following damage caused by radiation (gamma or UV) or desiccation. All species of the genus *Deinococcus* for which the characteristic has been tested have shown resistance to levels

of ionizing radiation to which they would never be exposed in the natural environment. The optimal growth temperatures of species of the genus *Deinococcus* fall across a broad range from 5°C to 50°C. The majority of species are reported to have optimal growth temperatures in the mesophilic range. Exceptions are the seven psychrophilic species *D. frigens*, *D. marmoris*, and *D.. saxicola* isolated from Antarctic soil or rocks (Hirsch et al. 2004), and *D. radiomollis*, *D. claudionis*, *D. altitudinis*, and *D. alpinitundrae* isolated from the treeline of alpine environments (Callegan et al. 2008).

Conclusions

The greater part of the earth is composed of permanently cold environments. Psychrophiles are numerically and functionally dominant in these environments because they have a wonderful ability to thrive in the cold. Species of psychrophiles are present in diverse bacterial genera according to phylogenetic classifications based on 16S ribosomal RNA sequence information. Those genera include both mesophiles and psychrophiles. At present, only the genus *Moritella* is composed of psychrophiles alone. Many new psychrophilic organisms that have unique abilities are expected to be isolated in the future and their diversity will expand.

Cross-References

- 5.1 Distribution of Piezophiles
- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
- 6.3 Diversity of Psychrophilic Bacteria from Sea Ice – and Glacial Ice Communities
- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats

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6.3 Diversity of Psychrophilic Bacteria from Sea Ice - and Glacial Ice Communities

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_6.3,

Introduction

Earth is primarily a cold, marine planet with 90% of the ocean's waters being at 5°C or lower. Frozen soils (permafrost), glaciers and ice sheets, polar sea ice, and snow cover make up 20% of the Earth's surface environments (Deming and Eicken 2007). A great diversity of microorganisms has been found in these habitats. However, only those that are adapted to life in the cold can be active in them and thus influence biogeochemical cycles.

Cold-adapted microbes are termed *psychrophiles* or cold-loving, having minimum, optimum and maximum growth temperatures at or below 0°C, 15°C, and 20°C, respectively or *psychrotolerant* (with growth maxima above 25°C but the capacity to grow to very low temperature (Morita 1975). Recently, additional definitions have been proposed, such as "moderate psychrophiles" with a minimum and maximum growth temperature at or below 0°C and 25°C (Helmke and Weyland 2004), "psychro-active" (organisms growing at or below −1°C, Laucks et al. 2005) and the terms "eurypsychrophile" and "stenopsychrophile" have been suggested (Caviocchiolli 2006 and references therein). Stenopsychrophile (formerly "true psychrophile") refers to a microorganism with a restricted growth-temperature range that cannot tolerate higher temperatures for growth. Eurypsychrophile (the formerly "psychrotolerant" or "psychrotroph") describes a microorganism that prefers permanently cold environments, but can also tolerate a wide range of temperatures reaching up into the mesophilic range (Caviociolli 2006). In this review we use the term *psychrophile* as a general term to describe a microorganism that grows in a cold environment. Specifically, we focus in this review on the diversity of psychrophiles found in the two major marine and terrestrial cold habitats for life on Earth – sea ice and glacier ice.

Marine psychrophiles play a globally significant role in biogeochemical cycling (Helmke and Weyland 2004). In the polar regions, they are important as processors of polar marine primary productivity (Legendre et al. 1992), which serves as the base for the entire polar food web, ultimately feeding krill, fish, whales, penguins, and seabirds. The rate of primary production varies greatly throughout Arctic and Antarctic marine waters and ice, and underlying environmental factors important to this process are investigated (Mock and Thomas 2005; Garneau et al. 2009; Kellogg and Deming 2009; Arrigo et al. 2010). Psychrophilic bacteria are of interest not only because they play important roles in organic carbon and elemental transformations throughout the polar food web (terrestrial and aquatic), but also because of their remarkable ability to thrive under extremely cold and salty conditions (Thomas and Dieckmann 2002). The enzymes and membranes that enable psychrophiles to live at low temperatures are of considerable interest for biotechnological and industrial applications (see Chap. 6.7 Psychrophilic Enzymes: Cool Responses to Chilly Problems).

Cold environments on Earth are extremely sensitive to global warming, especially those in the polar regions. As a result of increasing temperatures, the livelihood of larger organisms that perform critical ecological roles in the food chains of these cold environments (such as Polar Bears) is threatened. How microorganisms that also perform critical ecological roles might respond to these changes is an important and urgent question that requires attention (Kirchman et al. 2009). Furthermore, cold temperatures are prevalent in extraterrestrial environments, and especially in most outer planets and moons in our solar system. Therefore, from an astrobiological perspective, studying frigid environments on Earth can provide important clues and technology for understanding life that may exist elsewhere in our solar system, such as on Mars and Jupiter's moon, Europa.

In the following sections, we present what is known to date about the diversity of cultured and uncultured psychrophilic bacteria in sea ice and glacial ice and describe their habitats. We furthermore discuss briefly the adaptive strategies in *Psychrobacter ingrahamii*, a "true" psychrophile (i.e., stenopsychrophile) and discuss processes that could lead to extended longevity of microbes immured in glacial ice.

Sea Ice Communities

Ecological Aspects

One needs to appreciate the physics and chemistry of sea ice in order to understand the ecology and adaptation mechanisms of the bacteria that live within. Sea ice is one of the largest habitats in polar oceans and plays a crucial role in structuring the whole polar ecosystem (Eicken 1992). At its maximum extent, the ice covers 13% of the Earth surface with the largest expanse occurring in the Southern ocean where, during the winter months, 20 million square kilometers are covered by ice. In recent years, Arctic sea ice has shown a dramatic decrease in thickness and extent due to increasing overall temperatures in Arctic regions caused by global change (Stroeve et al. 2005; Serreze et al. 2007).

Seawater which typically contains about 34 g of dissolved salts and ions (mostly sodium, chloride, sulfate, magnesium, calcium and potassium) begins to freeze when temperatures drop below -1.86°C. Ice crystals begin to form and rise to the surface. These initial crystals (called frazil ice) are of varying shape, from plates to needles; and size, from $\leq$1.0 mm to 1.0 cm in length (Mock and Junge 2007 and references therein; also see Petrich and Eicken 2010 for an in-depth review on ice development, its micro and macrostructure).

Within hours, frazil ice crystals consolidate by wind and water motion to form loosely aggregated disks (called pancakes). After a few days of growth by accumulation of more and more ice crystals that form in the upper water column, pancakes can be several meters across and up to 50 cm thick. They freeze together forming a closed ice cover after 1–2 days (termed pack ice). As temperatures continue to decrease this pack ice thickens, not necessarily by the accumulation of more ice crystals, but by the growth of columnar ice at the ice–water interface. Columnar ice forms by the vertical elongation of frazil ice crystals. The proportion of frazil ice to columnar ice depends largely on the turbulence of the water in which it was formed. The more turbulent the water, the more frazil ice is usually found. Antarctic sea ice contains up to 80% frazil ice as it is formed under more turbulent conditions. In the Arctic, sea ice is formed under more calm conditions and contains up to 80% columnar ice. This difference is important for sea ice biology because frazil ice provides more habitable space for organisms than columnar ice (Spindler 1990).

When ice is formed from seawater, dissolved salts, air and other "impurities" in the seawater, including bacteria, inorganic and organic dissolved and particulate matter, are not incorporated into the freshwater ice crystals and instead are concentrated into a salty brine that persists as inclusions of pockets and channels within the ice or is released into the water below (Eicken 1992). These channels vary in size from a few micrometeres to several millimeters in diameter and represent the main habitat for all sea ice microorganisms (Fig. 6.3.1, Mock and Junge 2007).

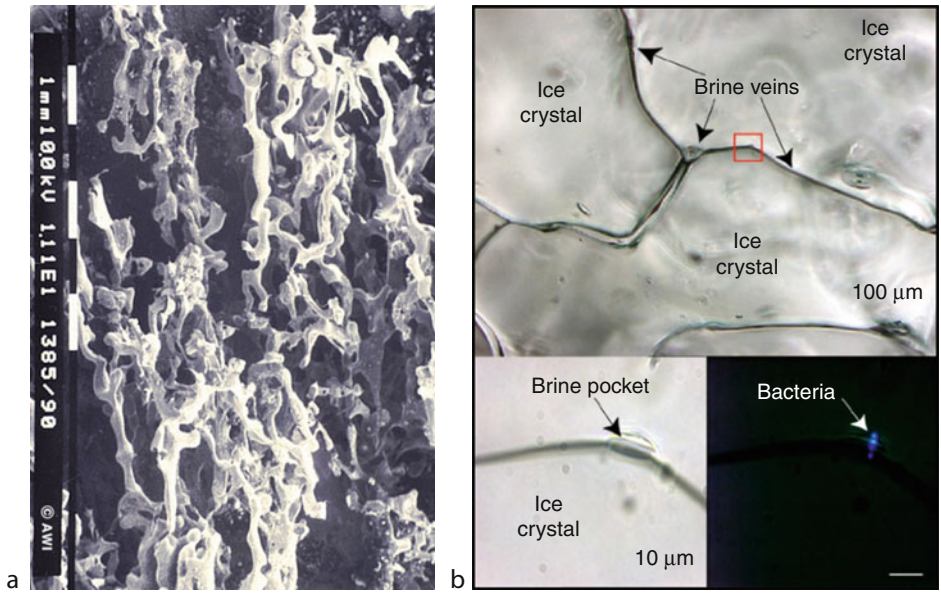

Fig. 6.3.1

(a) SEM picture of the brine channels system in columnar sea ice made visible by filling the system with epoxy resin under a vacuum. Picture by Alfred-Wegener Institute for Polar and Marine Research, Bremerhaven, Germany (Junge and Mock 2007, reprinted with permission from Springer Verlag). (b) In situ microscopic images of (a) ice crystals and brine pockets and (b) detail of a brine pocket in (a) that harbors bacteria stained with the blue DNA stain DAPI (Adapted from Junge et al. 2001)

Temperature determines the volume of brine channels and the concentration of salts within them; with decreasing temperatures, brine volumes decrease and salt content (salinity) increases. Thus, the coldest ice contains brine channels with the saltiest brines, and overall fewer, smaller and less interconnected channels than warmer ice. Ice at the sea ice–air interface is usually colder than ice in contact with the underlying water resulting in vertical temperature-(salinity and brine volume) gradients throughout the ice (*Fig. 6.3.2*). The ice is considered permeable at temperatures above −5°C (with corresponding bulk melt salinities of 5‰ [in situ salinities of 90‰] and brine volume fractions above 5%). Below −5°C sea ice is effectively impermeable with no convective fluid flow occurring (Golden et al. 1998).

The majority of sea ice is ephemeral, with melting during the summer season releasing the community into the underlying water. Melting forms ponds on the surface of the ice (mostly in the Arctic), which develop their own unique ice-algae assemblages and microbial communities (Brinkmeyer et al. 2004). When melting continues due to increasing water temperatures and solar irradiance on top of the ice, the ice gets thinner and more porous, and the majority melts completely. If ice survives the summer, refreezing occurs during the following winter that makes the ice even thicker. The longevity of the ice depends on the geographic location, wind and ocean currents. Sea ice of northern Greenland and the Canadian archipelago can be several years old with an average thickness of 6–8 m. The ice in the Southern Ocean is considerably

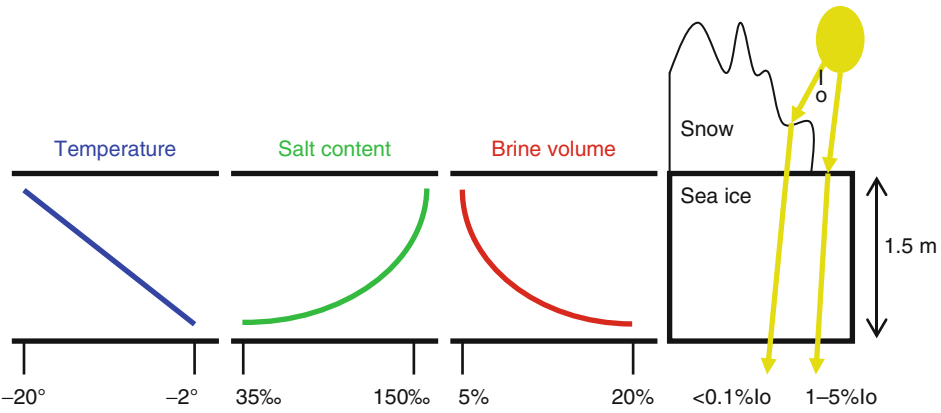

Fig. 6.3.2

Vertical gradients of temperature, salt content, brine volume and irradiance through sea ice. These general patterns may vary due to changes in temperature (Adapted from Mock and Junge 2007)

thinner with an average thickness of only 1 m. Such differences in physical properties of the ice also result in differences in the abundance, activity and composition of the microbial communities within (Mock and Junge 2007).

Psychrophilic bacteria that grow in sea ice must be adapted to cope with these ever-changing physical and chemical conditions of their environment after they are introduced into the ice as it forms. Along with salts, other dissolved impurities and particulate material, the cells are excluded from the newly formed ice crystals and reside in the brine channels (Junge et al. 2001). However, they may also become incorporated into the ice when attached to algae that adhere to ice crystals when they rise through the water as it freezes in autumn (Ackley and Sullivan 1994).

In the following sections we review the state of knowledge on the phylogenetic diversity of psychrophilic sea ice bacteria both from cultivation studies and whole community analyses. A discussion on the discovery of gas-vacuolate sea ice bacteria and the biogeography of sea ice bacteria follows. We end with a brief description of *Psychrobacter ingrahamii* as a representative of the stenopsychrophilic sea ice bacteria.

Phylogenetic Diversity of Psychrophilic Sea Ice Bacteria

Annually during spring and summer, extensive microbial communities (termed SIMCO for sea ice microbial community) develop within sea ice (for recent reviews see Mock and Thomas 2005, Mock and Junge 2007, Deming 2009). The SIMCO are usually dominated by ice-algae assemblages composed primarily of pennate diatoms (reviewed by Mock and Junge 2007). The populations within the ice are often so rich that the ice appears brown-green to the naked eye. Heterotrophic bacteria represent another major group within these communities (as are viruses; recently reviewed by Deming 2010), as evidenced by (1) measures of bacterial abundance, activity and production, (2) the microbial loop and (3) phylogenetically diverse

bacterial assemblages. Heterotrophic protozoa, amphipods, invertebrate larvae, copepods, euphausids such as krill, nematodes, turbellarians and some fishes are the larger protozoan and metazoan consumers found in sea ice (for reviews on these larger consumers see various chapters in Thomas and Dieckman 2010).

Over the last 2 decades, the phylogenetic diversity of sea ice bacteria has been studied extensively, mostly during spring and summer (e.g., Gosink and Staley 1993; Bowman et al. 1997; Brown and Bowman 2001; Junge et al. 2002, Brinkmayer et al. 2003) and more recently during winter as well (e.g., Brinkmayer et al. 2003; Junge et al. 2004; Collins et al. 2010). Efforts have mainly focused on polar Arctic and Antarctic sea ice, with some research performed in other sea ice-influenced water bodies such as the Baltic Sea (e.g., Petri and Imhoff 2001; Kaartokallio et al. 2008), Okhotsk Sea and Sea of Japan (Romanenko et al. 2008). Culturing efforts both in the Antarctic and Arctic have yielded new genera and species within the divisions of the Proteobacteria phylum including the Alphaproteobacteria, Betaproteobacteria and the Gammaproteobacteria classes, the Bacteriodetes phylum (formerly called

Table 6.3.1

Psychrophilic phyla, genera and species described from sea ice, with their temperature optima or range for growth (list amended from data compiled by Bowman J, personal communication and Deming 2010). Genera only represented by environmental sequences are in bold, genera only known from melt ponds and Baltic Sea ice are indicated by (mp) and (bs), respectively

Phyla and associated genera	Species	T_{opt} or T_{range} for growth (°C)	Reference(s)
Bacteria			
γ-proteobacteria:			
Acinetobacter	*Colwellia psychrerythraea*	9–16	D'Aoust and Kushner (1972), Bowman et al. (1998a), Huston et al. (2000)
Alteromonas	*Colwellia demingiae*	10–15	Bowman et al. (1998a)
Citrobacter	*Colwellia hornerae*	10	Bowman et al. (1998)
Colwellia	*Colwellia rossensis*	10	Bowman et al. (1998a)
Glacieocola	*Glaciecola pallidula*	13–14	Bowman et al. (1998b)
Halomonas	*Glaciecola punicea*	15–18	Bowman et al. (1998b)
Iceobacter	*Psychrobacter glacincola*	15–21	Bowman et al. (1998d)
Marinobacter	*Psychromonas boydii*	0–10	Auman et al. (2009)
Marinomonas	*Psychromonas ingrahamii*	−12 to 10	Auman et al. (2006)
Neptunomonas	*Shewanella gelidimarina*	16	Bowman et al. (1997b)
Oceanospirillum	*Shewanella frigidimarina*	20	Bowman et al. (1997b)
Pseudoalteromonas			
Pseudomonas			
Psychrobacter			
Shewanella			

Table 6.3.1 (Continued)

Phyla and associated genera	Species	T_{opt} or T_{range} for growth (°C)	Reference(s)
Terridinibacter			
Vibrio			
β-proteobacteria:			
Aquaspirillum (mp)	*Polaromonas vacuolatus*	10	Irgens et al. (1996)
Matsuebacter (mp)			
Rhodoferaz (mp)			
Ultramicrobacterium (mp)			
Comamonadaceae (bs)			
Hydrogenophaga (bs)			
α-proteobacteria:			
Octadecabacter	*Octadecabacter arcticus*	10	Gosink et al. (1997)
Roseobacter	*Octadecabacter antarcticus*	8	Gosink et al. (1997)
Ruegeria			
Sphingomonas			
Sulfitobacter			
Devosia (mp)			
Rhodobacter (mp)			
Loktanella (bs)			
Bacteroidetes (former CFB):			
Cellulophaga	*Flavobacterium gillisiae*	20	McCammon and Bowman (2000)
Cytophaga	*Gelidibacter algens*	15–18	Bowman et al. (1997c)
Cyclobacterium (mp)	*Polaribacter irgensii*	8–10	Gosink et al. (1998)
Flavobacterium	*Polaribacter franzmannii*	7	Gosink et al. (1998)
Flexibacteraceae (bs)	*Polaribacter filamentus*	10–12	Gosink et al. (1998)
Gelidibacter	*Psychroflexus torquis*	12	Bowman et al. (1998c)
Hymenobacter (mp)			
Lewinella			
Salegentibacter			
Polaribacter			
Psychroflexus			
Psychroserpens			
Green non-sulfur bacteria			
Planctomycetes			
Planctomycetales (mp)			
Verrucomicrobia			
Verrucomicrobium			
Prosthecobacter			

Table 6.3.1 (Continued)

Phyla and associated genera	Species	T_{opt} or T_{range} for growth (°C)	Reference(s)
Purple sulfur bacteria (bs)			
Firmicutes:			
Halobacillus	*Planomicrobium mcmeekinii*	0–37	Junge et al. (1998), Yoon et al. (2001)
Planomicrobium			
Actinobacteria:			
Corynebacterium	*Arthrobacter*	0–30	Junge et al. (1998)
Clavibacter	*Brachybacterium*	0–30	Junge et al. (1998)
ARCHAEA (only from winter ice)			Collins et al. (2010)
Crenarchaeota			
Marine Group I			
Euryarchaeota			
Group IIb			

the Cytophaga-Flexibacter-Bacteroides or CFB phylum) and the Actinobacteria phylum (see *Table 6.3.1* for a list of psychrophilic sea ice bacterial isolates described to date and associated references). Many of the described Arctic and Antarctic sea ice bacteria are stenopsychrophiles with growth optima or maxima below 15°C. Most bacteria isolated from sea ice have been observed to be pigmented, highly cold-adapted with some able to form gas vesicles (see below and Sullivan and Palmisano 1994; Grossi et al. 1984; Staley et al. 1989; Gosink and Staley 1995).

Sea ice bacteria have been found to be unusually easy to cultivate - with up to 60% of the total bacterial population being culturable (Junge et al. 2002; Brinkmayer et al. 2003). This stands in marked contrast to the culturability of most seawater bacteria (~0.01% of the total cell count, Amann et al. 1995) and might be due to the exceptionally high concentrations of labile dissolved organic matter (DOM) released by ice algae reported for sea ice brines in the Arctic, Antarctic and Baltic Sea (to exceed surface water concentration by factors of up to 500; Thomas and Mock 2005 and references therein). This likely explains why sea ice bacteria are so readily cultivated both in low and high nutrient media (Junge et al. 2002) and why groups commonly associated with marine algae are repeatedly found in sea ice bacterial culture collections.

Small subunit rRNA gene sequence analyses of whole communities have demonstrated an unexpectedly strong congruence with cultivation data. Mostly, the same phyla and genera were found to be present (Brown and Bowman 2001; Brinkmayer et al. 2003), except for a few clones grouping among the Verrucomicrobia, closest to the prosthecate aerobic genera *Verrucomicrobium* and *Prosthecobacter* (Brown and Bowman 2001). No evidence of uncultured bacterial clone groups such as SAR11, SAR86 or archaeal groups that commonly occur in oceanic non-polar and polar seawater samples had been found in the Arctic and Antarctic spring/summer ice or melt ponds studied (Brinkmeyer et al. 2004).

Molecular and cultivation studies on Arctic summer time ice-melt ponds revealed another group that had not been found previously in the interior of the ice. β-Proteobacterial genera known only from freshwater habitats were found to dominate in the mostly freshwater ponds (along with gram-positives species, α- and γ-Proteobacterial genera occurring in more saline ponds and Bacteriodetes members in sediment containing ponds; Brinkmeyer et al. 2004).

Anaerobic phototrophic purple sulpfur bacteria have also been found in the interior of Baltic sea ice (Petri and Imhoff 2001), indicating that oxygen deficient and anoxic zones are present in sea ice. High amounts of mucopolysaccharide gels and exopolymeric substances (EPS) are suggested to provide oxygen-depleted microhabitats for these species within the sea ice habitat (Mock and Thomas 2005). Other Baltic sea ice studies showed denitrification to occur in zones of ice with accumulated nitrite (Kaartokallio 2001). In Arctic sea ice, anaerobic bacterial denitrification with high numbers of anaerobic nitrate reducing bacteria and ammonium oxidation was found in zones with high levels of nitrate, ammonium ions and DOM (Rysgaard and Glud 2004; Rysgaard et al. 2008), which are characteristics of many sea ice habitats (reviewed by Thomas and Dieckmann 2002).

Archaea were found in low numbers (up to 4% of the population) in Arctic wintertime sea - ice samples (Junge et al. 2004) but not in any of the Spring- and Summer time Arctic and Antarctic ice samples cited above. More recently, the presence of Archaea in winter time sea ice was confirmed during a seasonal study of community composition over the course of the Arctic winter (Collins et al. 2010). Surprisingly it was also found that communities of Bacteria and Archaea in the ice resembled that in the underlying water. The microbial community consisted primarily of SAR-11 clade alpha Proteobacteria and Marine Group 1 Crenarcheaota, neither of which is known from Spring and Summer time sea ice. The wintertime ice bacterial clone library contained γ-proteobacteria from oligotrophic seawater clades (e.g., OM60, OM182) and no clones from γ-proteobacteria genera commonly detected in spring and summertime ice (e.g., *Colwellia, Psychrobacter*) were observed. It was concluded that selection during ice formation and mortality during winter played minor roles in the process of microbial succession that leads to distinctive spring and summer sea ice bacterial communities (Collins et al. 2010). Seasonal studies that explore the winter/spring/summer transitions are needed to explore further how distinctive summer and spring time communities develop but factors such as extensive algal growth resulting in increased highly labile DOM levels are likely determining factors.

Discovery of Gas Vacuolate Sea Ice Bacteria

Gas vesicles, which have been hypothesized to be an early organelle of prokaryotic motility (Staley 1980), are intracellular structures found in many aquatic Bacteria and Archaea (Walsby 1994). They appear as bright areas in cells that contain them when they are observed by light microscopy. To distinguish them from other bright, intracellular areas in cells, they need to be visualized by transmission electron microscopy. Each gas vacuole observed by TEM contains many subunits that are termed gas vesicles. The membranes of the gas vesicles consist of a hydrophobic protein with a molecular weight of about 7,500, which is the subunit of the gas vesicle. The subunits are arrayed in a manner that produces a central cylindrical structure capped at each end by conical tips.

The function of the gas vesicles is that they to provide buoyancy to the organism. This is accomplished by excluding water into the interior of the vesicles because of the hydrophobic

vesicle membrane. However, gases are freely permeable through the vesicle membranes so that gases in the ambient environment accumulate in the interior of the vesicle. As a result, the vesicles decrease the density of the cells thereby providing buoyancy.

Gas vacuolated bacteria are very common in freshwater habitats, especially those that have a thermally stratified water column. Typically in these habitats the gas vacuolated cyanobacteria that produce blooms in lakes are found in the surface waters where they receive abundant light for photosynthesis. In the deeper hypolimnion of stratified lakes, blooms of anoxygenic photosynthetic bacteria, many of which contain gas vacuoles, occur. Gas vacuolated bacteria and archaea are also found in the hypolimnion and even in freshwater sediments.

In 1989, marine gas vacuolate bacteria were reported from euphotic waters off the Palmer Peninsula (Irgens et al. 1989). Based on this discovery, it was hypothesized that gas vacuolate bacteria, which are typically found in stratified aquatic habitats might be indigenous to the SIMCO because it is a dramatic example of a stratified marine habitat in which underlying cold water is capped by the SIMCO ice layer.

To test this hypothesis, more intensive studies were performed at McMurdo Sound, where the sea ice community is more amenable to study. Sure enough, gas vacuolate bacteria were encountered, in some instances at very high concentrations relative to total cultivable bacteria, in the SIMCO layer, underlying frazil ice layer and water column (Staley et al 1989). It is still not known whether the gas vesicles are expressed when they are in the SIMCO layer but it is thought that they are likely expressed after the summer thaw when the sea ice microbial community is dispersed in the water column. The evidence for this is that the gas vacuolated strains were only isolated from samples taken from the surface waters and to depths down to 50 m whereas none were isolated from lower depths, i.e., 100, 200, and 500 m.

The taxonomy of the gas vacuolated bacteria followed their isolation. The first described gas vacuolated bacterium from Antarctica, *Polaromonas vacuolata*, is a member of the Proteobacteria (Irgens et al. 1996). Although *Polaromonas vacuolata*, is likely a member of the SIMCO, this bacterium was isolated from open waters during the summer months so its association with the SIMCO is uncertain. However, many gas vacuolated bacteria have been isolated from the SIMCO layer and identified.

The gas vacuolate bacteria isolated and described taxonomically from sea ice fall into two major phylogenetic groups, the phyla, Proteobacteria and Bacteroidetes (Staley et al 1989; Gosink and Staley 1995). Examples of gas-vacuolate sea ice bacteria that have been named belong to Proteobacterial genera, *Octadecabacter* (Gosink et al. 1997), *Psychromonas* (Auman et al. 2006; Auman et al. 2009) and the Bacteroidetes genus *Polaribacter* (Gosink et al. 1998).

Biogeography of Polar Sea Ice Bacteria

One of the reasons for studying the bi-polar distribution of sea ice bacteria, is to evaluate whether the same species are found at each pole. Baas-Becking's hypothesis (1934) that "everything is everywhere, the environment selects" indicates that polar sea ice bacteria should be cosmopolitan. Therefore, the two polar environments should contain the same species of bacteria. Staley has hypothesized that this is improbable and therefore psychrophilic bacteria from each pole should be endemic, separate species (Staley, ASM News; Staley and Gosink 1999). He reasoned that the great distances between the two poles and the difficulty of dispersing stenopsychrophiles, which quickly die at room temperature, across tropical areas through various means, such as animal vectors like the arctic tern or long transport via deep

ocean circulation under deep sea pressures, might make it improbable that the same species would be found at each pole.

To test this hypothesis, a number of strains of unique psychrophilic gas vacuolated bacteria were isolated from each pole and compared. The closest matches that were found were strains were from the genera *Octadecobacter* and *Polaribacter*. This indicates, that at the level of the genus, these bacteria are cosmopolitan in their distribution.

The 16S rRNA gene sequences of the two most closely related strains of *Octadecobacter* isolated from the two poles, were $<1\%$. Therefore, in order to determine whether they were separate species, DNA-DNA hybridization was performed. The reassociation value at 42% was well below the $>70\%$ required for them to be the same species. Therefore the new species were named *O. arcticus* and *O. antarcticus* to reflect the two different poles from which they were isolated (Gosink et al. 1997).

Likewise, four *Polaribacter* strains isolated from the two separate poles were also compared. The two most closely related strains based on DNA-DNA hybridization showed 34% relatedness, again significantly lower than the 70% required for them to be the same species (Gosink et al. 1998). Each strain was therefore named as a separate species of the genus.

Junge et al. (2002) reported on an Arctic isolate (*Shewanella frigidimarina*) which showed 100% sequence similarity to a *Shewanella frigidimarina* isolate from Antarctica, suggesting bipolar distribution for bacteria even at the species level and thus possibly reversing the earlier suggestion of endemism by Staley and Gosink (1999). However, DNA-DNA hybridization tests are needed to confirm this suggestion. Furthermore, despite the implication of its species name, *frigidimarina*, the type species of *Shewanella frigidimarina* is not a stenopsychrophile because its maximum temperature for growth is 30°C. In contrast the gas vacuolated sea ice bacteria tested for bipolar endemism were true stenopsychrophiles that do not grow above 10–15°C and cannot survive at room temperature and are therefore highly unlikely to survive transport across the equator.

Thus to date no definitive evidence of cosmopolitan bi-polar strains of stenopsychrophilic bacteria from the SIMCO habitat has yet been established. Multiple-locus sequence analysis (MLSA) is an alternative approach to resolving this question (Whitaker et al. 2002). This approach would require the isolation and comparison of several closely related strains of a presumed "species" from each pole for a more complete comparative analysis.

Are Sea Ice Bacteria "Good" Psychrophiles?

Not all organisms that grow in polar environments are steno-psychrophiles. For example, typical bacteria isolated from Antarctic soils may grow in the laboratory at temperatures above 25°C. This may be due to the large annual fluctuation in temperatures in soils. In contrast, polar marine environments are consistently cold. Temperatures in many areas do not exceed 2°C during the year and temperatures in the sea ice are colder. Therefore, in general, the sea ice environment has been an excellent source of psychrophilic bacteria. The question arises, are there features that are typically found in organisms that make them steno-psychrophiles?

Several attributes of bacteria make them good candidates for psychrophily. For example low growth temperatures provide a basis for predicting a psychrophilic life style. It should be noted that low temperature for growth as contrasted with low temperature for metabolism provides a better indicator of psychrophily. As with thermophiles, the maximum temperature for growth is the hallmark of thermophily. Therefore, using the same rationale, the lowest

temperature for growth should be the maxim for psychrophily. Furthermore, it is very difficult to accurately identify and assess in situ microbial activities in frozen habitats (for advances in this area see Junge et al. 2004 and 2006).

Low maximum growth temperature. One feature is that stenopsychrophiles have a low maximum temperature for growth. Most of the sea ice bacteria do not grow at room temperature and many do not grow at temperatures above 10°C. This feature is an important predictor for psychrophily, because most bacteria have a limited temperature range for growth. *E. coli* for example, grows at a low of 8°C and at a high of 44°C. Therefore its temperature range is 36°C. Likewise, extreme thermophiles, such as species in the genus *Pyrococcus*, which grow from about 80–110°C, have a growth temperature range of about 30°C.

Therefore, if we extrapolate this information to psychrophiles, if they have a maximum growth temperature of 10°C, they could actually grow at temperatures as low as −20°C or perhaps lower.

Compatible solute production. Another feature of a typical steno psychrophile is its ability to produce an intracellular solute to counterbalance the osmotic effect of higher salinities in brine pockets where organisms reside, that are encountered as sea ice freezes.

Habitat. The lowest temperatures encountered in sea ice occur in the winter months at the air–ice interface. Therefore, it would seem logical that organisms that are located in the upper layers of the sea ice are good candidates for psychrophily.

Low mol% G+C content. From a purely theoretical standpoint, DNA replication may be aided at low temperatures when there are fewer hydrogen bonds. DNA with a low G+C content would have more double-bonded AT nucleotides and fewer GC nucleotides thereby aiding in replication. Consistent with this argument is that several psychrophilic bacteria have mol% G+C contents below 50%. It should be noted, however, that some thermophilic bacteria also have mol% G+C values below 50%, e.g., in *Sulfolobus* species it ranges from about 35–42 mol%, so this pattern may not apply to thermophiles.

A good example for a sea ice bacterial species exhibiting all of the features listed above that are consistent with psychrophilic growth is *P. ingrahamii*. Its maximum growth temperature is 10°C. Therefore, its minimum growth temperature, in theory could be at least −20°C, if we accept the modest estimate of a 30°C temperature range for its growth. The lowest growth temperature recorded for this organism is −12°C (Breezee et al. 2008). However, the authors noted that it is likely that the organism can grow at even lower temperatures, but the conditions they used for lower temperatures resulted in freezing the cultures which resulted in cessation of growth.

P. ingrahamii was isolated from the upper half of a sea ice column in Elson lagoon, near Pt. Barrow, Alaska. This lagoon has a slightly higher salinity than typical seawater thereby also providing conditions to lower the freezing point of lagoon's water.

The mol% G+C of *P. ingrahamii* is 40.1, lower than that of many other members of the Gammaproteobacteria. Finally, genomic analyses of this organism indicate that it produces a compatible solute, betaine choline aiding its growth under high salinity conditions encountered in brine pockets in the ice (Riley et al. 2008).

Genomes of Psychrophilic Bacteria

Several genomes of psychrophilic bacteria have been published. They include *Colwellia psychroerythraea* 34H (Methé et al. 2005), *Idiomarina loihiensis* L2TR, *Pseudoalteromomas*

haloplanktis TAX 125 in addition to *Psychromonas ingrahamii* (Riley et al. 2008). For further information on this subject we refer the reader to an excellent review of the genomes of psychrophilic bacteria written by Bowman (2008).

Glacial Ice Microbiology

Ecological Aspects

The ice sheets of Greenland and Antarctica represent most of the glacial ice on Earth, which cover about 10% of Earths terrestrial surface and contain 77% of the freshwater on the planet (Miteva 2008). Temperate, high altitude non-polar glaciers are found all over the world (e.g., China has more than 46,000 glaciers) and represent an important freshwater source for millions of people. Glaciers can range from a few hundred meters to 4 km in depth, with temperatures increasing with depth (e.g., the glacier overlying Lake Vostok in Antarctica exhibits −60°C to −2°C from surface to base).

The physicochemical characteristics and structure of the polycrystalline glacier ice as a microbial habitat have recently received more attention where it is now understood that cells reside either in liquid filled veins (cells need to be less than 2 μm; *Fig. 6.3.3*; Mader et al. 2006) containing variable substrates suitable to support bacterial metabolism and survival or in the thin liquid films on the surface of mineral grains (see below; Miteva 2008). Furthermore, clear glacier ice and basal ice show significant differences (with basal ice containing much higher concentrations of inorganic and organic ions and minerals) resulting in significant differences in the microbial populations within (with much higher abundances in the latter and a possible function of basal ice microbes in geochemical cycling reactions within the ice,

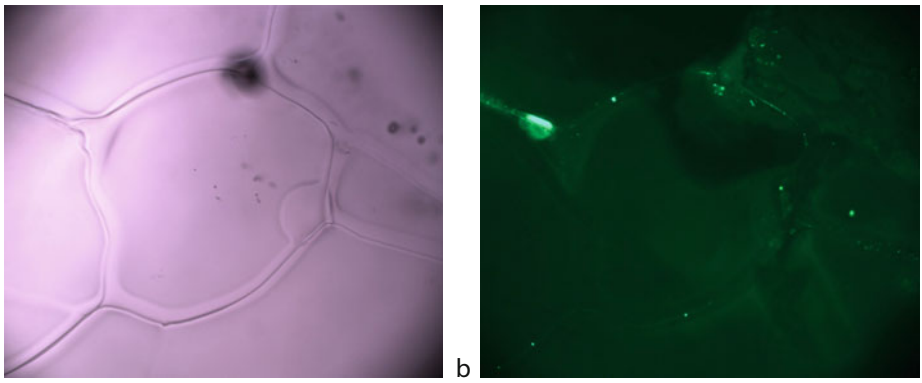

Fig. 6.3.3

Cryostage microscopic image at −20°C of microbial cells within an ice vein habitat. A thin section of glacial ice from the Taylor Glacier (McMurdo Dry Valleys, Antarctica) was mounted on a microscope slide and overlain with a saline solution of SYBR Green I. (a) Bright field image of the ice; (b) the same area under epifluorescence showing DNA-containing cells in the interstitial habitat between ice crystals. Each micrograph is ~1,000 mm in width. Images: K. Suematsu, visiting researcher, LSU

see Chap. 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats; Miteva 2008). In the following we focus on what is currently known about the diversity of glacial microbes, discuss the glacial environment as a habitat or archive and the longevity of microbes in ice.

Diversity of Microbial Species Immured in Glacial Ice

Glacial ice sheets entrap and preserve aerosolized biological material (i.e., insects, plant fragments, seeds, pollen grains, fungal spores and microorganisms) deposited in snowfall. The presence of viable bacteria and fungi in ancient glacier ice has been widely documented in polar and non-polar locations (e.g., Abyzov et al. 1993, 1998; Christner et al. 2000, 2003, 2006; Miteva and Brenchley 2005; Bidle et al. 2007; D'Elia et al. 2009; Miteva et al. 2009). Recent reviews by Priscu and Christner (2004) and Miteva (2008), and Christner et al. (2008c) and Priscu et al. (2008), provide good detailed overviews of the diversity of bacteria reported in a range of glacial and subglacial environments. Remarkably, many isolates obtained from geographically diverse glacier samples of polar and non-polar origin belong to the same bacterial genera (summarized in *Table 6.3.2*), including representatives of the Actinobacteria, Firmicutes, Proteobacteria and Bacteriodetes (Miteva 2008). Isolates of the Actinobacteria and Firmicutes are usually found to be predominant, followed by members of the Proteobacteria and Bacteroidetes. Studies that have succeeded in characterizing glacial microbial communities directly through culture-independent 16S rRNA based methods found sequences primarily relating to the same groups (Miteva 2008).

Microorganisms entrapped at increasing depth, and therefore, increasing age in glacier ice remain viable for hundreds of thousands (Abyzov et al. 1993, 1998; Christner et al. 2003, 2006; Miteva and Brenchley 2005) to perhaps millions of years (Bidle et al. 2007). Metagenomic analysis of a primarily bacterial assemblage in glacial ice from the German Alps indicates the potential for high metabolic diversity and molecular evidence for adaptations to increase survival at low temperature and during freezing (Simon et al. 2009). Some of the ice-related phenotypes found in species deposited in glacial environments may even have a role in precipitation generation and/or their precipitation from the atmosphere in snowfall (e.g., Christner et al. 2008a, b). Comprehensive studies of microbial assemblages immured chronologically within an ice core provide a unique approach to geobiology, e.g., by examining the influence of climate conditions on the species that were distributed in the atmosphere at different times in history (e.g., Christner et al. 2000; Miteva et al. 2009). Miteva et al. (2009) used a molecular approach to study the microbial assemblage in the GISP 2D (Greenland) ice core and found that higher bacterial and fungal diversity in ice deposited during cold climate conditions. Efforts to characterize the unique properties of psychrophiles from glaciers and other permanently frozen environments have increased significantly in recent years, and an assortment of properties have been identified that would be useful to cells that persist in a frozen matrix. Loveland-Curtze et al. (2010) described the species *Chryseobacterium greenlandense*, which was isolated from a depth of 3,043 m in the GISP 2D (Greenland) ice core. The small cells (<0.1 mm^3) of this species enable a high cell surface-to-volume ratio, which enhances nutrient uptake and diffusion of substances throughout the cell. A species in the genus *Chryseobacterium* has also been reported from a deep Antarctic ice core (3,519 m in the Vostok ice core) that has the ability to alter the physical structure of ice (Raymond et al. 2008). This microbe produces an extracellular protein which binds to the

Table 6.3.2

Examples of bacteria isolated from various polar and nonpolar glacial ices

Phylum	Genus	Source	Reference
Actinobacteria	*Arthrobacter*	Antarctica, China, Greenland, and New Zealand	Christner et al. (2000), Miteva et al. (2004), Foght et al. (2004), Xiang et al. (2005)
Actinobacteria	*Clavibacter*	China and Bolivia	Christner et al. (2000), Xiang et al. (2005)
Actinobacteria	*Mycobacterium*	Bolivia and Greenland	Christner et al. (2000), Miteva et al. (2004)
Alphaproteobacteria	*Methylobacterium*	Antarctica, Greenland, and China	Christner et al. (2000, 2001, 2008), Miteva et al. (2004, 2009)
Alphaproteobacteria	*Sphingomonas*	Antarctica, Greenland, China, and New Zealand	Christner et al. (2000, 2001, 2008), Foght et al. (2004), Miteva et al. (2004), Xiang et al. (2005), Miteva et al. (2009)
Gammaproteobacteria	*Acinetobacter*	Antarctica, Bolivia, and China	Christner et al. (2000), Xiang et al. (2005)
Bacteroidetes	*Chryseobacterium*	Antarctica and Greenland	Raymond et al. (2008), Loveland-Curtze et al. (2010)
Bacteroidetes	*Flavobacterium*	Antarctica, China, and New Zealand	Christner et al. (2000), Zhu et al. (2003), Foght et al. (2004), Xiang et al. (2005), Zhang et al. (2006)
Firmicutes	*Bacillus*	Antarctica, Bolivia, China, and Greenland	Abyzov et al. (1993), Christner et al. (2000), Miteva and Brenchley (2005), Xiang et al. (2005), Miteva et al. (2009)
Firmicutes	*Exiguobacterium*	Bolivia, Greenland, and India	Christner et al. (2000), Miteva et al. (2004), Chaturvedi and Shivaji (2006)
Firmicutes	*Paenibacillus*	Antarctica, Bolivia, China, Greenland, and New Zealand	Christner et al. (2000, 2001), Foght et al. (2004), Miteva et al. (2004), Xiang et al. (2005)
Firmicutes	*Plannococcus*	Bolivia and China	Christner et al. (2000), Xiang et al. (2005)

prism faces of ice crystals and prevents recrystallization, i.e., an ice-binding protein. Bacteria in the genus *Psychrobacter* have been recovered frequently in enrichments targeting heterotrophs in icy environments (e.g., Gilichinsky et al. 2003, 2005; Bakermans et al. 2003, 2006; Mosier et al. 2007). In particular, studies of *Psychrobacter* species have provided valuable insight into their growth (Bakermans et al. 2003) and gene and protein expression patterns (Bakermans et al. 2007; Bergholz et al. 2009) at subzero temperatures, as well as their capability to metabolize within an ice matrix (Christner 2002; Amato and Christner 2009a).

Glacial Ice: Archive or Habitat?

It was initially assumed that cells surviving within ancient glacial ice did so in a state of anabiosis (e.g., Abyzov et al. 1998). In freshwater and saline ice, various studies have shown that cells are physically located in the aqueous interstitial veins that exist at ice grain boundaries (Junge et al. 2004; Mader et al. 2006; Amato et al. 2009b; *Figs. 6.3.1* and *6.3.3*), supporting Price's (2000) hypothesis that ice veins provide a habitat for cells in glacial ice. Sub-nanometer thin films of liquid water have been reported down to temperatures of −196°C (Pearson and Derbyshire 1974); however, the increased ionic concentration of unfrozen water at decreasing temperatures in ice likely constrains biological activity by creating chemical conditions incompatible with a microorganism's physiology. Laboratory studies have provided a new perspective on the potential for functional biological processes under frozen conditions (Carpenter et al. 2000; Rivkina et al. 2000; Christner 2002; Amato et al. 2009b). As of yet, the low temperature limit for metabolism is not known and is probably <-40°C (e.g., Junge et al. 2006; Panikov et al. 2006; Amato and Christner 2009). Hence, the physiological potential for metabolism under conditions in glacial ice has been established.

Analysis of the gases entrapped in ice cores from Antarctica, Bolivia, and Greenland have revealed ice horizons where the CO_2, N_2O and CH_4 concentrations are significantly elevated above atmospheric concentrations, and a number of investigators have concluded that these anomalies are the result of in situ microbial activity (Sowers 2001; Campen et al. 2003; Tung et al. 2006). Gas anomalies are commonly observed in basal ice, and Souchez et al. (1995) suggest that anomalously high CO_2 and CH_4 concentrations entrapped within GRIP silty ice may have originated from "flow-induced mixing"; however, in situ production of these gases still remains a plausible explanation (e.g., Tung et al. 2006). If microbial consortia remain metabolically active in the ice, then the World's glaciers represent active biomes, substantially expanding the known realm for life in the biosphere.

Limits to Longevity in Glacial Ice

For a microorganism to remain viable over an extended period of frozen dormancy, the macromolecular damage (e.g., to DNA) incurred by the cell must not exceed a level where effective repair is no longer possible. Viable microorganisms have been recovered from ancient glacial ice (Christner et al. 2003; Miteva and Brenchley 2005; Bidle et al. 2007) and permafrost (Vishnivetskaya et al. 2006; Gilichinsky et al. 2007; Johnson et al. 2007) samples ranging from hundreds-of-thousands to millions of years old. McKay (2001) predicted that background ionizing radiation in terrestrial permafrost (i.e., from the decay of potassium-40, thorium-232, and uranium-238) would inactivate dormant bacteria in 10–100 million years. In *Fig. 6.3.4*, we predicted cellular damage based on the introduction of lethal DNA double-stranded breaks (DSB) to the genome from ionizing radiation dosages in the Antarctic subsurface and "low background" environments (10^{-3} to 10^{-4} Gy y^{-1}; Luckey 1991). The susceptibility of bacteria to ionizing radiation is expressed as the number of DNA DSB at the D_{37}, which is the ionizing radiation dosage at which 37% of the cells survive, which on average, is sufficient to inactivate a single colony-forming unit of the irradiated population (Battista 1997). These data and related calculations imply that it would take 300,000–3,000,000 years of exposure to acquire an ionizing radiation dosage equivalent to the D_{37} of *Escherichia coli*, but a radiation-resistant bacterium such as *Deinococcus radiodurans* could remain in the ice for

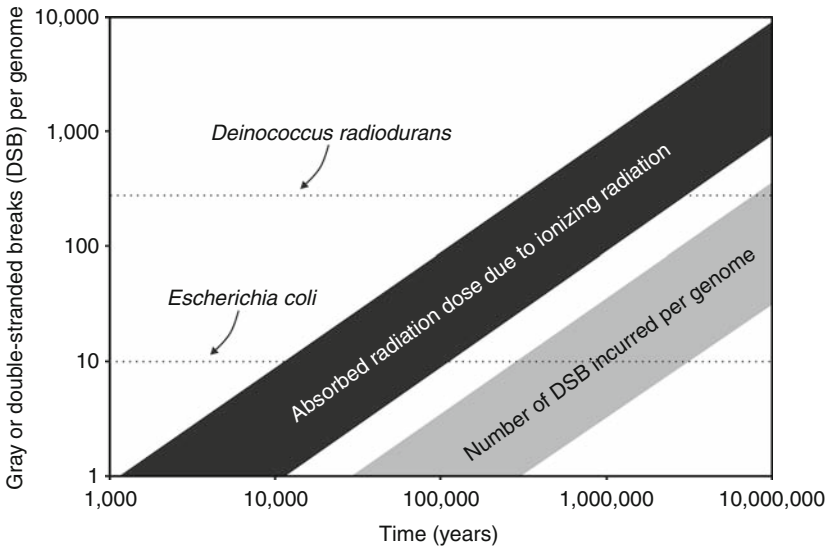

Fig. 6.3.4

Estimated number of DNA double-stranded breaks (DSB) in a microbial genome over time from ionizing radiation sources within glacial ice. Data range estimates for dosage from Luckey (1991). Values for DNA DSB damage due to ionizing radiation dosage (1 DSB per 30 Gy) from Battista (1997). The dotted horizontal lines are examples for the number of DNA DSB at the D37 for Escherichia coli and Deinococcus radiodurans

>8,250,000 years before reaching the D_{37} (*Fig. 6.3.4*). Our calculations are consistent with the exponential decline in average community DNA size ($t_{1/2}$ = 1.1 million years) observed by Bidle et al. (2007) in a time sequence of ice samples up to 8 million years old. It is important to note that these estimates assume that microorganisms are metabolically dormant when entrapped within frozen matrices, and therefore, are incapable of repairing cellular damage as it is incurred.

Christner (2002a) demonstrated the ability of bacteria to conduct DNA synthesis at −15°C and hypothesized that the metabolic activity observed was directed towards repairing damage sustained during the freezing process (i.e., DNA breakage) or for maintenance metabolism (Price and Sowers 2004). Johnson et al. (2007) provided evidence for DNA repair in permafrost samples up to 600,000 years old and concluded that "bacteria with an active DNA repair mechanism are most likely to persevere." If laboratory measurements of microbial metabolism under frozen conditions are a true reflection of their physiological potential in natural icy environments, a slow metabolic rate may be sufficient to offset cellular damage, allowing survival in frozen substrates for extended time frames. In contrast, ionizing radiation-resistant bacteria, which are often also resistant to UV radiation and desiccation (i.e., Rainey et al. 2005), might be capable of remaining viable in the absence of metabolism due to their extremely efficient DNA repair mechanisms (e.g., Battista 1997). Testing the ionizing radiation tolerances of viable microorganisms recovered from ancient glacial ice would provide a means to formulate hypotheses regarding the particular means of survival (i.e., dormancy and efficient DNA repair versus metabolic activity and repair under frozen conditions) used by species that survive for geological timeframes in ice.

Conclusions

Sea ice and glacier ice harbor unique microbial communities with many steno- and euro psychrophilic members. These psychrophiles exhibit a rich diversity with many of the same groups represented in both habitats indicating that environmental conditions in the ice (temperature, concentration of solutes, etc.) select for certain types of organisms. It has now been shown that psychrophilic microbes residing both in saline and freshwater ice veins might not be merely persisting but can also carry out low levels of metabolic activity. For glacial ice microbes, this could possibly allow for DNA damage repair while being immured in the ice over thousands to hundreds of thousands and possibly even millions of years.

Cross-References

- 6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens
- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats
- 6.7 Psychrophilic Enzymes: Cool Responses to Chilly Problems
- 9.4 Genetics, Genomics, Evolution

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6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens

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Introduction

Food preservation has long relied on using a variety of processes to control the growth of microbial organisms, helping to preserve food quality and food safety. These processes include techniques such as acidification, anaerobic packaging, heat treatments, increased salinity, and cold storage. The comparatively recent advent of and ready access to refrigeration has been critical in the control of numerous microbial species in food products. However, in recent years, it has become increasingly apparent that a subset of these foodborne organisms is capable of growing at refrigeration temperatures. Growth under cold conditions leads to enrichment of these organisms when foods are contaminated with low or even undetectable titers of these psychrotolerant pathogens. Additionally, some of these organisms are also capable of causing disease, which following cold "enrichment" can lead to outbreaks and sporadic cases of disease. The increased awareness of these psychrotolerant pathogens, coupled with the consumer's desire for minimal food processing, has resulted in a significant amount of research to determine what organisms can grow in a variety of foods using various combinations of food preservation processes. While most of the diseases caused by these organisms involve self-limiting gastroenteritis, more severe illnesses, such as listeriosis and botulism, are possible and include a significant risk of fatality.

The extraordinary adaptability of these pathogenic strains provides a unique opportunity to study the mechanisms of regulation accompanying growth over a very wide range of temperatures. Current research in these human psychrotolerant pathogens identifies many of the same type of adaptation mechanisms, including membrane alterations and osmolyte accumulation that are the hallmarks of psychrotolerant organisms. Our understanding of the mechanisms by which these pathogens grow in cold temperatures is limited. While the incidence of the diseases caused by these psychrotolerant pathogens is rare compared to those caused by *Salmonella* and *E. coli*, the view that these organisms encompass an emerging class of pathogens, adapting to stressful environments, encourages in-depth examination of these organisms for better understanding of the mechanisms of stress adaptation and to help identify other organisms that might also adapt to this new niche.

In this chapter, five species of bacteria, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Clostridium botulinum*, and *Aeromonas* spp., are discussed both from the psychrotolerance perspective including the characteristics of growth (*Table 6.4.1*) and mechanisms of adaptation (*Table 6.4.2*) and from the pathogen perspective. While other organisms, specifically *Salmonella* spp., are capable of growth at equivalent temperatures, the species reviewed in this chapter are those with established associations between refrigerated food and disease incidence.

Listeria monocytogenes

Listeria monocytogenes is an aerobic Gram-positive pathogenic bacterium that causes listeriosis, a serious invasive disease in both humans and animals (Datta 2003; Cossart and Toledo-Arana 2008). *Listeria monocytogenes* has been known for more than a century, but it was recognized as an important foodborne pathogen in humans only in the 1970s (Park et al. 2004). The great majority (>99%) of human listeriosis cases results from the consumption of contaminated food products (Mead et al. 1999; Hain et al. 2006). Individuals with immuno-compromising conditions, including the elderly and pregnant women, are at a higher risk for invasive listeriosis,

Table 6.4.1

Summary of salient characteristics of psychrotolerant pathogens, [a]some of which vary based on strain type as discussed in this article

Species	Average Genome size (Mb)	Growth			Diseases
		Temp (°C)	Salinity (%)	pH	
Listeria monocytogenes	2.9	−1.5 to 45	Up to 10	4.4–9.4	Self-limiting gastroenteritis Invasive listeriosis
Yersinia enterocolitica	4.68	0–44	Up to 5	4.2–10	Self-limiting gastroenteritis/ yersiniosis
Bacillus cereus	5.4	4–55	Up to 10	4.3–9.3	Self-limiting gastroenteritis
Clostridium botulinum	3.9	3.3–50[a]	Up to 5 or 10[a]	4.6–8.3	Botulism
Aeromonas spp.	4.7	0–55[a]	Up to 4	6.0–8.5	Gastroenteritis? Septicemia Wound infections

whereas gastroenteritis, caused by *L. monocytogenes*, also affects healthy individuals (Datta 2003; Cossart and Toledo-Arana 2008). Invasive listeriosis diseases include septicemia, meningitis, and meningoencephalitis. In addition, the capability of *L. monocytogenes* to cross the placenta in pregnant women leads to premature birth, miscarriages, and neonatal listeriosis (Datta 2003; Cossart and Toledo-Arana 2008). Invasive listeriosis has a high fatality rate (20%) and is an enormous burden on society (Mead et al. 1999). In 2008, the CDC Foodborne Diseases Active Surveillance Network (FoodNet) recorded 135 listeriosis cases among 18,499 cases caused by bacterial foodborne diseases which accounts for 0.29 cases per 100,000 people in the United States.

One of the important characteristics of *L. monocytogenes* is its ability to grow at high salinity and/or temperatures much below its optimum growth temperature (35–37°C) (Junttila et al. 1988; Datta 2003; Chaturongakul et al. 2008). Certain strains of *L. monocytogenes* have been shown to grow, albeit slowly, at temperatures around 0°C (Walker et al. 1990). Growth of *L. monocytogenes* in brain-heart infusion medium (BHI) at 37°C and 5°C is shown in *Fig. 6.4.1*. Post-pasteurization contamination that can occur during food processing, when followed by cold storage, will then lead to the enrichment of the bacteria making it difficult to ensure the safety of refrigerated ready-to-eat food products (Datta 2003).

Physiological alterations affecting growth and survival of microorganism at low temperature include reduction of membrane fluidity, slow mobility of transcriptional and translational machinery, intracellular ice crystal formation, cold-denaturation, and impaired protein folding (D'Amico et al. 2002; Laksanalamai et al. 2009). Molecular adaptations underlying growth and survival at low temperature of *L. monocytogenes* have been studied including modification of cellular structures, expression of specific proteins, and regulation of transcription and translation.

Membrane Modification Systems

Organisms across the three domains of life (Eukaryotes, Bacteria, and Archaea) (Pace 2009) face a reduction of membrane fluidity when they are grown at low temperature due to the

Table 6.4.2

A list of genes identified from published literature that were then analyzed for their presence in the other species based on CMR searches (Peterson et al. 2001). Yes indicates the presence of homologs; No indicates no significant homology identified

Cold adaptation	Genes	Organism				
Mechanism		*L. monocytogenes*	*Y. enterocolitica*	*B. cereus*	*C. botulinum*	*Aeromonas* spp.
Cold shock protein synthesis	*cspA*	Yes	Yes (CspA1 and CspA2)	Yes	Yes	Yes
	cspB	Yes	Yes	Yes	Yes	Possible
	cspD	Yes	Yes	Yes	No	Yes
Membrane modification	*bkd* operon	Yes	No	Yes	No	No
	des	No	Yes	Yes	No	Possible
Compatible solute acc-umulation	*betL*	Yes	No	Yes	Yes	No
	gbuABC operon	Yes	No	No	No	No
	opu operon	Yes	No	Yes	Yes	No
	proU operon	No	Yes	No	No	yes
	proP	No	Yes	Yes	No	No
Alternative sigma factor	*sigB*	Yes	No	Yes	No	No
	sigL	Yes	No	Yes	No	No
RNA chaperoning	Numerous	Yes	Yes	Yes	Yes	Yes

reversible change of a liquid crystalline state to a gel-like state (Russell 1990; Aguilar et al. 2001; D'Amico et al. 2002; Cavicchioli 2006; Badaoui Najjar et al. 2007; Laksanalamai et al. 2009). Rigidity of the cell membrane could interfere with membrane or membrane-associated protein functions. Maintaining membrane fluidity is therefore an important process for organisms to cope with cold environments. Since fatty acid alterations play a crucial role in membrane adaptation, three modes of adaptation have been described including changes in compositions, chain length, and the degree of unsaturation of membrane fatty acids (Suutari and Laakso 1994; Annous et al. 1997). No protein homologs involved in the modification of lipid saturation have been identified in *L. monocytogenes*, although these proteins and their homologs are commonly found in most bacteria and archaea (Cavicchioli 2006; Chan and Wiedmann 2009). Fatty acid and chain length alterations have been shown to be important in cold adaptation of *L. monocytogenes*, (Annous et al. 1997; Chan and Wiedmann 2009). Several forms of branched chain fatty acids (BCFA), including anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, and iso-$C_{15:0}$ are typically found when *L. monocytogenes* is grown at 37°C (Annous et al. 1997). The melting point of anteiso-branch chain fatty acids appears to be lower than their analogous iso-forms, providing

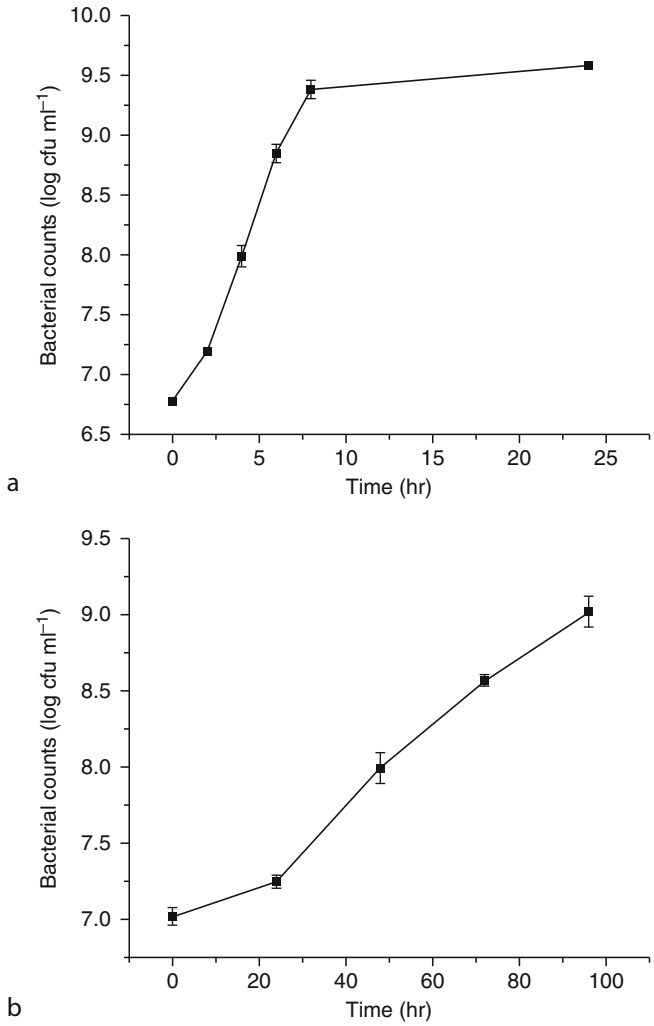

Fig. 6.4.1

Growth of *Listeria monocytogenes* (LS411) in brain-heart infusion (BHI) medium at 37°C (a) and 5°C (b)

more cell membrane fluidity (Chan and Wiedmann 2009). The fatty acid composition of *L. monocytogenes* grown at low temperature is dominated by the low melting point anteiso-$C_{15:0}$ forms (Annous et al. 1997). When *B. subtilis* and *L. monocytogenes* were grown in cold environments, α-keto acid dehydrogenases, encoded by the *bkd* operon, catalyze the degradation of branched chain amino acids, enabling cold adaptation. The products of the branch-chain amino acid degradation are acyl-CoA intermediates, used in membrane lipid synthesis. In *B. subtilis*, 2-methylbutyryl-CoA, one of the products from α-keto acid dehydrogenase, is used to synthesize anteiso-branched chain fatty acids (Nickel et al. 2004). In addition, mutations in the *bkd* operon in *L. monocytogenes* exhibit a cold sensitive phenotype (Zhu et al. 2005).

The reduction of fatty acid chain length, in addition to the alteration of fatty acid composition has been implicated in listerial cold adaption. When the temperature declines, a $C_{15:0}$ fatty acid is unable to elongate since the fatty acid synthetase, which adds two carbon units, is inhibited, leading to shorter fatty acid chains (Annous et al. 1997).

Membrane Transports and Compatible Solutes

Membrane transport systems facilitating transportation of compatible solutes play a crucial role in *L. monocytogenes* cold adaptation. Compatible solutes are small and highly soluble compounds that protect or stabilize cellular components exposed to stress conditions without significantly interfering with their functions (Yancey et al. 1982; Baskakov and Bolen 1998; Angelidis and Smith 2003a). Compatible solutes, such as glycine betaine and carnitine, function as osmoprotectants but have no effects on promoting growth at temperatures between 30°C and 37°C in the absence of osmotic stress (Bayles and Wilkinson 2000). However, the ability of these molecules to stimulate growth at temperatures between 4°C and 7°C supports their role as cryoprotectants (Bayles and Wilkinson 2000). The accumulation of glycine betaine and carnitine is dependent on transport systems as *L. monocytogenes* is unable to synthesize these compounds. Although there are homologs of glycine betaine synthesis genes in the listerial genome, such as glycine betaine aldehyde dehydrogenase and a type III alcohol dehydrogenase, these genes appear to be disabled, resulting in the inability of *L. monocytogenes* to synthesize glycine betaine (Bayles and Wilkinson 2000).

Glycine betaine is a primary compatible solute in *L. monocytogenes* and can be transported into cells via two glycine betaine transport systems, glycine betaine porter I (*betL*) and glycine betaine porter II (*gbuABC* operon) (Wemekamp-Kamphuis et al. 2002; Chan and Wiedmann 2009). Glycine betaine porter I is a Na^+ glycine betaine symporter, whereas glycine betaine porter II is an ATP binding cassette (ABC) transporter (Mendum and Smith 2002). Several lines of evidence indicate that the ATP-dependent glycine betaine porter II is a primary transport system for glycine betaine during cold adaptation that can be activated by osmotic and cold stresses (Ko et al. 1994; Gerhardt et al. 2000; Mendum and Smith 2002). L-carnitine is also a compatible solute implicated in *L. monocytogenes* psychrotolerance. The ABC transporter, OpuC, is encoded by the *opuCABCD* operon and is primarily responsible for L-carnitine transport (Fraser et al. 2000; Angelidis et al. 2002; Angelidis and Smith 2003a, b). Similar to the glycine betaine porter II, both osmotic and cold stresses activate OpuC-mediated carnitine transport (Angelidis and Smith 2003b). In the absence of both ATP-transport systems, the Na^+ glycine betaine symporter acts as the sole transporter, accumulating betaine at low level. BetL, Gbu, and OpuC appear to be the only psychrotolerant transport systems in *L. monocytogenes* as no other transporters function at 4°C (Angelidis and Smith 2003a).

In *L. monocytogenes*, oligopeptides are transported into cells by the oligopeptide permease, a specific type of ABC transport systems (Borezee et al. 2000). This transport system is encoded by an *opp* operon, consisting of 5 genes, *oppA*, *oppB*, *oppC*, *oppD*, *oppF*, and allows peptides of up to 8 amino acids to be transported into cells. The oligopeptides are subsequently hydrolyzed by peptidases, providing amino acids essential for *L. monocytogenes* growth. The inability of an *oppA* mutant to grow at 5°C, whereas the complemented strain is able to grow, revealed that OppA-mediated transport of oligopeptides is required by *L. monocytogenes* for growth at low temperature (Borezee et al. 2000; Chan and Wiedmann 2009).

Macromolecules

Exposure of microorganisms to a rapid downshift of temperature or cold shock leads to the induction of a set of proteins known as cold shock proteins (Csps) (Graumann and Marahiel 1996; Ermolenko and Makhatadze 2002). Cold shock responses have been characterized in both Gram-positive and Gram-negative bacteria and Csps are found throughout the domain bacteria with a wide range of growth temperature from psychrophilic to thermophilic bacteria (Jones et al. 1987; Graumann et al. 1996; Graumann and Marahiel 1998). This finding suggests that Csps may be implicated in a variety of cellular functions and stress responses, including cold adaptation. Interestingly, the cold shock domain (CSD) found in eukaryotic nucleic-acid-binding proteins also contain amino acid sequences similar to those of Csps (Graumann and Marahiel 1998; Giaquinto et al. 2007). The number of Csp homologs involved in cold adaptation varies among different species of bacteria. For example, nine Csp homologs are found in *Escherichia coli* while five homologs are found in *Bacillus subtilis* (Yamanaka et al. 1999; Giaquinto et al. 2007; Schmid et al. 2009). Based on the similarity between the CSD and Csp, Csps have been shown to function as a nucleic acid chaperone, facilitating transcription, translation, and/or replication (Ermolenko and Makhatadze 2002; Chan and Wiedmann 2009; Schmid et al. 2009) during cold stress. Although the mechanism of Csp action in *L. monocytogenes* remains to be determined, three Csps, CspA, CspB, and CspD, implicated in cold adaptation have been recently characterized (Schmid et al. 2009). Although at least one of the Csps is required for *B. subtilis* growth at 37°C, all three *L. monocytogenes* Csp homologs are dispensable at optimal growth temperatures (Schmid et al. 2009). Among all Csps, CspA appears to be the most critical for growth at low temperature as a *cspA* deletion mutant fails to grow at low temperatures (4°C, 10°C). The growth of a *cspD* deletion mutant is significantly reduced at 4°C, while no cold growth defect has been observed in *cspB* deletion mutant in the presence of wild type *cspA* and *cspD* (Schmid et al. 2009). This result indicated that Csps play important roles in *L. monocytogenes* growth at low temperature.

Ferritin, an 18 kDa Csp encoded by *fri*, is found in *L. monocytogenes*, and the nonpathogenic *Listeria innocua* appears to be implicated in cold adaptation (Chan and Wiedmann 2009). Although the mechanism for iron transport in *L. monocytogenes* has not been elucidated, it has been proposed that ferritin may be involved in iron storage, providing iron during low temperature growth (Olsen et al. 2005; Chan and Wiedmann 2009). Several lines of evidence indicate that ferritin is crucial for growth at low temperatures. Ferritin was induced when *L. monocytogenes* was transferred from 30°C to 5°C (Hebraud and Guzzo 2000). Additionally, ferritin is involved in temperature stress adaptation as a *fri* mutant of *L. monocytogenes* shows reduced growth at 4°C and survival is decreased at 45°C (Dussurget et al. 2005).

Gene and Protein Expression Machinery Systems

Transcriptional and translational controls of gene and protein expression also play a crucial role in cold adaptation. Transcriptome analysis of cold-shocked *B. subtilis* revealed that many genes involved in cold adaptation are upregulated whereas those involved in biosynthesis are repressed (Kaan et al. 2002). In addition, proteome analysis of *B. subtilis* under cold stress also displayed various upregulated stress proteins (Kaan et al. 2002; Brigulla et al. 2003). With advances in genomic, proteomic, and bioinformatic tools, several gene and protein expressions control mechanisms underlying cold adaptation of *L. monocytogenes* have been proposed and studied.

Sigma (σ) factors are protein subunits involved in bacterial transcriptional controls, recognizing promoters and leading the core RNA polymerase to initiate transcription (Helmann and Chamberlin 1988). Bacteria possess several σ factors to alter gene expression in response to changing environmental conditions (Helmann and Chamberlin 1988; Kroos et al. 1999; Chan and Wiedmann 2009). The genome sequence of *L. monocytogenes* EGD-e revealed four alternative σ factors, σ^B, σ^C, σ^H, and σ^L (Chaturongakul et al. 2008; Glaser et al. 2001). σ^B, encoded by *sigB*, has been identified as an alternative sigma factor for stress responses in many Gram-positive bacteria including *L. monocytogenes* (Becker et al. 1998; Fouet et al. 2000; Wemekamp-Kamphuis et al. 2002; Kazmierczak et al. 2003; Suzuki et al. 2007; Chaturongakul et al. 2008; Giotis et al. 2008). Several lines of evidence indicated that σ^B may be involved in cold stress responses in *L. monocytogenes* (Chan and Wiedmann 2009). The transcript of the *rsbV* promoter, which is σ^B-dependent, was induced when *L. monocytogenes* was shifted from 37°C to 8°C for 2 h. Further characterization of several genes implicated in cold growth has shown that the expression of genes, such as *fri*, or *opuCA*, is σ^B-dependent (Chan and Wiedmann 2009). In addition, the ability of *L. monocytogenes sigB* mutant to accumulate compatible solutes, such as betaine or carnitine, was abolished (Becker et al. 1998). In addition to σ^B, characterization of a listerial σ^L has revealed an important role in cold adaption. Several genes involved in cold responses, including *oppA*, appear to be under the control of σ^L (Raimann et al. 2009).

E. coli and *B. subtilis* possess DEAD box RNA helicases (RNA chaperone) or cold-induced proteins (Cshs) that serve as RNA chaperones to initiate the translation of certain Csps at low temperature (Hunger et al. 2006). These RNA helicases contain a short conserved amino acid motif (Asp-Glu-Ala-Asp) known as the DEAD box (Turner et al. 2007). It has been proposed that when organisms are exposed to cold shock, unfavorable mRNA secondary structures are formed, resulting in the inhibition of translation. DEAD box of the RNA helicases are capable of reversing these mRNA secondary structure by binding to the cold box, which is a conserved transcribed region located upstream of the gene. Csps can subsequently bind to the single stranded RNA to prevent refolding before the translation can occur (Hunger et al. 2006). Microarray analysis showed that transcripts of DEAD box RNA helicase genes are induced in *L. monocytogenes* grown at 4°C, suggesting that the DEAD box RNA helicases may be involved in cold adaptation of *L. monocytogenes*.

Yersinia enterocolitica

Yersinia enterocolitica is a Gram-negative, facultative anaerobic, enteric bacterium associated with a number of serious illnesses including gastroenteritis (yersiniosis), septicemia, and reactive arthritis (Lal et al. 2003). As a species, *Y. enterocolitica* represents a very heterogeneous group comprised of several biotypes, especially when compared to its relatives, *Y. pestis* and *Y. pseudotuberculosis* (Robins-Browne 1997). *Yersinia enterocolitica* is commonly associated with pigs and can be readily isolated from pig carcasses during slaughter, though isolation of pathogenic biotypes is rare (Fredriksson-Ahomaa et al. 2006). The most common biotype globally associated with human disease is biotype 4. However, in the U.S., most human clinical isolates are biotype 1B, which appears to be the most virulent biotype and is not common outside the U.S (Robins-Browne 1997). Most outbreaks of *Y. enterocolitica* have been linked to milk and milk products; outbreaks have also been linked to pork products, such as pork cheese, bean sprouts, and tofu (Robins-Browne 1997). There have even been reports linking

Y. enterocolitica infection to a water source, though this is rare (Robins-Browne 1997). Between 2% and 5% of gastroenteritis diseases globally are caused by *Y. enterocolitica*, typically affecting children (Kapperud 1991; Lal et al. 2003; Perdikogianni et al. 2006). The symptoms associated with yersiniosis can often mimic appendicitis, resulting in the possibility of unnecessary appendectomies (Perdikogianni et al. 2006). In the United States, the incidence of yersiniosis in 2008 was 0.36 cases per 100,000 people (CDC 2009). Disease incidence from *Y. enterocolitica* is more common than that of the spore-formers and *Aeromonas* and is comparable to that of *L. monocytogenes*, stimulating more study on its mechanisms of cold adaptation.

Growth

The *Y. enterocolitica* growth profile is somewhat unique among pathogens as its optimal growth temperature is between 25°C and 30°C, rather than 37°C (Schofield 1992). Complicating its control, like all of the bacteria discussed in this chapter, is its ability to grow, albeit slowly, at temperatures as low as 0°C (Kapperud 1991). This cold growth has been established on a variety of food matrices including vacuum-packed meat, pork, beef, boiled eggs, seafood, milk, and tofu (Robins-Browne 1997). Additionally, *Y. enterocolitica* has been found to grow better on cooked than raw foods and can survive in frozen foods even with repeated freeze-thaw cycles (Robins-Browne 1997). Overall, *Y. enterocolitica* is a comparatively hardy bacterium capable of growth from pH 4 to pH 10 and in the presence of up to 5% NaCl, though this latter condition inhibits growth in food products (Robins-Browne 1997). Multiple factors have been implicated in *Y. enterocolitica* cold adaptation with similarities to those systems identified in other psychrotolerant pathogens. For example, *Y. enterocolitica* modifies its membrane fatty acid composition, switching from predominantly saturated fatty acids at temperatures of 30°C and above to largely unsaturated fatty acids at 10°C and below (Goverde et al. 1994). Other putative cold-adaptive mechanisms include transporters, cold shock proteins, and alterations in motility.

General Mechanisms of Cold Adaptation

Transposon mutagenesis has been used to identify the genes necessary for cold growth. This led to the discovery of *pnp*, a polynucleotide phosphorylase (PNPase) that when mutated resulted in reduced growth at 5°C but not 30°C (Goverde et al. 1998). Successful complementation of the mutation resulted in restoration of 5°C growth and PNPase activity. Screening of a transposon promoter reporter library identified 42 transcriptional units that were altered when cultures were grown at 10°C vs. 30°C (Bresolin et al. 2006b). The genes encompassed previously characterized cold adaptation genes, such as *cspA* and *cspB*, as well as genes encoding proteins with no established role, including putative regulatory proteins, outer membrane proteins, and hypothetical proteins. Other genes included functions in well established metabolic pathways. However, no genes identified in this study were indispensible for low temperature growth (Bresolin et al. 2006b). This study also identified a significant number of genes involved in motility and chemotaxis, and subsequent transposon-based studies identified temperature dependent regulation of chemotaxis and motility genes (Bresolin et al. 2006b; Bresolin et al. 2008). Further characterization of the temperature dependent expression profile showed that

expression increased as temperature decreased from 37°C to 20°C. This was then followed by a reduction in expression at 6°C that was still well above the background levels seen at 37°C (Bresolin et al. 2008). This upregulation of chemotaxis and motility genes raises the question as to how motility and chemotaxis can benefit a bacterium at cold temperatures.

Glycine Betaine Transporters

Like *L. monocytogenes*, *Y. enterocolitica* accumulates glycine betaine in response to cold temperatures. However, the components of the systems differ from *L. monocytogenes* using variant genes, encoded by *proP* and *proU* operon (Annamalai and Venkitanarayanan 2009). The *proP* gene encodes a glycine/proline permease that lacks homology to the *Listeria* transporters. The *proU* operon is comprised of three genes, *proV*, *proW*, and *proX*, and, based on homology to *E. coli* proteins, appears to encode an ABC (ATP binding cassette) transporter. This transporter is functionally similar to the listerial glycine betaine porter II (*gbuABC*), which also shares limited amino acid sequence homology with the subunits of this system. In particular, GbuA and GbuB are homologous to ProV and ProW, respectively (∼45% identity and ∼60% similarity). However, the periplasmic binding proteins, encoded by *gbuC* and *proU*, show little, if any, homology with only 25% identity and 42% similarity. This is more striking as intraspecies comparisons of different *L. monocytogenes* strains showed somewhat stronger conservation of GbuC (>96% identity) than GbuA and GbuB (87–90% identity). This suggests the possibility of either convergent evolution or a branching in the evolution of the two functional approaches to adapt to their respective niches. Interestingly, loss of either system, which each cause reductions in glycine betaine uptake, has no effect on growth at 4°C (Annamalai and Venkitanarayanan 2009). However, inactivation of both systems results in a complete inability to acquire glycine betaine and a significant reduction in growth at 4°C (Annamalai and Venkitanarayanan 2009). Similar results were also observed when *Y. enterocolitica* was grown under osmotic stress. These data show that either system is sufficient to permit low temperature growth and that the redundancy in transporters underlines the importance of glycine betaine uptake in *Y. enterocolitica* cold adaptation and osmotic stress.

Cold Shock Proteins

Characterization of cold shock proteins in *Y. enterocolitica* led to the identification of a tandem gene duplication of *cspA* leading to two nearly identical genes (96.7%) (Neuhaus et al. 1999). This gene set was found to have one transcriptional start site that produced two transcripts, a monocistronic one containing *cspA1* and a bicistronic one encoding *cspA1* and *cspA2* (Neuhaus et al. 1999). Interestingly, this study also showed that the two transcripts exhibit different expression profiles. Both genes were largely unexpressed at 30°C; however, a shift in temperature to 10°C led to an immediate 300-fold induction of the bicistronic message whereas the *cspA1* transcript showed slower and less substantial response (30-fold increase) (Neuhaus et al. 1999). Both transcripts were undetectable after 2 h of cold exposure supporting the notion that these genes are involved in the initial cold shock response and not adaptation to prolonged growth at low temperatures. The reduction in detectable transcript was caused by degradation of the mRNA via PNPase which appears to replace RNase II at reduced temperatures (Neuhaus et al. 2000). A PNPase mutant was found to have delayed degradation of the

cspA transcript that correlated with a delayed initiation of exponential growth (Neuhaus et al. 2000). This degradation is dependent on cleavage of an AGUAAA sequence found within the transcript (Neuhaus et al. 2003). Modification of the sequence to AGUCCC blocked cleavage of the transcript, delaying degradation of the transcript and the onset of exponential growth. PNPase and RNase II are part of the degradosome that cleaves the sequence and facilitates further degradation of the transcript (Neuhaus et al. 2003). This degradation frees ribosomes, previously heavily occupied by *cspA1/cspA2* transcript, to focus on the synthesis of other proteins critical for the return to exponential growth (Neuhaus et al. 2000; Neuhaus et al. 2003). Interestingly, temperature profiling of the expression of the two transcripts showed that the bicistronic transcript was expressed in increasing amounts as temperature decreased from 20°C to 0°C, whereas the monocistronic message was most highly expressed at 15°C and 20°C (Neuhaus et al. 1999). These data suggest that the roles these two highly conserved proteins play in cold adaptation differ to some degree.

Environmental Adaptation and Pathogenicity

Yersinia enterocolitica is also capable of producing a heat-stable toxin, although the likelihood of expression at refrigeration temperatures is unclear. Expression of this toxin is maximal at ambient temperatures and has not been detected in milk cultures at refrigeration temperatures; however, toxin production has been identified in media cultures, such as TSB, at 4°C (Olsvik and Kapperud 1982; Schofield 1992). This leaves the possibility that *Y. enterocolitica* could produce toxin in some refrigerated food products and, in such circumstances, cooking would likely be insufficient to eliminate food toxicity.

Some of the adaptations observed to permit cold growth may have evolved to allow survival in an insect host. Transposon work identified a putative toxin subunit whose expression was increased at reduced temperatures and whole cell extracts of *Y. enterocolitica* cultured at 10°C were found to have insecticidal activity (Bresolin et al. 2006a, b). A knockout mutation of *tcaA* was found to negate the insecticidal activity associated with low temperature *Y. enterocolitica* cultures. Furthermore, the *tcaA* region contains ten ORFs, many homologous to other toxin-encoding genes characterized in other *Yersinia* spp. and *Photorhabdus luminescens* (Bresolin et al. 2006a). These data suggest the possibility of an insect reservoir for *Y. enterocolitica*. However, the possibility exists that these toxins evolved for analogous mammalian systems to those, as yet, unidentified insect systems (Bresolin et al. 2006a).

Finally, *Y. enterocolitica* strains are known to carry virulence plasmids (Lepka et al. 2009). Two novel plasmids have been identified in a biotype 1a strain (Lepka et al. 2009). This biotype is typically nonpathogenic; however, strains have been identified that have gained a pathogenic phenotype that appears to be associated with acquired virulence plasmids (Lepka et al. 2009). One of these plasmids is of particular interest as it contains an ORF that is expressed at 4°C but not above 27°C (Lepka et al. 2009). While this is likely due to an environmental niche other than the pathogenic one, it is likely that this type of adaptation would also enable better survival during refrigeration.

Spore-Forming Bacteria

Spore-forming bacteria are a fundamental concern in the public health sector as processes that are used to ensure food safety may not be successful at eliminating spores. Additionally, in

some cases, the heat treatments used to eradicate vegetative cells can initiate spore germination. Determination of shelf life then becomes a balancing act between how long a food potentially contaminated with spores from pathogenic organisms can be stored before enough growth and toxin production pose a risk to illness and/or death. Reduction of storage temperatures typically abolishes growth of many of these bacteria. However, a few species are capable of growing even at refrigeration temperatures. The growth rates of these organisms can be affected by numerous variables including pH, salinity, food matrices, and lysozyme content. Most research to date has focused on characterizing the interaction of these variables and how they affect storage parameters which translate into a reduced or extended shelf-life. Two bacteria of concern are *Clostridium botulinum*, the causative agent of botulism, and *Bacillus cereus*, which causes a diarrheal emetic-foodborne disease.

Clostridium botulinum

Clostridium botulinum is a Gram-positive obligate anaerobe capable of forming spores which are resistant to heat treatments used in food preservation, e.g., pasteurization. This species of bacteria represents a diverse cluster of organisms categorized into four phenotypic groups (I–IV) (Collins and East 1998). Groups I and II are of particular concern, causing more than 1,000 outbreaks between 1969 and 1998, as they are capable of producing the botulinum toxin, which can result in lethal paralysis in humans (Hinderink et al. 2009; Carlin et al. 2000). The botulinim neurotoxin, produced by the members of serotypes A through G, causes a flaccid paralysis and is considered to be the most potent of known toxins in terms of its exceptionally low LD50 (Chen et al. 2008; Hinderink et al. 2009; Osborne et al. 2007; Peck 2009). These two groups can also be divided as proteolytic (Group I) and non-proteolytic (Group II) subsets. Proteolytic strains cannot grow at temperatures below 10°C and, therefore, can be easily controlled by refrigeration (Hinderink et al. 2009). However, while there is variability, most non-proteolytic strains are able to grow at refrigeration temperatures, below 10°C, and growth has been detected as low as 3°C (Graham et al. 1997; Roberts and Hobbs 1968). Additionally, studies have shown that these strains are producing botulinum toxin when grown at refrigeration temperatures (Graham et al. 1997; Roberts and Hobbs 1968; Chen et al. 2008). qPCR studies have shown that the *bot* transcript is produced at similar levels in cultures grown at 10°C and 30°C, although there are differences in the dynamics of transcription (Chen et al. 2008). This work has shown that *bot* expression at 10°C peaked late in exponential phase and was maintained well into stationary phase, as opposed to peaking during early stationary phase but was followed by a sharp decline in expression at 30°C. These data suggest the possibility of alternate regulation based on temperature. However, little is known about the mechanisms by which *C. botulinum* grows at cold temperatures. In fact, characterization of the temperatures at which *C. botulinum* can be cultured is still an evolving topic and has been for decades (Roberts and Hobbs 1968; Graham et al. 1997; Hinderink et al. 2009). Typical food preservation techniques are successful at killing the vegetative cells; however, the spores persist and their germination can often be triggered by prophylactic heat treatment. Research has shown that the conditions under which spores are formed can affect their germinability (Evans et al. 1997). Spores formed at ambient temperatures (20°C) or above were more capable of germination than those formed at reduced temperatures. Additionally, the storage conditions affected spore germination, which was affected by the combination of storage time and temperatures at which cells formed, stored, and germinated (Evans et al. 1997). For example,

spores formed at 20°C and then frozen overnight or for 1 week had higher levels of germination at 10°C than fresh spores. However, spores stored for a month were comparable to fresh spores in their ability to germinate. Conversely, spores formed at 10°C showed limited germination upon induction.

While many studies have clarified the conditions that affect *C. botulinum* growth at reduced temperatures, little, if any, work has been done to characterize the specific molecular components and their roles that allow such growth to occur. It is likely that, based on mechanisms in other related psychrotolerant pathogens, membrane fatty acid components are altered to increase fluidity and that RNA chaperones are involved but there are differences in the genomic organization as well as amino acid similarity of potential homologs which could change the functional role these homologs play in *C. botulinum*.

Bacillus cereus

Bacillus cereus is a Gram-positive psychrotolerant facultative anaerobe that, like *C. botulinum*, is capable of forming spores that resist many treatments used in food preservation. Studies have shown that this bacterium is capable of growing at 5°C (Christiansson et al. 1989; van Netten et al. 1990; Choma et al. 2000). After ingestion, *B. cereus* causes a self-limiting disease typical of food poisoning illnesses with symptoms including diarrhea and vomiting. The bacterium is widespread in the environment and can be found associated with a variety of foods, including milk, grains, and meat products. The prevalence of *B. cereus* in the environment and food products poses an additional level of concern in food safety as the spores can readily attach to food processing equipment, germinate, grow, and resporulate leading to a protracted contamination of food products produced from an affected production line (Andersson et al. 1995). This also leads to difficulties in adequately cleaning all equipment that comes in contact with contaminated food products. *B. cereus* has been shown to grow at reduced temperatures (below 10°C) in a variety of foods including vegetable products, ground beef, and dairy products (Christiansson et al. 1989; van Netten et al. 1990; Choma et al. 2000). In one study, *B, cereus* grew at 8°C in milk; although toxin production was found to be low when cultures were grown without aeration (Christiansson et al. 1989). In a study evaluating 17 psychrotolerant strains, seven showed toxicity comparable to those associated with food poisoning strains (90% inhibition of protein synthesis in Vero cells) and six other strains were able to produce enough toxin to result in $\geq$20% inhibition, supporting the notion that these strains could pose a food safety problem (Stenfors and Granum 2001). And, while toxin production at reduced temperatures was not evaluated in this study, a second study demonstrated toxin production as low as 12°C, the lowest evaluated temperature (Stenfors and Granum 2001; Finlay et al. 2000). In fact, refrigerated storage has been linked to one *B. cereus* outbreak among four children, resulting in one death (Dierick et al. 2005). While a variety of factors, including pH, nisin, and variations in heat treatments can help control *B. cereus*, each of these methods have their own unique considerations when deciding which ones are appropriate for a given food as prolonged heat treatments and acidification can affect food quality (Beuchat et al. 1997; Valero et al. 2003). Additionally, a study evaluating the effect of nisin on growth at reduced temperature found an initial control of growth but found that this protective effect was lost in 6–14 days depending on temperature and nisin concentration, suggesting nisin degradation (Beuchat et al. 1997).

While many studies have evaluated the conditions at which *B. cereus* can grow, little is known about the precise mechanisms which enable the growth of this organism at

temperatures as low as 5°C. However, unlike *C. botulinum*, research has identified two paths that are likely to contribute to cold adaptation. These mechanisms may also be applicable to *C. botulinum* as they are both members of the Firmicutes phylum. The first mechanism observed for *B. cereus* was a shift in glucose metabolism pathways (Chung et al. 1976). When grown at 32°C, *B. cereus* reduced the pH of the medium and readily formed spores. However, when the culture temperature was adjusted to 7°C, sporulation was reduced and pH of the medium was found to increase, even though final viable counts were higher than those at the warmer temperature and glucose concentration was comparably reduced. These observations led to further evaluation of glucose catabolism at different culture temperatures. These studies indicated that at 32°C glucose was predominately metabolized by the glycolytic/EMP pathway. However, as temperature was reduced to 20°C and then 7°C, increasing amounts of glucose were metabolized via the pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle also showed increased activity, based on the accumulation of TCA cycle intermediates. It is tempting to speculate that the difference in metabolic pathways used for reduced temperature growth could also explain why *B. cereus* strains produce low amounts of toxin at similar temperatures but without aeration as the PP pathway and TCA cycle would increase the demand for oxygen.

A second set of studies identified a group of four proteins whose expression is upregulated when cultures are grown at 7°C (Mayr et al. 1996). Three of these were expressed poorly or not at all at 30°C but were highly expressed at 7°C. The most highly expressed of these three proteins was designated CspA. Four other proteins, clustered around CspA in 2D-PAGE analysis, were found to be expressed at 7°C and one of these was upregulated during cold growth. These four proteins copurifiied in a fraction with an apparent molecular weight of 60 kDa during isolation of CspA (7.4 kDa), suggesting the possibility of a protein complex. Immunoblot studies show that all four of these proteins cross-reacted with polyclonal antibodies against CspA. Sequencing of the genes encoding these four proteins showed a high degree of identity with the CspA gene, suggesting these proteins comprised a family of proteins all containing a putative DNA binding motif. Homologs of these genes are present in all *B. cereus* strains, as well as in other species such as *L. monocytogenes*. Further analysis of the *cspA* gene identified three nucleotides at positions 4, 9, and 124 that varied consistently with the psychrotolerant *B. cereus* strains compared to the mesophilic strains (Francis et al. 1998). The role these substitutions may play in cold adaptation is unknown; however, these differences were used to develop a PCR-based assay to readily identity psychrotolerant *B. cereus* isolated from food sources. Further evaluation of two of these proteins identified that CspA and CspE were capable of binding ssDNA and that these two proteins were capable of forming homodimers (Mayr et al. 1996). However, the precise functional role(s) of these proteins has yet to be elucidated. As members of this cluster also contain RNA binding motifs they have the potential to serve as RNA chaperones or as transcriptional regulators among a myriad of possibilities.

Some confusion exists as to whether psychrotolerant *B. cereus* strains are a true subset of the *cereus* species or represent a new species within the *B. cereus* group, *B. weihenstephanensis.* Based on differences in *cspA*, 16S rRNA genes, 23S rRNA genes, and the 16S–23S rRNA genes spacer regions, Lechner et al. identified the psychrotolerant members of the *B. cereus* group as a new species, *B. weihenstephanensis* (Lechner et al. 1998). However, subsequent work involving 26 *B. cereus* strains from a variety of sources revealed that the species split, as determined by *cspA*, and 16S rDNA PCR did not always coincide with psychrotolerant phenotypes (Stenfors and Granum 2001). While the PCR assay identified mesophilic and psychrotolerant organisms did correlate with phenotypic growth profiles, most of the strains (19 of 26) appeared as both mesophilic and psychrophilic organisms according to the dual 16S rDNA PCR products. Those

dual positive strains that also tested positive for the psychrophile *cspA* gene variant were classified as psychrotolerant organisms by phenotypic analysis. However, a subset of these dual 16S rDNA positive strains lacked the psychrophile *cspA* gene variant but still retained the ability to grow at 6°C. These data suggest that while the two species do exist, there are intermediates that do not fall neatly into one category or the other (Stenfors and Granum 2001).

Other Mechanisms of Cold Adaptation

While little is known specifically about the mechanisms used by *C. botulinum* and *B. cereus* to permit growth at refrigeration temperatures, some postulations can be made based on related species, such as *B. subtilis*, which has been better characterized though its low growth temperatures are not as low as refrigeration conditions. Adaptations in *B. subtilis* occur through alterations to improve membrane fluidity, a ribosome rescue system, and RNA helicases.

The first of these mechanisms has been fairly well characterized in *B. subtilis* and includes a molecular sensor two-component regulatory system, encoded by *desK* and *desR* that induces the transcription of *des*, a desaturase that converts membrane lipids from saturated fatty acids to unsaturated fatty acids (Mansilla et al. 2004). However, this upregulation is transient as *des* transcription is blocked after prolonged growth at the reduced temperature despite continued expression of *desK* and *desR*. A second feature altering membrane fluidity is the synthesis of anteiso-branched-chain fatty acids utilizing ketoacids, derived from isoleucine (Mansilla et al. 2004). Deprivation of isoleucine or the addition of unsaturated fatty acids altered activation of *des* with the former inducing and the latter blocking expression (Klein et al. 1999; Aguilar et al. 2001; Mansilla et al. 2004).

RNA stability mechanisms have also been implicated in cold adaptation of *B. subtilis*. Two DEAD box RNA helicases, CshA and CshB, have been implicated in cold adaptation helping to correct secondary structural abnormalities induced by reduced temperatures, with both genes showing cold-induced expression and a mutant with severely reduced expression showed a reduction in growth after cold shock with no differences in 37°C growth prior to shock (Beckering et al. 2002; Hunger et al. 2006). Double deletion mutations could not be studied as they were lethal (Hunger et al. 2006). Additionally, fluorescence resonance energy transfer (FRET) measurements showed interaction between CshB and CspB, a cold shock protein. CspB, along with CspC and CspD, comprise a family of cold shock proteins in *B. subtilis* also present in *B. cereus*. At least one of these proteins is required for growth, and double deletion mutants showed reduction in growth at low and high temperatures (Weber et al. 2001a, b). The *B. subtilis* Csp family of proteins appears to be involved in RNA stabilization as they colocalize with nucleoids during transcription and a *cspB cspC* double deletion mutant could be complemented by the *E. coli* translation initiation factor, IF1 (Weber et al. 2001a, b). Homologs of CshB and CshA are present in *B. cereus* and proteins with limited similarity are present in *C. botulinum* that could play similar roles in cold adaptation in these two species. However, the cold-induced *B. cereus* CspA protein, while similar to these Csps, appears to be unique to *B. cereus*. RNA stabilization also occurs through secondary structures found in the 3′ ends of some cold-induced transcripts which show no promoter induction at reduced temperatures despite increased levels of transcript (Nickel et al. 2004). Predicted secondary structures were postulated to play a role in this stabilization, however, as other sequences that were not cold-inducible have similar structures, other factors may also be involved (Nickel et al. 2004).

In addition to RNA stability, an adaptation mechanism involving the SsrA-SmpB ribosome rescue system has been characterized in *B. subtilis* (Shin and Price 2007). Deletion of either *ssrA* or *smpB* resulted in severe reductions in growth at low (16°C) and high (52°C) temperatures with minimal alterations were observed at 37°C. The five gene cluster is found largely only within *Bacillales* though not in all; *B. cereus* has the cluster conserved, while *L. monocytogenes* and *C. botulinum* have homologs of *smpB* and *ssrA* but not within the same five gene cluster (Shin and Price 2007). The question remains whether the cluster organization is integral to the cold adaptation response or if the genes themselves are the only components critical to adaptation.

Finally, a macroarray transcriptional analysis of cold-shocked *B. subtilis* identified 31 cold-induced genes, including previously characterized cold-induced genes such as the *des* system. Other cold-induced genes included ribosomal proteins, a putative elongation factor, initiation factors, ABC transporters, and an unknown protein (Beckering et al. 2002). An additional 79 genes showed reduction in transcription, mostly involved in amino acid, tRNA, purine and pyrimidine synthesis, and in basic metabolism. These systems functionally match other characterized cold adaptation mechanisms, which alter membrane lipid profiles and utilize RNA chaperones among other adaptive mechanisms and this functional similarity suggests that comparable mechanisms are present in *B. cereus* and *C. botulinum*, though the genes may vary.

Aeromonas spp.

Aeromonas spp. are Gram-negative, rod-shaped, facultative anaerobic bacteria classified into the family *Vibrionaceae* (Mary et al. 2002; Imbert and Gancel 2004; Janda and Abbott 2010). The organisms can be isolated from a variety of environments, such as food products, water distribution systems, or aquatic animals. (Edberg et al. 2007; Janda and Abbott 2010). Members of the genus *Aeromonas* have been recognized as an important pathogen in both human and animals. Particularly, *Aeromonas hydrophila* is included in the Candidate Contaminant List of the U.S. Environmental Protection Agency (US-EPA) under the Safe Drinking Act amendment of 1996 due to its ability to grow in water distribution systems (Edberg et al. 2007). While *Aeromonas* spp. can cause septicemia in immuno-compromised individuals, serious wound infections can also occur among healthy populations (Janda and Abbott 2010). In spite of evidence regarding *Aeromonas*-associated diarrhea cases, the etiologic role of the bacteria causing gastroenteritis still remains unclear (Knochel 1990; Janda and Abbott 2010).

The psychrotolerant nature of several strains of *Aeromonas* spp. has been well documented, leading to the public health concerns related to food processing and storage (Knochel 1990). Certain strains of *A. hydrophila*, isolated from various foods, have been shown to produce haemolysin at 10°C but not at 37°C (Wilcox et al. 1992). The growth of *Aeromonas* spp. in a variety of foods, such as seafood, raw milk or meats, increases 1–3 logs at 5°C and numerous motile *Aeromonas* spp. strains are able to grow at 5°C in different salt concentrations (Knochel 1990; Janda and Abbott 2010). In addition, *A. hydrophila* is also capable of growing, albeit slowly, at −2°C in vacuum-packed meat (Gill and Reichel 1989).

Unlike *L. monocytogenes* and *Y. enterocolitica*, the mechanisms underlying survival and growth of *Aeromonas* spp. at low temperature remains to be elucidated. However, emerging evidence has revealed some factors that may be responsible for cold adaptation. Cold shock proteins (Csps) in *A. hydrophila* were identified based on a PCR method (Francis and Stewart 1997; Imbert and Gancel 2004). Shifting temperature from 30°C to lower temperatures

resulted in the synthesis of several Csps detected by 2-dimentional polyacrylamide gel electrophoresis. In addition, more Csps are synthesized when cultures were shifted from 30°C to 5°C than from 30°C to 20°C (Imbert and Gancel 2004). Cold acclimation proteins (Caps) have also been identified from cultures undergoing a prolonged cold growth period. Like a psychrotolerant bacterium, *Arthrobacter globiformis*, the level of housekeeping protein expression in *A. hydrophila* did not significantly change in response to downshifted temperatures (Imbert and Gancel 2004). This may indicate a more specific adaptation to psychrophily as compared with a mesophilic bacterium, *E. coli*, to respond to cold environment (Hebraud and Potier 1999; Imbert and Gancel 2004). While more evidence regarding Csp and Caps such as that of *L. monocytogenes*, and *Y. enterocolitica* is required, it appears that these proteins may play a significant role in *Aeromonas* spp. cold adaptation.

Conclusions

Pathogens have generally evolved with an optimum growth temperature corresponding to their hosts. However, a subset of these pathogens has been shown to be psychrotolerant, capable of low temperature survival and growth. This enhanced capability of growth at refrigeration temperatures is, therefore, a major concern for public health.

Studies of psychrotolerant pathogens using genetic techniques, such as mutagenesis or complementation, have revealed a variety of genes and mechanisms responsible for cold adaptation. Cold shock proteins are present in most organisms across the three domains of life. Several cold shock proteins clearly contribute to cold adaptation of psychrotolerant pathogens indicated by the dependence of cold growth on cold shock proteins. Compatible solutes have also been shown to play a significant role in the growth of organisms at both ends of the temperature spectra. Although these psychrotolerant pathogens appear unable to synthesize these compatible solutes, they have evolved new transport systems to import these molecules into the cells. Finally, cell membranes undergo modification, changing the lipid composition and shortening and desaturating fatty acid chains, to maintain membrane fluidity.

The combination of high salinity and low temperature has been used in food processing and preservative for centuries. However, the recent discovery of the interplay between salt and cold tolerance in organisms, such as in *L. monocytogenes*, is a major concern as these conditions can lead to enrichment of psychrophilic bacteria. Better understanding of the mechanisms of cold adaptation in these pathogens will result in improved controls of these foodborne diseases.

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6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_6.5,

Introduction

Low temperature environments, and particularly those in polar regions, have long been a source of fascination for explorers, naturalists, and scientists. The remoteness and apparent severity of these regions continues to draw attention from scientists and nonscientists alike: the biological scientists are particularly attracted by the scope for investigating biological processes at the margins of the "biological envelope." Issues such as long-term survival of microbial cells (in ice cores), the structure and function of cryptic lithic communities, and the ability of organisms to adapt to temperatures approaching the freezing point of CO_2 all contribute to the fascination of this field of study.

There has been much discussion in past literature on the most appropriate terminology for the classification of organisms inhabiting cold environments: the terms "psychrotroph," "psychrophile," "psychrotolerant," and "cold-active" are all used to imply the ability to survive and grow at low temperatures, and are variously linked to particular physiological properties and/or cardinal growth temperature ranges. For the purposes of simplicity, in this chapter we use the term "psychrophile" in a generic sense to incorporate all organisms inhabiting cold environments, and the other terms as they have been employed in the relevant reference literature.

Psychrophilic microorganisms have long proved to be a fertile subject for microbial taxonomists, ecologists, physiologists, and geneticists. "Early" culture-dependent studies yielded a range of cold-active microbial isolates, which underpinned extensive physiological and biochemical studies on cold adaptation and led to an understanding of complex temperature-responsive processes such as solute accumulation, membrane lipid thermoadaptation, and temperature-controlled gene expression. Through the 1980s, psychrophilic protein structures were added to a catalog of mesophilic and thermophilic homologs, and comparative studies have led to a comprehensive understanding of the relationships between protein stability (and instability) and catalytic function.

Early microbiology studies, relying almost exclusively on microscopic and culture-dependent methods, showed a wide range of bacterial and lower eukaryotic species in psychrophilic habitats. However, in comparative terms, such studies generally supported the dogma that microbial diversity and environmental extreme were inversely correlated. With the development and widespread application of new molecular ecological methods in the 1990s, the microbial "landscape" of cold (and most other) environments changed dramatically. Phylotypic surveys clearly demonstrated that the dominant microorganisms in psychrophilic habitats were generally not those which had been identified by earlier culture-dependent studies. A series of fairly recent phylogenetic studies of soils from both poles suggest that microbial diversity is much greater than ever appreciated, and that a very high proportion of the extant organisms have little or no record of culturing.

It is estimated that 80% of the earth's biosphere displays an average temperature of 15°C, including many marine ecosystems. Specialized psychrophilic habitats include permafrost sediments, glaciers, Antarctic lakes, and deep marine ecosystems, and the microbiology of these sites has been discussed in other chapters. This chapter focuses on the microbial diversity associated with terrestrial soil biotopes in Antarctica (including maritime, the Dry Valleys and lithic communities), and Arctic habitats (alpine and Arctic tundra). Although the cold deserts in the Southern Hemisphere have been extensively studied, there is a lack of published research on soil ecosystems in the dry deserts of the Northern Hemisphere, including the Tibetan Plateau and Himalayan Cold Deserts, and future studies on this specific ecological niche are warranted.

Antarctica

Antarctica is the Earth's fifth largest continent, with a total landmass of approximately 14 million km^2, and is situated south of the Antarctic Circle surrounded by the Southern Ocean. Over 98% of the Antarctic continent is covered in ice and the average thickness of the ice-shelf is 1.6 km. Antarctica is the coldest, driest continent and experiences severe environmental and climatic conditions, including extremely low temperatures, low atmospheric humidity, low liquid water availability, and periods of high incident radiation coupled to long periods of complete darkness. The lack of liquid water severely restricts both macro- and microscopic life, and higher eukaryotes are restricted to the more northerly latitudes of the Antarctic Peninsula. Despite the severity of the climate, microbial communities have adapted to inhabit specialized environmental niches including desiccated mineral soils, nutrient-rich ornithogenic soils, ice (glacial and sea ice), ice-covered lakes, rocks, and geothermally heated soils. Several novel species have been isolated from terrestrial Antarctic habitats and a comprehensive list is provided in *Table 6.5.1*.

Maritime and Peninsula

Antarctic Peninsula soils contain complex communities of algae, mosses, lichens and liverworts, although the total macro- and micro-species diversity is low. Fungi are likely to play a fundamental role in these ecosystems as decomposers, and are essential for the development of soil structure and the transformation of soil nutrients (Wicklow and Söderström 1997). Fungal taxa which have been detected, include basidiomycetes, oomycetes and ascomycetes. The most prolific ascomycetes are yeasts and the dominant genera include *Aureobasidium, Candida, Cryptococcus* and *Rhodotorula* (Atlas et al. 1978).

In the warmer northerly latitudes of the Antarctic Peninsula and the offshore Signy and Marion Islands, the most common and widespread terrestrial ecosystem are fellfields. Fellfield soils are characteristically either moist with a high silt content or are drier, sand/gritty ash and are dominated by cryptogamic vegetation (plants that reproduce by spores) (Cowan and Ah Tow 2004). The distribution of microbial species within fellfield soils is nonhomogenous, with cyanobacterial species predominantly colonizing the upper 1 mm of soil, while nonmotile species occur at the periphery of frost polygons (Davey and Clarke 1991). Large microbial populations are associated with the plant communities in these soils. The organisms typically identified include the cyanobacterial species *Phormidium autumnale* and *Pseudoanabaena catenata,* as well as the diatom *Pinnularia borealis.* Smaller populations of *Achnanthes lapponica, Clamydomonas chlorostellata, Cosmarium undulatum, Cylindrocystis rebissonii, Netrium* sp., *Nostoc* sp. and *Planktospheaerella terrestris* have also been detected.

The coastal regions of Antarctica are more "temperate" compared to the severe climatic conditions experienced by the Antarctic Peninsula, as the maritime climate is more oceanic with smaller temperature fluctuations. The maritime regions discussed in this chapter include the western Antarctic Peninsula, South Orkney Islands, South Shetland and King George Islands, Prince Edward Islands, McDonald Island, South Georgia Island and Ross Island, most of which are located in the Southern Ocean and in the sub-Antarctic zone. Typically, maritime soils have a high water content, are rich in organic nutrients predominantly of marine origin (penguin guano and macroalgae dispersed by the wind), and are colonized by flowering plants and cryptogams (algae, lichens, and mosses) (Ludley and Robinson 2008), although maritime soils

Table 6.5.1

Novel microbial species isolated from soil ecosystems in Antarctica

Species	Taxonomic affiliation	Source	Growth range (°C)	Reference
Brevibacillus levickii	*Actinobacteria*	Geothermal soils, Mt Melbourne	15–55	Allan et al. (2005)
Cryobacterium psychrophilum	*Actinobacteria*	Antarctic soil	<18	Suzuki et al. (1997)
Friedmanniella antarctica	*Actinobacteria*	Sandstone, McMurdo Dry Valleys	18–25	Schumann et al. (1997)
Micromonospora endolithica	*Actinobacteria*	Sandstone, McMurdo Dry Valleys	8–39	Hirsch et al. (2004)
Modestobacter multiseptatus	*Actinobacteria*	Soil Asgard Range, Transantarctic Mountains	0–28	Mevs et al. (2000)
Pseudonocardia antarctica	*Actinobacteria*	Cyanobacterial mat, McMurdo Valley	7–38	Prabahar et al. (2004)
Streptomyces hypolithicus	*Actinobacteria*	Hypolithic community, Miers Valley	22–30	Le Roes-Hill et al. (2009)
Aequorivita sublithincola	*Bacteroidetes*	Quartz rock	2–25	Bowman and Nichols (2002)
Flavobacterium antarcticum	*Bacteroidetes*	Penguin habitat, King George Island	5–24	Yi et al. (2005a)
Flavobacterium segetis	*Bacteroidetes*	Penguin habitat, King George Island	5–21	Yi and Chun (2006)
Flavobacterium weaverense	*Bacteroidetes*	Soil, Weaver Peninsula	5–19	Yi and Chun (2006)
Sejongia antarctica	*Bacteroidetes*	Penguin habitat, King George Island	4–28	Yi et al. (2005)
Sejongia jeonii	*Bacteroidetes*	Moss sample, King George Island	4–31	Yi et al. (2005)
Alicyclobacillus pohliae	*Firmicutes*	Geothermal soil, Mt Melbourne	42–60	Imperio et al. (2008)
Aneurinibacillus terranovensis	*Firmicutes*	Geothermal soils, Mt Melbourne	20–55	Allan et al. (2005)
Sporosarcina antarctica	*Firmicutes*	Soil, King George Island	0–23	Yu et al. (2008)
Pseudoalteromonas antarctica	*γ-Proteobacteria*	Mud, Admiralty Bay, King George Island	4–30	Bozal et al. (1997)
Pseudomonas guinea	*γ-Proteobacteria*	South Shetland Islands	−4 to 30	Bozal et al. (2007)
Psychrobacter frigidicola	*γ-Proteobacteria*	Penguin colony, Vestfold Hills	−18* to 22	Bowman et al. (1996)
Psychrobacter urativorans	*γ-Proteobacteria*	Penguin colony, Vestfold Hills	−10* to 25	Bowman et al. (1996)

Table 6.5.1 (Continued)

Species	Taxonomic affiliation	Source	Growth range (°C)	Reference
Shewanella livingstonensis	*γ-Proteobacteria*	Sediment, Johnson's Dock, Livingston Island	4–20	Bozal et al. (2002)
Shewanella vesiculosa	*γ-Proteobacteria*	Sediment, Deception Island	−4 to 30	Bozal et al. (2009)
Eukaryotic species				
Cryptococcus albidosimilis	*Basidiomycota*	Soil, Linnaeua Terrace	4 to <20	Vishniac and Kurtzman (1992)
Cryptococcus antarcticus	*Basidiomycota*	Soil, University Valley	4 to <20	Vishniac and Kurtzman (1992)
Cryptococcus consortionis	*Basidiomycota*	Ross Desert	4–23	Vishniac (1985)
Cryptococcus lupi	*Basidiomycota*	Dolerite gravel, South Victoria Land	4 to <25	Baharaeen and Vishniac (1982)
Cryptococcus socialis	*Basidiomycota*	Ross Desert	4–23	Vishniac (1985)
Cryptococcus statzelliae	*Basidiomycota*	Soil, Lichen Valley, Vestvold Hills	<22	Thomas-Hall et al. (2002)
Dioszegia antarctica	*Basidiomycota*	Taylor Valley, South Victoria Land	<20	Connell et al. (2010)
Dioszegia cryoxerica	*Basidiomycota*	Taylor Valley, South Victoria Land	<20	Connell et al. (2010)

*Theoretical minimum growth temperature

are largely populated by algal, bacterial and fungal communities. The presence of nutrients contributes to the development of rich heterotrophic communities and several genera have been cultured from maritime sites. Using culture-dependant methods (epifluorescence microscopy and plate counts) *Arthrobacter, Corynbacterium* and *Micrococcus* species have been isolated from the volcanic ash-based soils of Deception Island (Cameron and Benoit 1970), while micrococci, *Aerobacter, Flavobacterium* and *Streptomyces* species have been detected in the organic-rich coastal soils of Cape Bird, Ross Island. Also using culture-dependant methods, the microbial diversity at 13 sites from King George Island lithogenic soils was assessed. Small, rod-shaped bacteria were found to be dominant at all the sites and accounted for more than 60% of the total population. Interestingly, of the 134 microorganisms isolated only 13 fungal species were identified. Most strains (both bacterial and fungal) were classified as psychrotolerant (able to grow at 5°C) and over half were viable at 30°C. While the isolates clustered into four multi-strain clusters, only a single cluster (the *Pseudomonas fluorescens* group) could be identified on the API database. Three strains belonging to single-member clusters were found to be related to *Agrobacterium radiobacterium, Pasteurella* sp. and *Sphingobacterium multivorum* species, respectively. Less than one third of the strains isolated in the study were identifiable according to published methods, which may be a reflection of the apparent novelty of the microorganisms colonizing Antarctica (Zdanowski and Wêgleñski 2001).

While traditional culture-based studies are invaluable in assessing the diversity of psychrophilic environments, the main disadvantage of such methods is that many of the slow growing

or fastidious microorganisms will be missed (Smith et al. 2006). Therefore, by using culture-independent, metagenomic methods to study microbial diversity, the more rare microorganisms can typically be detected. These techniques were applied in a recent study of five sites ornithogenic from the Ross Sea region, which included four mineral and one ornithogenic site(s) (Aislabie et al. 2008). In this study 16S rRNA gene clone libraries were constructed and over a thousand clones were analyzed and grouped into phylotypes based on restriction fragment length polymorphism (RFLP) patterns. Phylogenetic analysis revealed that the majority of the taxa were rare. Clones derived from the ornithogenic site were most closely related to endospore-formers (including *Clostridium acidiurici, Oceanobacillus profundus* and *Sporosarcina aquimarina*), while the mineral soil clones were most similar to other uncultured environmental clones. Dominant phyla identified included *Acidobacteria*, *Actinobacteria* (represented by the genera *Arthrobacter, Friedmanniella* and *Rubrobacter*), *Bacteroidetes* ("Chitinophaga," *Gillisia* and *Hymenobacter*), *Deinococcus-Thermus, Firmicutes, Proteobacteria* (*Psychrobacter*) and several species of *Cyanobacteria* (Aislabie et al. 2008). Although cyanobacteria have been identified in some maritime zones, as was shown on Ross Island, they are not the main phototrophs in these regions, unlike in the Antarctic Peninsula fellfields. Mosses and lichens are the most important primary producers and may in turn be colonized by microbial epiphytes and form the basis of the food web (Vincent 1988).

Many studies investigating the fungal diversity of maritime sites have focused on decomposing *Basidiomata* species and the majority of the strains isolated thus far are psychrotrophic. Several basidiomycete species have been identified from the South Georgia, Prince Edward and McDonald Islands, including *Dacrymyces stillatus* and *Trametes versicolor* (both from timber) (Pegler et al. 1980). *Collybia, Galerina* and *Omphalina* species were isolated from the grass litter which accumulated at the base of tussock pedestals on South Georgia Island (Smith 1994). Similarly, *Coprinus* and *Galerina* species have been cultured from a bryophyte bog, while strains of *Coprinus martini* have been found to be associated with the dwarf shrub *Acaena magellnica* (Ludley and Robinson 2008). On Ross Island diverse fungal populations including *Alternaria, Aspergillus, Aureobasidium, Cladosporium, Chrysosporium, Epicoccum, Mortierella, Penicillium, Phialophora* and *Trichoderma* species have been identified (Vincent 1988).

Continental

The temperatures experienced in the oasis regions of continental Antarctica are similar to those that occur in the Dry Valleys. However, continental Antarctica is only ice-free during the Antarctic winter, and there are increased levels of precipitation (Walton 1984). In the Schirmacher Oasis and Queen Maud Land soils, the dominant bacterial species present were found to be Gram-negative rods (Shivaji et al. 1989). Based on biochemical and physiological tests these isolates were assigned to the genus *Pseudomonas*. Most of the pseudomonads identified were psychrotrophs (capable of growth from 4°C to 30°C) and halotolerant (grew in the presence of 5.8% NaCl). Eight strains produced a fluorescent green pigment and were tentatively classified as *P. fluorecens* species, while the remaining two strains were possibly either *P. putida* or *P. syringae* species (Shivaji et al. 1989). Other Gram-negative bacilli, which have been isolated from continental soils include *Achromobacteria* and *Alcaligenes* species, while halotolerant *Planococcus* species have been characterized from saline continental soils (Vincent 1988). The microbial community present in ornithogenic soils from an Adélie penguin colony, Vestfold Hills, was found to be dominated by large populations of Gram-negative coccoid

bacteria (Bowman et al. 1996). Based on phospholipid and lipid profiles most were found to belong to the genus *Psychrobacter*. Members of this genus are psychrotolerant/psychrophilic and halotolerant, which may account for their ubiquitous distribution in many Antarctic marine and terrestrial habitats (Bowman et al. 1996).

The bacterial communities present in soil samples collected from Marble Point, Wright Valley (the Dry Valleys are discussed separately in The Dry Valleys) and Victoria Land were analyzed by metagenomic (16S rRNA gene clone libraries) and traditional culturing techniques. Marble Point (which had the highest soil moisture content of 45%) had the highest number of culturable heterotrophs, while relatively few microorganisms were cultured from Mt. Fleming, which had no detectible liquid soil moisture. Seventy-one bacterial isolates and 728 clones were grouped by RFLP patterns. Dominant ribotypes belonged to phyla which have previously been identified from Antarctic soils including *Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteris* and *Thermus-Deinococcus*. Several clones/bacterial isolates were most similar to phylotypes previously isolated from Antarctic sources, such as cryptoendolithic cyanobacterial communities from Beacon sandstone (Aislabie et al. 2006).

The fungal diversity of historic sites (Discovery Hut, Cape Evans Hut and Cape Royds Hut, Ross Island) and "pristine" sites (Lake Fryxell Basin, Mt. Fleming and Allan Hills) has been compared by both culturing and molecular methods (Arenz et al. 2006). Historic sites were found to be dominated by *Cadophora, Cladosporium* and *Geomyces* species, while the most common taxa at the pristine sites were cladosporioides. Denaturing gradient gel electrophoresis (DGGE) analysis detected 28 taxa not identified by culturing including four taxa with less than 50% similarity to ITS sequences on the GenBank database. BLAST analysis of the ITS sequences identified 39 fungal genera, of which 74% were filamentous ascomycetes, 21% basidiomycetous yeasts, 1% ascomycete yeasts and 1% zygomycetes. The dominant genera present were *Cadophora* (21%), *Geomyces* (14%), *Cladosporium* (13%) and *Cryptococcus* (12%). *Cadophora* species were found to be most prevalent in soil and wood samples. Filamentous fungi and yeasts were equally represented at pristine sites (Arenz et al. 2006). Another study found that *Aureobasidium* species were detected in 25% of the Victoria Land sites investigated, while mosses from Victoria Land have been shown to be colonized by *Rhodotorula minuta* species (Tosi et al. 2002; Ludley and Robinson 2008).

The Dry Valleys

Less than 2% of the Antarctic continent is ice-free and the Dry Valleys of Eastern Antarctica account for 0.03% (4,800 km^2) of the total land area. The Dry Valleys are considered to be the Earth's coldest, driest desert and experience extreme climatic conditions (low humidity, strong winds and low precipitation). In addition, the region experiences wide temperature fluctuations and repeated freeze-thaw cycling. The soils contain little organic matter, have a high salt concentration and the water content is low (0.5–2% wt) (Cowan and Ah Tow 2004). It has been proposed that the survival of microorganisms in Dry Valley mineral soils is dependent on their hydration state, the presence of compatible solutes, as well as their ability to alter their metabolism to synthesize cryoprotectants (Cameron 1969). Early studies found that both the species and biomass levels were low, with microbial cell counts in mineral soils ranging from 10^2 to 10^4 per gram soil. However, a subsequent study found that based on *in situ* ATP analysis the microbial biomass levels were three to four orders of magnitude greater than previously

reported (Cowan et al. 2002). Primary production in the Dry Valleys is limited as there are no vascular plant species and until recently it was proposed that the ecosystem was primarily driven by abiotic rather than biotic factors. However, it has now become apparent that photosynthetic microorganisms may play an important role in these habitats. Cyanobacteria present in Dry Valley mineral soils contribute significantly to the microbial diversity, and have been shown to increase both the stability of soil and the nutrient concentration (by nitrogen fixation). The dominant species identified in early studies included *Calothrix*, *Nostoc* and *Scytonema* species (Vincent 1988). From several recent metagenomic studies employing methods such as ARISA, T-RFLP and 16S rRNA gene clone libraries (Wood et al. 2008; Pointing et al. 2009) it is apparent that cyanobacterial diversity is more complex and varied than originally described. Several soil habitats in the Beacon and Miers Valleys have been analyzed, and included dry and moist soils, hypoliths, lake soils and microbial mats. By comparing the diversity of cyanobacteria at the different sites the role of aquatic cyanobacteria as a source of organic matter in terrestrial communities could be investigated. Soil samples from the Beacon Valley were found to be lacking in cyanobacterial signals and the authors proposed that the absence of lakes and ponds in Beacon Valley was a contributing factor. Conversely, multiple cyanobacterial species were detected in Miers Valley soil (4–27 ATLs in the ARISA profiles). Notably, a significant number (29–58%) of the ARISA fragment lengths found in aquatic cyanobacterial mats were also present in soil and hypolith samples. It was concluded that lacustrine and hydroterrestrial cyanobacteria play an important role in structuring the soil communities at these sites. The occurrence of the same species in aquatic and terrestrial habits in the Miers Valley was confirmed by 16S rRNA gene clone libraries, and *Chroococcales, Nostocales* and *Oscillatoriales* phylotypes were indentified in the soil samples (Wood et al. 2008).

Despite the abundance of cyanobacteria, most microorganisms isolated from Dry Valley mineral soils are aerobic heterotrophs which are predominantly psychrotrophic, as psychrotrophs are better adapted to survive the temperature fluctuations experienced at the soil-air interface. Chromogenic bacteria are routinely found at the soil surface, while nonpigmented bacteria occur mainly below the soil surface (Cameron et al. 1970; Cowan and Ah Tow 2004). Early studies reported that coryneforms were the most abundant bacteria isolated from Dry Valley soils, of which the majority (85%) were strains of *Corynebacterium sepedonicum* (Cameron et al. 1972).

Actinobacterial populations appear to be cosmopolitan in Antarctic soils and have been identified as the dominant phylotype in both cultured-based and metagenomic studies. A recent study focusing on the diversity of actinobacteria present in Miers Valley mineral soils isolated *Nocardia, Pseudonocardia* and *Steptomyces* species. All three genera cultured in this study had previously been isolated from Dry Valley soils (Cameron 1972; Prabahar et al. 2004) and *Streptomyces* species accounted for over 80% of the strains isolated (Babalola et al. 2009). Similarly, *Arthrobacter*, *Friedmanniella* and *Rubrobacter* species have been isolated from the Wright Valley (Aislabie et al. 2006). Members of the class *Actinobacteria* were also amongst the heterotrophic microbial populations identified in early culture-based studies on McMurdo Dry Valley soils. The bacterial populations isolated in these studies included *Arthrobacter, Corynebacterium, Micrococcus, Nocardia and Streptomyces* species (Cameron 1972). Subsequently, novel species belonging to these genera have been identified from diverse Antarctic habitats. Several *Flavobacterium* species have been isolated including *Flavobacterium gillisiae* (sea ice from Prydz Bay), *F. hibernum* (oligotrophic freshwater lake, Vestfold Hills), *F. tegetincola* (cyanobacterial mat), and *F. xanthum* (formerly "*Cytophaga xantha*" Inoue and Komagata, 1976, mud pool near Syowa Station). "*Flavobacterium salegens*" was originally

misclassified as a *Flavobacterium* species and was later elevated to the novel genus *Salegentibacter as Salegentibacter salegens* (type species). This strain was isolated from Organic Lake, a meromictic hypersaline lake in the Vestfold Hills (Dobson et al. 1993; McCammon et al. 1998; McCammon and Bowman 2000). Novel *Arthrobacter* species have been characterized from Antarctica and include *Arthrobacter ardleyensi* (lake sediment), *A. flavus* (pond, McMurdo Dry Valley), *A. gangotriensis* (penguin rookery soil), *A. kerguelensis* (sea water Kerguelen Islands), *A. psychrochitiniphilus* (Adélie penguin guano) and *A. roseus* (cyanobacterial mat) (Reddy et al. 2000, 2002; Gupta et al. 2004; Chen et al. 2005; Wang et al. 2009). Several pseudomonads have been characterized from Antarctic aquatic habitats: *P. antarctica, P. meridian*, *P. proteolytica* (all from cyanobacterial mats) and *P. pelagia* (green algae) (Reddy et al. 2004; Hwang et al. 2009). *Planococcus antarcticus* was isolated from a cyanobacterial mat in Antarctica (Reddy et al. 2002).

Smith et al. (2006) conducted an extensive metagenomic survey of three diverse soil biotopes occurring in the Dry Valleys. Analysis of the 16S rRNA gene clone libraries revealed that a high proportion of the phylotypes were classified as "uncultured" and relatively few phylotypes were related to identifiable taxa. Members of the classes *Acidobacteria, Actinobacteria* and *Bacteroidetes* were found at all three sites. In addition, phylotypes related to *Chloroflexi, Cyanobacteria,* α-*Proteobacteria,* β-*Proteobacteria* and *Verrucomicrobia* were identified at selected sites. In general, the sequence identities to known phylotypes (on the GenBank database) were low (>91%), which suggests that Dry Valleys mineral soils are a source of novel species and genera (similar findings have been reported elsewhere; Wood et al. 2008; Babalola et al. 2009). The largest represented phylotypes were *Actinobacteria* (26%), *Acidobacteria* (16%) and *Cyanobacteria* (13%), although cyanobacteria were only detected in the fine gravel soil from Penance Pass, a high-altitude site between the Miers and Shangri La Valleys. The identification of actinobacteria at all sites is consistent with findings from culture-based studies (Buckley and Schmidt 2002). Surprisingly, several taxa which are frequently isolated in culture-based studies such as *Achromobacter, Bacillus, Corynebacterium, Micrococcus, Planococcus* and *Pseudomonas* were not represented in the 16S rRNA gene clone libraries. Conversely, the genera *Acidobacteria, Bacteroidetes* and *Verricumicrobia*, which are not routinely represented in culture-dependent studies, were widely distributed in the clone libraries (Smith et al. 2006).

Yeasts appear to be relatively abundant in the surface horizons of certain Antarctic soils (Cameron 1972), and although yeasts are the only fungal type detected at many sites within the Dry Valleys, they only account for a small percentage of the total heterotrophs present at many sites (McMurdo Station 10% of the total heterotrophic population, Strand Moraines 0.5%, and Wheeler Valley 0.001%) (Vincent 1988). While filamentous and single-cell fungi have been isolated from different Antarctic soil types, only yeasts are known to be endemic to the drier polar desert soils (Vishniac and Klinger 1986; Vishniac 1993). Culture-independent molecular methods have detected yeast (*Malassezia* species) and members of the genus *Hohenbuehelia* (this genus includes wood decomposers and nematophagous fungi) in Dry Valley soils (Ludley and Robinson 2008). Fell et al. (2006) detected yeast belonging to the genus *Trichosporon* and an unidentified clade similar to *Termitomyces*-fungi in McMurdo Dry Valley mineral soils (Fell et al. 2006), while diverse populations of cosmopolitan fungi have been isolated from McMurdo Dry Valley lakes, as well as from areas associated with human activity (Baublis et al. 1991). *Aureobasidium* species have been isolated from Dry Valleys, Strand Moraines and McMurdo Sound (Ludley and Robinson 2008). Connell and coworkers investigated the distribution of filamentous and nonfilamentous

yeast-like fungi in Taylor Valley mineral soils. The distribution of filamentous fungi appeared to be closely associated with soil pH (dominant in alkaline soils) and moisture content. Conversely, there did not appear to be an apparent link between the distribution of yeasts and soil conditions, and yeasts were found to colonize a broader range of habitats (Connell et al. 2006).

Lithic Communities

Porous translucent rocks are a specialized microhabitat for microbial colonization and offer physical protection from UV radiation and scouring by wind. Additionally, rocks trap moisture and act as a water source (Cockell et al. 2003; Cockell and Stokes 2004). In the Dry Valleys, fine-grained beacon sandstone is most often colonized, although quartz rock and limestone may also be inhabited (Friedmann 1993; Cowan and Ah Tow 2004). Lithic communities are classified by the specific environmental niche they reside in, and hypoliths, chasmoliths, and cryptoendoliths are discussed below.

Hypoliths are photosynthetic microbial communities that colonize the underside of translucent rocks and stones (Broady 1981b; Thomas 2005). Although the rock itself limits the amount of radiation that can penetrate, it has been shown that photosynthesis can occur at irradiance levels less than 0.1% of the total incident light (Thomas 2005) and the productivity of hypolithic communities may be equal, or greater, to that of the above-ground biomass (Cockell and Stokes 2004). The most commonly observed type of hypolithon are green and/or red pigmented cyanobacterial layers that occur at the rock-soil interface colonizing the base and sides of rocks. Hypolithic communities are dominated by microthallate cyanobacteria including *Leptolyngbya* and *Phormidium* species, as well as *Chroococcidiopsis*- and *Synechococcus*-like cells (Smith et al. 2000; Cockell and Stokes 2004; Pointing et al. 2009). Vestfold Hills hypolithic communities have been studied by cultured-based and metagenomic methods (Smith et al. 2000). Heterotrophic bacteria detected in this study included α- and γ-*Proteobacteria*, members of the order *Planctomycetales* and actinobacterial species. Several bacterial taxa were detected in the hypolithon and the underlying soil including *Achromobacter, Arthrobacter, Gelidibacter, Janibacter, Micrococcus, Pseudomonas, Psychrobacter, Rhodococcus* and *Stenotrophomonas* species. Bacterial phylotypes present in the clone libraries which were not detected by culturing methods included the actinobacterial genera *Acidimicrobium, Microthrix* and *Rubrobacter*. Cyanobacterial communities were found to be dominated by *Chroococcidiopsis* and *Synechococcus* phylotypes; however, the overall cyanobacterial species diversity detected was low (Smith et al. 2000).

Chasmoendoliths inhabit cracks in weathering rocks, which are common in the ice-free areas where repeated freeze-thaw cycling causes fracturing of the rocks (Broady 1981; Cowan and Ah Tow 2004). Chasmoendolithic communities are colonized by endolithic lichens (fungal mycobionts with the phycobiont *Trebouxia*) associated with cyanobacteria, typically *Chroococcidiopsis* or *Gloeocapsa* species (Nienow and Friedmann 1993). A recent study investigating the microbial diversity present in different lithic communities in McKelvey Valley (McMurdo Dry Valleys) found that chasmoliths and endoliths only occurred in above-ground sandstone. While both bacterial and eukaryal phylotypes were detected, eukaryotic (fungal and algal) signatures accounted for $<5\%$ of the recoverable phylotypes. Algal diversity was limited to two closely related groups within Trebouxiophyceae and a single *Bracteococcus* phylotype. Fungal phylotypes identified belonged to the classes *Dothideomycetes*, *Sordariomycetes* (both Ascomycota) and *Cystobasidiomycetes* (Basidiomycota). Microscopic and molecular analysis

found that *Chroococcidiopsis*-like cyanobacterial morphotypes were the dominant bacterial phylotype present (Pointing et al. 2009) (*Fig. 6.5.1*).

Cryptoendolithic organisms reside in the interstices of crystalline rocks and microbial colonization is dependent on the geological features of the rock substrate (Friedmann and Ocampo 1976). Two types of cryptoendolithic communities have been identified, namely, lichen- and cyanobacterium-dominated assemblages and the communities have clear zonal stratification. Lichen-dominated communities typically have an upper lichenized zone of

Fig. 6.5.1

(a) Scott Base, Marion Island; (b) Beacon sandstones in University Valley, East Antarctica. The pinkish coloration derives from accelerated weathering, largely caused by cryptoendolithic microbial populations; (c) hypolithic microbial community on beach sandstone; (d) penguin colony; (e) Tibertan desert; (f) Arctic terrestrial habitat. Attribution (a) L Rohr (b) DA Cowan (c) M Stevens, Adelaide, AU (d) SC Cary, University of Waikato, (e) S Pointing, University of Hong Kong (f) L Ovreas, University of Bergen, Norway NZ

mycorrhizal fungi associated with the green algal symbiont *Trebouxia*, while the lower zone is dominated by cyanobacteria. Heterotrophic organisms frequently identified from these communities include Gram-positive cocci and several actinobacterial genera (Siebert and Hirsch 1988; Nienow and Friedmann 1993). The microbial biodiversity present in McMurdo Dry Valley sandstone cyanobacterium- and lichen-dominated cryptoendolithic communities has been assessed by molecular methods. Phylogenetic analysis revealed that no phylotypes were shared between the cyanobacterium- and lichen-dominated communities. Within the lichen-dominated community three dominant phylotypes were identified: a fungal phylotype (*Texosporium sancti-jacobi* species), a green algal phylotype (*Trebouxia jamesii* species) and a chloroplast phylotype with 90% sequence identity to *Koliella sempervirens* chloroplast. A third of the clones from the cyanobacterium-dominated library were related to other cyanobacterial sequences from Antarctic lake-ice covers and sublithic soils. These clones were related to *Plectonema* species (filamentous cyanobacterium). The second phylotype contained clones which were most similar to *Blastomonas* species (α-Proteobacteria), while the third dominant phylotype contained clones which were distantly related to the genus *Deinococcus* (de la Torre et al. 2003).

The Arctic

The Arctic Circle includes parts of Alaska, Canada, Europe, Greenland, Iceland and Russia, and is dominated by the Arctic Ocean (*Fig. 6.5.3*). Arctic terrestrial habitats are estimated to cover more than 7 million km^2. The physical geography of the Arctic includes polar deserts in the "high Arctic," permafrost areas, tundra and alpine soils. Minerals and fossil fuels present in Arctic soils can support diverse populations of organisms, both macro- and microscopic, many of which are capable of cold adaption. The Arctic Circle experiences long, dark winters broken by short summer periods (maybe less than 6 weeks per annum) when the average temperature during the day is above 0°C (Heal 2000; Callaghan et al. 2010).

In addition to the extremely low average temperatures, several other factors place stress on cold-adapted organisms in the Arctic, including soils varying from a flooded state during the spring thaw to periods of drought during the late summer as a consequence of inconsistent rainfalls. Tundra soils are further stressed by the occurrence of freeze-thaw cycles disrupting the community structure, while permafrost and persistent snow coverage place additional stress on soil communities (Callaghan et al. 2010).

The tundra is devoid of higher vascular plants (tree growth is restricted as a consequence of low temperatures) and these areas are vegetated by dwarf shrubs, lichens and mosses. Northern Hemisphere tundra is divided into two subgroups, namely Arctic tundra and alpine tundra (the latter is discussed in Alpine tundra). Arctic tundra is found further north (north of the Taiga Belt) and is characterized by the absence of trees (due to the presence of permafrost), whereas alpine tundra lacks trees as a consequence of high altitude (*Fig. 6.5.3*) (*Table 6.5.2*).

Arctic Tundra

Microorganisms inhabiting tundra soils are challenged by low temperatures, extended periods in frozen conditions, limited nutrients, low pH and high water content (as a consequence of

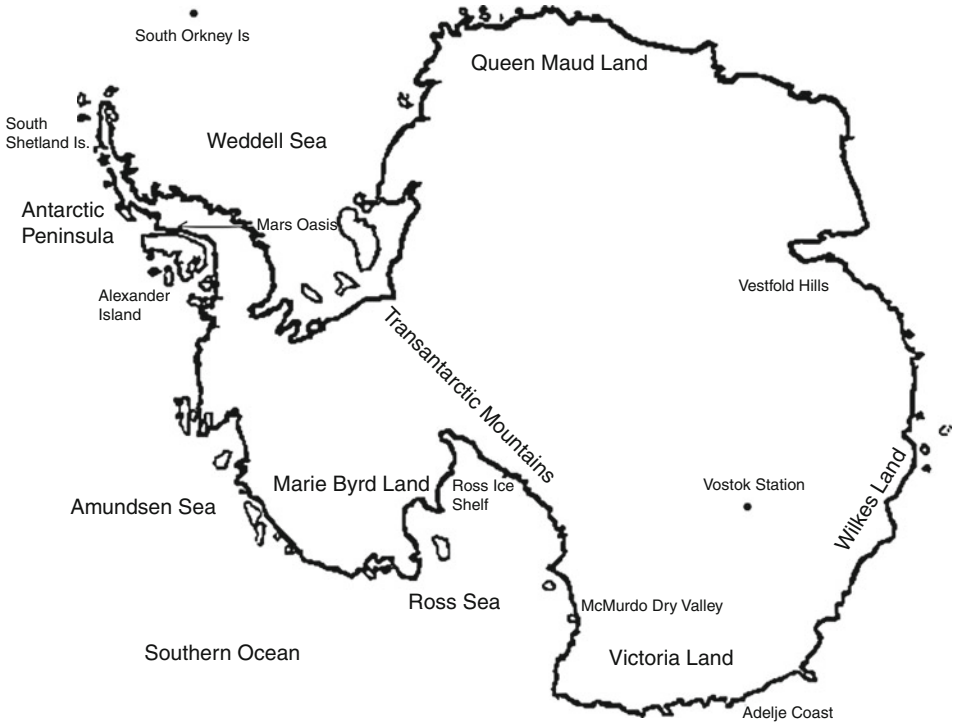

Fig. 6.5.2
Continental Antarctica and offshore islands

inadequate drainage). These stressful conditions, combined with the large reservoirs of stored organic carbon in the Arctic tundra, are believed to favor the growth of psychrophilic methanotrophs (Wei et al. 2003). Methanogens have been isolated from spagnum peat bogs of boreal and tundra soils of Western Siberia and North Russia, as well as acidic, oligotrophic forest soils in Northern Germany. Analysis of the 16S rRNA gene revealed that the isolates represented previously uncharacterized species from two novel genera, namely, *Methylocella* (*Methylocella palustris*, *M. silvestris*, and *Methylocella tundra*) and *Methylocapsa* (*Methylocapsa acidiphila*) (Trotsenko and Khmelenina 2005). These genera are related to the genera *Methylosinus* (which has previously been observed in Arctic soils (Wartiainen et al. 2003) and *Methylocystis*. Similarly, *Methylobacter psychrophilus* has been observed in Canadian Arctic tundra soil (Omelchenko et al. 1996; Tourova et al. 1999; Pacheco-Oliver et al. 2002). In a Canadian-based study, *M. luteus* and *M. fundripaludum* were isolated from Eureka soil, Ellesmere Island, Nunavut. *M. luteus* and *M. fundripaludum* were identified as the dominating methane-degrading bacteria at 25°C and 4°C, respectively (Martineau et al. 2008).

A number of studies have isolated microorganisms capable of biodegradative processes from Arctic tundra soils. While the majority of the studies focussed on bacteria, algal and fungal (discussed below) representatives have also been shown to contribute to biodegration in these polar soils. *Acetobacterium tundra* is a novel acetogenic organism (most closely related to *Acetobacterium paludosum*) that was isolated from slightly acidic (pH of 6.1) tundra wetland soil of the Polar Ural, the northern most region (latitude 65°N) of the Ural, which forms the

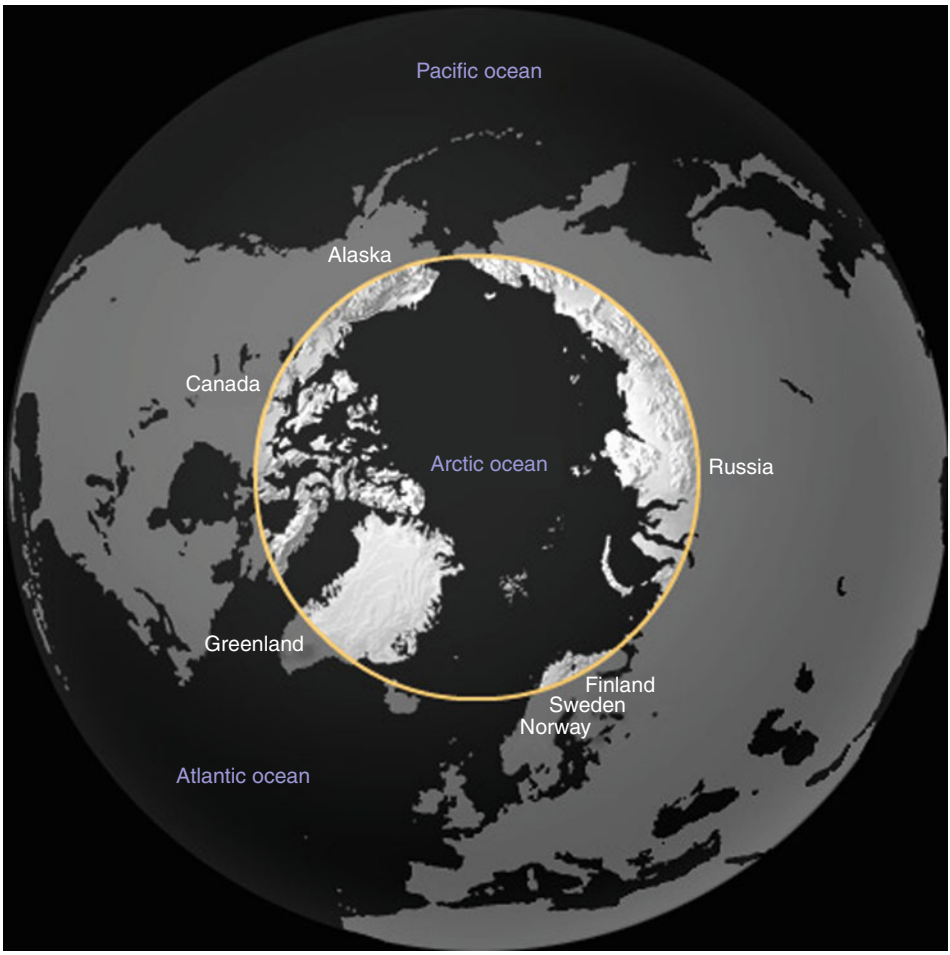

Fig. 6.5.3
The Arctic Circle (Oracle Education Foundation, 2010)

boundary between Europe and Asia (Simankova et al. 2000). Additionally, a study on Siberian tundra soils identified a lipolytic psychrotrophic bacterium belonging to the genus *Acinetobacter* (Wei et al. 2003; Suziki et al. 2009). As in Antarctica, bacteria play a key role in the decomposition of guano in the Arctic, and the nutrient-rich, ornithogenic tundra soils provide a specialized habitat for microbial colonization. Guano samples collected from Little Auk, Spitsbergen were found to be dominated by members of the phylum *Firmicutes*, as well as the families *Flavobacteriaceae* and *Moraxellaceae* (Zmuda-Baranowska et al. 2008). Two psychrotolerant strains closely related to *Pedobacter cryoconitis* and *P. himalayensis* were isolated from soils and decaying lichen from forests in the Finish Arctic. The strains were capable of growth between −4°C and 30°C, and produced large quantities of extracellular polymeric substances (Männistö et al. 2008a). Several studies conducted on tundra soils have focused on hydrocarbon-contaminated sites. Labbé and co-workers (2007) compared the diversity of hydrocarbon-degrading bacteria in pristine and contaminated soils from Tyrol,

Table 6.5.2

Novel microbial species isolated from soil ecosystems in the Arctic and alpine tundra

Species	Taxonomic affiliation	Source	Growth temperature (°C)	Reference
Edaphobacter aggregans	*Acidobacteria*	Forest soil, Bavaria	15–37	Koch et al. (2008)
Edaphobacter modestus	*Acidobacteria*	Alpine rendzina, Bavaria	15–30	Koch et al. (2008)
Actinoalloteichus spitiensis	*Actinobacteria*	Cold desert, Himalayas	20–37	Singla et al. (2005)
Agrococcus lahaulensis	*Actinobacteria*	Cold desert, Himalayas	25–33	Mayilraj et al. (2006b)
Arthrobacter alpinus	*Actinobacteria*	Alpine soil, Austria	1–25	Zhang et al. (2009c)
Arthrobacter psychrophenolicus	*Actinobacteria*	Austrian alpine ice cave	1–25	Margesin et al. (2004)
Rhodococcus kroppenstedtii	*Actinobacteria*	Cold desert, Himalayas	10–37	Mayilraj et al. (2006b)
Dyadobacter psychrophilus	*Bacteroidetes*	Soil, South Tyrol, Italy	1–30	Zhang et al. (2009c)
Mucilaginibacter gracilis	*Bacteroidetes*	Western Siberia	2–33	Pankratov et al. (2007)
Mucilaginibacter paludis	*Bacteroidetes*	Western Siberia	2–33	Pankratov et al. (2007)
Pedobacter cryoconitis	*Bacteroidetes*	Alpine glacier cryoconite	1–25	Margesin et al. (2003)
Rhodonellum psychrophilum	*Bacteroidetes*	Ikaite tufa columns, Ikka Fjord	0–22	Schmidt et al. (2006)
Deinococcus alpintundrae	*Deinococcus-Thermus*	Mount Evans, USA	5–25	Callegan et al. (2008)
Deinococcus altitudinis	*Deinococcus-Thermus*	Mount Evans, USA	5–25	Callegan et al. (2008)
Paenibacillus tundrae	*Firmicutes*	Alaskan tundra	13–37	Nelson et al. (2009)
Paenibacillus xylanexedens	*Firmicutes*	Alaskan tundra	13–32	Nelson et al. (2009)
Planococcus stackebrandtii	*Firmicutes*	Soil, Himachal Pradesh, India	15–30	Mayilraj et al. (2005)
Acidisoma sibiricum	*α-Proteobacteria*	Siberian Wetland	2–30	Belova et al. (2009)
Acidisoma tundrae	*α-Proteobacteria*	Siberian Wetland	2–30	Belova et al. (2009)
Asticcacaulis benevestitus	*α-Proteobacteria*	Tundra wetland soil, Russia	4–28	Vasilyeva et al. (2006)

Table 6.5.2 (Continued)

Species	Taxonomic affiliation	Source	Growth temperature (°C)	Reference
Methylocella tundra	*α-Proteobacteria*	Acidic tundra peatlands	5–30	Dedysh et al. (2004)
Sphingopyxis bauzanensis	*α-Proteobacteria*	Soil, South Tyrol, Italy	1–25	Zhang et al. (2009)
Luteimonas terricola	*γ-Proteobacteria*	Soil, South Tyrol, Italy	1–25	Zhang et al. (2009)
Eukaryotic species				
Candida linzhiensis	*Ascomycota*	Linzhi District, Tibet, China	<28	Wu and Bai (2006)
Candida tibetensis	*Ascomycota*	Linzhi District, Tibet, China	<28	Wu and Bai (2006)
Mrakiella cryoconiti	*Basidiomycota*	Siberian sediment and cryoconite	1–20	Margesin and Fell (2008)
Rhodotorula glacialis	*Basidiomycota*	Glacier cryoconite	1–20	Margesin et al. (2007)
Rhodotorula psychrophenolica	*Basidiomycota*	Mud, glacier foot	1–20	Margesin et al. (2007)

western Austria (600–2,900 m above sea level). There was a wide variation in the pH of the alpine soils (pH ranged from 4.8 to 9.2), and the organic matter content was less than 2%. The authors identified complex communities of oil-degrading, cold-adapted heterotrophs. Phylogenetic investigation revealed the presence of members of the order *Actinobacteria*, as well as α-, β- and γ-*Proteobacteria* (Labbé et al. 2007). In a similar comparative molecular study on oil-contaminated versus pristine soils from various alpine sites in Tyrol, phylotypes related to *Acinetobacter* sp., *Mycobacterium* sp., *P. putida* and *Rhodococcus* sp. were identified through 16S rRNA gene sequence analysis (Margesin et al. 2003b). Similarly, several phenol-degrading species such as *A. psychrophenolicus, Pseudomonas* and *Rhodococcus* species have been isolated from contaminated alpine soils (Margesin et al. 2003, 2005, 2007).

Samoylov Island, Lena Delta, Siberia, is situated in a continuous permafrost zone and the upper soil layer (0–23 cm) remains frozen for 8 months of the year. Tundra soils from the island were analyzed by *in situ* hybridization with rRNA gene-targeted oligonucleotide probes. *Archaea*, *Proteobacteria* (α-, β- and γ-subclasses) and members of the *Cytophaga-Flavobacterium* cluster were detected (Kobabe et al. 2004); however, the dominant species identified in another study were related to γ-*Proteobacteria* (Nemergut et al. 2005). Several of the dominant phyla identified by Kobabe and co-workers have been detected in other studies conducted at other sites in the Arctic tundra including Lapland (Männistö and Häggblom 2006; Männistö et al. 2008). Lapland is situated mainly in the Arctic Circle and includes parts of Finland, Norway, Russia and Sweden. In an extensive study aimed at characterizing the bacterial populations of the Finnish Lapland, several oligotrophic, lichen-governed forest soils were sampled. Nearly 200 forest and tundra soil strains were identified through partial 16S rRNA gene sequence analysis. The isolates belonged to six phylogenetic lineages: *Actinobacteria* (*Rhodococcus* and *Streptomyces*), *Bacteroidetes* (*Pedobacter*), α-*Proteobacteria*

(*Sphingomonas*), β-*Proteobacteria* (*Burkholderia, Collimonas* and *Duganella/Janthinobacterium*), and *γ-Proteobacteria* (*Frateuria, Pseudomonas* and *Yersinia)*, and the low G+C Gram-positive bacteria *(Bacillus* and *Paenibacillus*). The predominant bacterial group was the *Proteobacteria* (Männistö and Häggblom 2006). The β-*Proteobacteria* were found to be more prevalent during the spring thaws, whereas *Acidobacteria* species were the most abundant during winter (Männistö et al. 2008).

Many of the dominant microbial species identified in traditional culture-based studies have also been detected in culture-independent metagenomic studies. In a Canadian study, analysis of soil samples from Kuujjuaq, Quebec, and Alert, Nunavut, identified strains belonging to the α-, β-, γ-, and δ-*Proteobacteria*, as well as high G+C Gram-positive bacteria. Species represented included *Arthrobacter globiformis*, *Cellulomonas* sp., *Clavibacter xyli, Halomonas pantelleriense, Nocardioides jensenii, Pelobacter carbinolicus, P. propionicus, Terrebacter* sp., *Variovorax paradoxus, Williamsia murale* and *Xanthomonas axonopodis*, as well as an isolate putatively identified as *Afipia* genospecies 13 (Juck et al. 2000). Similarly, Nemergut et al. (2005) investigated the microbial diversity present in pristine Canadian Arctic soils. Analysis of the 16S rRNA gene indentified phylotypes related to *Acidobacteria, Actinobacteria, Bacteriodetes* and *Proteobacteria* species (Nemergut et al. 2005). In a separate study, over 1,000 bacterial strains belonging to diverse microbial taxa including *Arthrobacter, Bacillus, Firmicutes, Paenibacillus* and *Pseudomonas* were isolated from sub-boreal forest soils in Canada (Axelrood et al. 2002).

As has been mentioned above, fungi have been shown to participate in the biodegradation of hydrocarbons in polar environments, and several species have been isolated from contaminated alpine soils. Petroleum contaminated Arctic soils have been shown to contain hydrocarbon degrading psychrotrophic Urediniomycete species as well as *Trichosporon dulcitum* (order Tremellales) (Margesin et al. 2005). Similarly, Basidiomycetous yeast strains isolated from alpine soils and glacier cryoconite were able to degrade phenol-related mono-aromatic compounds at low temperatures (Bergauer et al. 2005). These strains included members of the classes Hymenomycetes (*Cryptococcus*) and Urediniomycetes (*Mastigobasidium, Rhodosporium*, *Rhodotorula*, and *Sporobolomyces*) (Margesin et al. 2007). *Yarrowa lipolytica*, an oil-degrading yeast able to reproduce at subzero temperatures was isolated from alpine soils (Margesin and Schinner 1997). In another study, psychrophilic and cold-tolerant alpine yeasts were isolated and analyzed for their ability to degrade phenol and other phenol-related mono-aromatic compounds (Bergauer et al. (2005). The fungi were isolated from soil and sediment samples collected from diverse environmental sites, including an alpine oil-shale mine, an ice cave, glacier cryconites and a railway area at the Brenner Pass in Austria. The fungal species were identified by ITS sequence analysis and belonged to two classes of basidiomycetous yeast, namely, Hymenomycetes and Urediniomycetes. Within the Hymenomycetes, two *Filobasidiales* species were present (*Cryptococcus terreus* and *C. terricola), w*hile within the Urediniomycetes the *Sporidiobolus* clade was represented by *Rhodosporidium lusitaniae* and *Sporobolomyces roseus.* Similarly, the *Microbotryum* clade was represented by *Mastigobasidium intermedium*, *R. creatinivora* and *R. ingeniosa.* Additionally, 12 strains of *Microbotryomycetidae* could not be identified to the species level, although teliospores were observed for one of the strains, a characteristic of teleomorphic yeasts in both the *Microbotryum* and *Sporidiobolus* clades. Although none of the 32 strains utilized any of the highly volatile mono-aromatic compounds as the sole carbon source, non/low volatile aromatic compounds were degraded. The *R. creatinivora* strains demonstrated the greatest degradative capacity, and were able to degrade up to seven compounds (Bergauer et al. 2005).

An extensive study was conducted in the mid-1960s as part of the *International Biological Program* (IBP). This study covered a large geographical area including Alaska, Siberian and Scandinavian Arctic tundra, subarctic tundra as well as alpine tundra in Europe and Northern America. Within the 33 tundra areas investigated, about a hundred fungal genera were identified. The most prevalent genera were *Chrysosporium*, *Cladosporium*, *Mortierella* and *Penicillium*. Similarly, in a more recent study, tundra soil samples were collected from the southern part of Bellsund, Spitsbergen (where the mean annual temperature is –5°C). Fungal species identified in this study included *Alternaria alternate, Arthrobotrys oligospora, Aspergillus versicolor, Botrytis cinera, Chrysosporium pannorum, Cladosporium cladosporoides, C. herbarum, Cylindrocarpon magnusianum, Epicoccum purpurascens, Mortierella alpine, M. minutissim, Mycelia sterilia, Oidodendron cerealis, Phialophora fastigiata, Phoma exigua* var *exigua, Sclerotium* sp. and *Trichosporella cerebriformis*. Additionally, several *Penicillium* species (*Penicillium chrysogeum, P.cyclopium, P.expansum, P. islandicum, P.lanosum*, and *P. verrucosum v*ar *cyclopium*) were isolated (Kurek et al. 2007).

The distribution of root-associated Ascomycota fungi from the high Arctic tundra of Alexandra Fiord, Canada, was studied by nuclear rDNA sequence analysis. Strains affiliated to the orders *Dothideales* and *Helotiales* (including *Phialocephala fortinii* and *Rhizoscyphus ericae* species) were identified (Fujimura et al. 2008). Similarly, *Cryptosporiopsis radicicola* and *P. fortinii* (both species are root endophytes) are commonly found in the roots of alpine and Arctic plants. In addition to *Phoma* species (*P. herbarum* and *P. tropica*), a more recent study conducted at this site isolated *Acremonium strictum, B. cinerea, C. oxysporum, Exophiala mansonii, Placodiplodia* sp. and *Preussia* species (Robinson et al. 1998). The genus *Geomyces* (which includes several species that were formally classified as *Chrysosporium* species) are psychrophilic fungi widely distributed in Arctic permafrost and have been shown to form ericoid mycorrhizae with the roots of alpine Ericales species. *Geomyces* species have also been isolated from Antarctica (Hambleton and Sigler 2008).

Alpine Tundra

Alpine Tundra is defined as the high-lying regions (between 1,800 and 2,500 m above sea level), which occur above mountainous forests, while the sub-alpine belt includes the forest-tundra region (Löve 1970). The climatic conditions of the European Alpine region differ from that of the Arctic tundra in that it experiences higher maximum and lower minimum temperatures, and has increased levels of precipitation and humidity. Additionally, wider temperature fluctuations lead to frequent freeze-thaw cycles, and these regions are also subject to elevated levels of solar radiation (Margesin et al. 2004; Margesin 2007).

A study on the high alpine soils and glacial cryonites in the Eastern and Western Alps of Europe (between 2,000 and 4,000 m above sea level), identified 48 Gram-negative isolates belonging to four genera: *Pseudomonas* (41 isolates), *Flavobacterium* (4), *Xanthomonas* (2) and *Aeromonas* (1). Identification of the strains to the species level further classified the pseudomonads as *P. aeruginosa, P. fluorescens, P. paucimobilis*, and *P. pickettii* species. Similarly, the *Xantomonas* strains were both identified as *Xantomonas maltophilia* and the single *Aeromonas* was a strain of the *Aeromonas hydrophila* species group. Although the flavobacteria were not identified to the species level, the four strains were all non-motile, oxidase-positive, glucose oxidizers (Schinner et al. 1992). Paleoecological materials found in ancient rodent-burrows in permafrost layers includes seeds, plant organs, hair, insects, remains of small animals and feces

(Zanina 2009) and may be inhabited by complex microbial populations. A study was undertaken by Skatkhov et al. (2008) to characterize the well-preserved microbial communities associated with the fossilized seeds of higher plants (radiocarbon dated to be 32,000 years old) obtained from the late Pleistocene deposits in the Kolyma lowland, Russia. The seeds were identified as *Poa* spp. (bluegrass), *Potentilla nivea* (snow cinquefoil) and *Silene stenophylla* (narrow leaved campion). Molecular identification based on 16S rRNA gene analysis identified bacterial species belonging to the genera *Agreia, Arthrobacter, Clavibacter, Devosia, Micrococcus, Paenibacillus, Pseudomonas, Rhodoglobus, Roseomonas, Sanguibacter, Sinorhizobium Sphingomonas, Subtercola* and *Variovorax*. Additionally, filamentous fungal species such as *Aspergillus sclerotiorum, Coelomycetes* sp., *C. sphaerospermum, Geomyces pannorum, P. aurantiogriseum, P. granulatum, P. fastigiata, P. crystallifera, P. herbarum* and *P. nebulosa* were isolated from the same fossilized seed samples (Skatkhov et al. 2008).

Diverse microbial communities have been detected in alpine tundra sites in the USA. Niwot Ridge lies at the edge of the Indian Peaks Wilderness in the Colorado Rocky Mountains. The ridge lies above 3,000 m elevation and is characterized by the presence of many glacial bodies, permafrost and extensive alpine tundra. The mean temperature in January can reach as low as −13.2°C (Niwot Ridge Long Term Ecological Research Site 2007). Alpine meadow and bare-ground sites at Niwot Ridge were analyzed for microbial diversity during a 2-year study. *Acinetobacter*, *Actinomycetes, Arthrobacter, Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus* and *Pseudomonas* species were identified (Mancinelli 1984). Sequences related to the phyla *Chloroflexi* and *Verrucomicrobia* have also been identified in the tundra soils from Niwot Ridge (Mancinelli 1984).

Caves found in cold mountainous alpine areas provide an ideal environmental niche for cold-adapted microorganisms. Typically, the internal temperature of these caves remains close to 0°C and as such freeze-thaw cycling does not occur (Gounot 1999). Bacteria often observed in such caves display coryneform morphology, such as psychrophilic *Arthrobacter* species. Arthrobacter appear to be widely distributed in psychrophilic environments and have been isolated from subterranean cave silts, Arctic soils and Antarctica (Moiroud and Gounot 1969; Gounot 1976; Gounot 1999; Loveland-Curtze et al. 1999; Juck et al. 2000; Reddy et al. 2000, 2002; Stibor et al. 2003). The novel species *A. psychrophenolicus* was isolated from a carbonate-rich deposit in an Austrian alpine ice cave (Margesin et al. 2004).

Conclusion

Despite the vast number of studies contributing to the knowledge of microbial life in cold terrestrial habitats, considerably more research is required to understand the structure and function of microbial communities in these environments. The current status of such research is largely limited to an analysis of species distribution and even these studies are limited by the depth of sequence analysis and the inherent uncertainties associated with metagenomic phylogenetics. Functional studies, which potentially encompass both community-wide processes and the function of individual members of such communities, are extremely limited, both in scope and depth. The application of modern transcriptomic methods, particularly in conjunction with high-throughput nucleotide sequencing, offers huge scope for expanding our understanding of the roles of microbial community members and their functional responses to microenvironmental changes. Nevertheless, such data must still be supported by more traditional quantitative analyses (e.g., fixation and turnover rates).

The rise of metagenomics has re-energized microbial ecology. The evidence for a resurgence of interest in the microbiology of psychrophilic habitats is evident by the wave of publications over the past decade on the structure, composition, and function of psychrophilic, particularly polar, communities. From this growing understanding come a range of new perspectives: the role of psychrophiles in bioremediation and xenobiotic degradation, the sensitivity of psychrophilic communities to climate change and the value of these organisms as a pool of novel genes and gene products. We propose with some confidence that these and other as yet unconsidered perspectives on psychrophilic organisms will continue to drive research in this field.

Cross-References

- 1.1 Prologue: Definition, Categories, Distribution, Origin and Evolution, Pioneering Studies, and Emerging Fields of Extremophiles
- 5.1 Distribution of Piezophiles
- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
- 6.2 Taxonomy of Psychrophiles
- 6.3 Diversity of Psychrophilic Bacteria from Sea Ice-and Glacial Ice Communities
- 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)

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6.6 Psychrophiles: Genetics, Genomics, Evolution

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_6.6,

Introduction

From the deepest depths of the ocean to the highest alpine peaks of the mountains, from the darkness of subterranean caves to the intense radiation of the upper atmosphere, and from the Northern to the Southern polar extremes, over two thirds of the Earth's biosphere is dominated by cold habitats. In these cold zones, psychrophilic microorganisms thrive, actively metabolizing at temperatures as low as –20°C, surviving at –45°C (Margesin and Schinner 1999; Feller and Gerday 2003; Cavicchioli 2006) and in the process driving critical global biogeochemical cycles. Yet, despite the fundamental role that these organisms play within the cold biosphere, relatively little is known about their identity, their physiology, how they have evolved, and the biogeochemical processes they perform.

The classic definition of the term, psychrophile, which derives from the Greek words ψυχρος (psukhros, cold) and φιλειν (philein, to love), is for an organism with an optimal growth temperature (T_{opt}) lower than 15°C. However, this definition has important limitations (see, e.g., Feller and Gerday 2003; Cavicchioli 2006). One of the problems with using T_{opt} as a means of defining a psychrophile is that T_{opt} is not known for most microorganisms present in naturally cold environments because, like most environmental microorganisms, the great bulk are not amenable to laboratory cultivation. Moreover, T_{opt} is dependent on growth media and other environmental parameters, making it difficult to extrapolate from laboratory studies back to a natural environmental setting.

While some issues of cultivatablity have been overcome by the use of cultivation-independent approaches (see section Future Prospects in this chapter), and provide information regarding the diversity, relative abundance, and physiology of psychrophilic microbial communities, it is not possible to infer T_{opt} of individual members from this type of data.

The classic definition of a psychrophile also fails to effectively consider the effects of temperature on the rates of enzymatic activity. In effect, cells will continue to grow more rapidly at increasingly higher temperatures until a critical process in the cell becomes sufficiently compromised. As such, many of the autochthonous bacterial and archaeal isolates that dominate permanently cold environments have their T_{opt} and upper temperature limit for growth (T_{max}) well beyond the environmental temperature they would naturally encounter. T_{opt} and $T_{max,}$ therefore, define the unnaturally high temperatures that psychrophiles can tolerate, thereby achieving fastest rates of growth and upper temperature limits tolerated for growth, respectively. Consistent with this view, analyses of molecular markers and physiological responses of psychrophiles have revealed that cells are often stressed when growing at temperatures around T_{opt} (Feller and Gerday 2003; Cavicchioli 2006). In other words, while psychrophiles can grow faster at temperatures above what they typically experience in the natural environment, in many cases they are growing under suboptimal conditions and experiencing heat stress.

An improved way of describing organisms isolated from cold environments is to use the terms "eurypsychrophile" and "stenopsychrophile"(Feller and Gerday 2003; Cavicchioli 2006), where eury and steno are derived from the Greek words ευρυς (broad) and στενος (narrow), respectively, and are terms that are widely used in the field of Ecology. Using these derivations, eurypsychrophiles are organisms that grow in low temperature environments but can tolerate a much wider temperature range extending beyond the 15°C limit imposed in the classical definition. Conversely, stenopsychrophiles have a restricted growth temperature range with a T_{opt} of $\leq$15°C. Good examples of eurypsychrophiles that are numerically abundant in their respective cold environments and thereby demonstrate ecological competitiveness are the

marine bacterium *Sphingopyxis alaskensis* and the Antarctic hypersaline-lake archaeon, *Halorubrum lacusprofundi* (Cavicchioli 2006).

While eury and steno help to classify psychrophiles based on their growth temperature properties, there remains a lack of knowledge defining the molecular properties that enable eurypsychrophiles to tolerate temperatures that stenopsychrophiles cannot (and conversely, the factors that limit the ability of stenopsychrophiles to grow at higher temperatures). In the coming years it will be useful to address these issues within similar classes of microorganisms (e.g., methanogens) with representative eurypsychrophiles (e.g., *Methanococcoides burtonii*) and stenopsychrophiles (e.g., *Methanogenium frigidum*), and between representatives of different ecotypes of eurypsychrophiles and stenopsychrophiles (e.g., sea-ice versus marine planktonic bacteria). Achieving this would expand our understanding of the molecular basis of cold adaptation and the evolutionary paths that have produced these two psychrophilic subclasses.

In addition to naturally cold habitats, some artificial environments support the growth of microorganisms in the cold. Refrigerated appliances and frozen products can harbor potential pathogens such as *Listeria* (Tasara and Stephan 2006). However, microorganisms isolated from artificially cold environments, such as human pathogens, tend to "prefer" hotter environments (e.g., the human body) where they compete effectively and cause disease. As such these types of microorganisms are not psychrophiles, but tolerate cold environments. It will be insightful to assess the molecular and physiological responses of these microorganisms to growth at low temperature, and compare their responses to psychrophiles from naturally cold environments.

This chapter focuses on advances that have been gained through genomic and functional genomic analyses of psychrophilic bacteria and archaea.

Genomes of Cold Adapted Bacteria and Archaea

Currently (in October 2009) the genomes of 28 psychrophilic bacteria have been completed, of which 14 have been published. Most of these isolates are from the Gammaproteobacteria (20 of 28 genomes), with half of all completed bacterial psychrophile genomes belonging to the Alteromonadales (14 genomes) (*Table 6.6.1*).

Sequencing of a further 21 genomes of psychrophilic bacteria is currently underway (*Table 6.6.2*). In addition, genome sequence data for four members of the Archaea is presently available (*Table 6.6.3*). Together, these 53 completed genomes cover six different phyla of the domains Bacteria and Archaea, but even these represent only a small fraction of the phylogenetic diversity existing in low temperature environments. A number of major findings arising from the published psychrophile genomes are detailed below.

Bacterial Genomes

Psychromonas ingrahamii 37 was isolated from Arctic sea ice and is capable of exponential growth at −12°C. The genome contains a single circular chromosome of 4.56 Mb (Riley et al. 2008). The G+C content is 40.1% and there are 3,742 protein coding genes. Ten RNA clusters and 86 tRNA genes are present, along with 81 genes with horizontal gene transfer potential such as transposases and integrases. *P. ingrahamii* has five sigma factors to moderate gene expression by RNA polymerase and 61 regulators of Cgdp, which control motility, adhesion

Table 6.6.1

Completed genome sequences of psychrophilic bacteria

Genome name	Genome publication	Origin of strain	Phylogenetic group	Size (Mb)	Genes
Flavobacterium psychrophilum JIP02/86	Duchaud et al. (2007)	Fish pathogen	Bacteroidetes, Flavobacteria, Flavobacteriales	2.86	2,505
Bacillus cereus cytotoxis NVH 391–98	None yet	Soil	Firmicutes, Bacilli, Bacillales	4.09	4,250
Bacillus weihenstephanensis KBAB4	None yet	Soil	Firmicutes, Bacilli, Bacillales	5.87	5,983
Exiguobacterium sibiricum 255–15	Rodrigues et al. (2008)	Permafrost sediment in Siberia	Firmicutes, Bacilli, Bacillales	3.04	3,151
Leuconostoc citreum KM20	Kim et al. (2008)	The Korean food kimchi	Firmicutes, Bacilli, Lactobacillales	1.90	1,902
Methylocella silvestris BL2	None yet	Acidic forest cambisol	Proteobacteria, Alphaproteobacteria, Rhizobiales	4.31	3,971
Rhodoferax ferrireducens T118	Risso et al. (2009)	Aquifer sediment	Proteobacteria, Betaproteobacteria, Burkholderiales	4.97	4,561
Desulfotalea psychrophila LSv54	Rabus et al. (2004)	Marine sediments off Svalbard	Proteobacteria, Deltaproteobacteria, Desulfobacterales	3.66	3,332
Aeromonas salmonicida salmonicida A449	Reith et al. (2008)	Pathogen of brown trout	Proteobacteria, Gammaproteobacteria, Aeromonadales	5.04	4,609
Colwellia psychrerythraea 34H	Methe et al. (2005)	Arctic marine sediments	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.37	5,066
Idiomarina loihiensis L2TR	Hou et al. (2004)	Marine hydrothermal vent	Proteobacteria, Gammaproteobacteria, Alteromonadales	2.84	2,706
Pseudoalteromonas haloplanktis TAC125	Medigue et al. (2005)	Antarctic coastal seawater	Proteobacteria, Gammaproteobacteria, Alteromonadales	3.85	3,634
Psychromonas ingrahamii 37	Riley et al. (2008)	Sea ice, Northern Alaska	Proteobacteria, Gammaproteobacteria, Alteromonadales	4.56	3,877
Saccharophagus degradans 2–40	Weiner et al. (2010)	Decaying salt marsh cord grass	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.06	4,114

Table 6.6.1 (Continued)

Genome name	Genome publication	Origin of strain	Phylogenetic group	Size (Mb)	Genes
Shewanella baltica OS185	None yet	Baltic Sea	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.31	4,618
Shewanella baltica OS223	None yet	Baltic Sea	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.36	4,622
Shewanella halifaxensis HAW-EB4	None yet	Atlantic Ocean	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.23	4,462
Shewanella loihica PV-4	None yet	Hydrothermal vent	Proteobacteria, Gammaproteobacteria, Alteromonadales	4.60	4,011
Shewanella pealeana ATCC 700345	None yet	Colonizing the squid *Loligo pealei*	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.17	4,434
Shewanella piezotolerans WP3	Wang et al. (2008)	sediment, marine deep sea	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.40	4,944
Shewanella sediminis HAW-EB3	None yet	Marine sediment	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.52	4,666
Shewanella violacea DSS12	None yet*	Deep sea mud	Proteobacteria, Gammaproteobacteria, Alteromonadales	4.90	
Shewanella woodyi ATCC 51908	None yet	Deep marine sediment	Proteobacteria, Gammaproteobacteria, Alteromonadales	5.94	5,085
Psychrobacter arcticus 273–4	None yet	Siberian permafrost, Russia	Proteobacteria, Gammaproteobacteria, Pseudomonadales	2.65	2,215
Psychrobacter cryohalolentis K5	None yet	Siberian permafrost, Russia	Proteobacteria, Gammaproteobacteria, Pseudomonadales	3.10	2,582
Psychrobacter sp. PRwf-1	None yet	From the snapper *Lutjanus vivanus*	Proteobacteria, Gammaproteobacteria, Pseudomonadales	3.00	2,481
Aliivibrio salmonicida LFI1238	Hjerde et al. (2008)	Pathogen of Atlantic cod *Gadus mortua*	Proteobacteria, Gammaproteobacteria, Vibrionales	4.66	4,075
Photobacterium profundum SS9	Vezzi et al. (2005)	2,500m depth from the Sulu Trough	Proteobacteria, Gammaproteobacteria, Vibrionales	6.40	5,754

*Possibly published in Japanese in the journal Idenshi Igaku in 2003.

Table 6.6.2

Current genome sequencing projects for psychrophilic bacteria

Genome name	Sequencing center	Status	Habitat/source	Phylogenetic group	Genes	Size (Mb)
Aequorivita antarctica SW49	DOE JGI, DSMZ	In progress	Marine	Bacteroidetes, Flavobacteria, Flavobacteriales		
Aequorivita sublithincola QSSC9–3	DOE JGI, DSMZ	In progress	Marine	Bacteroidetes, Flavobacteria, Flavobacteriales		
Agreia sp. PHSC20C1	Desert Research Institute, JCVI	Draft available	Surface water off the Western Antarctic Penninsula	Actinobacteria, Actinobacteria, Actinomycetales	2,718	2.77
Colwellia sp. MT41	JCVI	In progress	Fresh water, deep sea	Proteobacteria, Gammaproteobacteria, Alteromonadales		
Gillisia limnaea R-8282	DOE JGI, DSMZ	In progress	Microbial mats in Lake Fryxell, Antarctica	Bacteroidetes, Flavobacteria, Flavobacteriales		
Glaciecola sp. HTCC 2999	JCVI	Draft available	Fresh water	Proteobacteria, Gammaproteobacteria, Alteromonadales	2,290	2.52
Hymenobacter roseosalivarius AA-718	DOE JGI	In progress	Antarctic soil and sandstone	Bacteroidetes, Sphingobacteria, Sphingobacteriales		
Leuconostoc gasicomitatum LMG 18811	University of Helsinki, Finland	In progress	Modified-atmosphere packaged, tomato-marinated broiler meat strips	Firmicutes, Bacilli, Lactobacillales		
Leuconostoc kimchii IMSNU11154	European Consortium	In progress	Kimchi	Firmicutes, Bacilli, Lactobacillales		
Marinobacter sp. ELB17	JCVI, Princeton Univ	Draft available	Permanently ice-covered lake, Antarctica	Proteobacteria, Gammaproteobacteria, Alteromonadales	4,908	4.89

Methylocapsa acidiphila B2T	DOE JGI	In progress	Acidic Sphagnum peat bog	Proteobacteria, Alphaproteobacteria, Rhizobiales		
Mucilaginibacter paludis TPT56	DOE JGI	In progress	Rice paddies	Bacteroidetes, Sphingobacteria, Sphingobacteriales		
Octadecabacter antarcticus 307	ICBM, JCVI	In progress	McMurdo Sound, Antarctica	Proteobacteria, Alphaproteobacteria, Rhodobacterales		
Octadecabacter arcticus 238	JCVI	In progress	350 km offshore off Deadhorse, Alaska	Proteobacteria, Alphaproteobacteria, Rhodobacterales		
Oleispira antarctica RB-8	Max Planck Institute	In progress	Antarctic marine	Proteobacteria, Gammaproteobacteria, Oceanospirillales		
Photobacterium profundum 3TCK	JCVI, Scripps Institute of Oceanography	Draft available	San Diego Bay	Proteobacteria, Gammaproteobacteria, Vibrionales	5,728	6.11
Polaribacter filamentus	Integrated Genomics Inc.	In progress	Fresh water	Bacteroidetes, Flavobacteria, Flavobacteriales		
Polaribacter irgensii 23-P	Desert Research Institute, JCVI	Draft available	Surface waters, Antarctica	Bacteroidetes, Flavobacteria, Flavobacteriales	2,602	2.75
Psychroflexus torquis ATCC 700755	JCVI, Univ of Tasmania	Draft available	Sea-ice algal assemblage, Antarctica	Bacteroidetes, Flavobacteria, Flavobacteriales	6,835	6.01
Psychromonas sp. CNPT3	JCVI, Scripps Institute of Oceanography	Draft available	Central north Pacific Ocean at a depth of 5,800 m	Proteobacteria, Gammaproteobacteria, Alteromonadales	2,829	2.95
Rhodoferax antarcticus Ant.Br	Arizona State Uni	In progress	Microbial mat	Proteobacteria, Betaproteobacteria, Burkholderiales		

Draft genomes are available at http://img.jgi.doe.gov/cgi-bin/pub/main.cgi

Table 6.6.3

Genome sequences for psychrophilic archaea

Genome	Sequencing status	Genome publication	Native environment	Phylogenetic group	Size (Mb)	Genes
Cenarchaeum symbiosum A	Closed	Hallam et al. (2006)	Symbiont of marine sponge off Californian Coast	Crenarchaeota – Ammonia Oxidation Clade	2.05	2,066
Halorubrum lacusprofundi ATCC 49239	Closed	None yet	Deep Lake, Antarctica	Euryarchaeota – Halobacteria	3.69	3,725
Methanococcoides burtonii DSM 6242	Closed	Saunders et al. (2003); Allen et al. (2009)	Ace Lake, Antarctica	Euryarchaeota – Methanomicrobia	2.58	2,506
Methanogenium frigidum Ace-2	Draft	Saunders et al. (2003)	Ace Lake, Antarctica	Euryarchaeota – Methanomicrobia	2–2.5*	

*Estimated genome size based on draft genome.

factors, fimbriae, and biofilm formation. Sixteen glycosyltransferases are present, and it is postulated that extracellular polysaccharide production may help lower the freezing point in the vicinity of the cell and play an important role in cell survival at such low temperatures. Other genes involved in temperature adaptation include a large number encoding cold shock proteins (Csps) (12 genes), heat shock proteins (9 genes), and chaperones (13 genes) along with a polyunsaturase for fatty acid modification. Production and transport of the osmolyte betaine choline is genomically encoded, facilitating osmotic control when the sea ice freezes. Unexpectedly, the proteins of *P. ingrahamii* were more similar to those of *Vibrio cholerae* than to its closer relatives the Shewanellaceae or Collwelliaceae (*Table 6.6.1*).

Photobacterium profundum SS9 is a Gammaproteobacterium that was isolated at a depth of 2,500 m. The genome is composed of a 4.1 Mb major circular chromosome, a 2.2 Mb minor circular chromosome, and an 80 kb circular plasmid (Vezzi et al. 2005). All but one of the 15 rRNA operons are present on the major chromosome, and the rRNA gene copies exhibit high intragenomic variation (up to 5.13% for the 16S gene) possibly indicating that the various operons operate under distinct physiological conditions. There are a very high number of tRNA genes (164 genes). Transposon sequences were found at higher frequency on the minor chromosome, suggesting the major chromosome is more stable while the minor chromosome may function as more of a "genetic melting pot." *P. profundum* has been adopted as a model piezophile, and much research has focused on its adaptations to life at high pressure rather than at cold temperature; for example, it has been observed that proteins for degradation of polymers such as chitin and cellulose are expressed at depth, while the stress response proteins are upregulated at atmospheric pressure (*Table 6.6.4*).

Shewanella piezotolerans WP3 is an iron-reducing bacterium belonging to the Gammaproteobacteria. It was isolated from sediment at a depth of 1,914 m in the western Pacific Ocean

Table 6.6.4

Proteomic and transcriptomic studies assessing cold adaptation

Study	Organism	Growth temperature (°C)	Transcriptomics	Proteomics
Bakermans et al. (2007)	*P. cryohalolentis*	16, 4, −4		2-DE
Berger et al. (1996)	*A. globiformus*	25, 4		2-DE, immunoblotting
Bergholz et al. (2009)	*P. arcticus*	22, 17, 0, −6	Microarray	
Campanaro et al. (2010)	*M. burtonii*	23, 4	Microarray	
Gao et al. (2006)	*S. oneidensis*	30→15, 30→8	Microarray	
Goodchild et al. (2004a)	*M. burtonii*	23, 4		2-DE, MS/MS
Goodchild et al. (2004b)	*M. burtonii*	4		LC/LC, MS/MS
Goodchild et al. (2005)	*M. burtonii*	23, 4		ICAT, LC/LC, MS/MS
Kawamoto et al. (2007)	*S. livingstonensis*	18, 4	Quantitative RT-PCR	MALDI-TOF-MS
Qiu et al. (2006)	*E. sibiricum*	25, 4		MALDI-TOF-MS
Rodrigues et al. (2008)	*E. sibiricum*	39, 28, 10, −2.5	Microarray	
Saunders et al. (2005)	*M. burtonii*	4		LC/LC, MS/MS
Saunders et al. (2006)	*M. burtonii*	23, 4		LC/LC, MS/MS
Seo et al. (2004)	*B. psychrosaccharolyticus*	30, 30→15, 30→0		2-DE, MS
Ting et al. (2010)	*S. alaskensis*	30, 10		Metabolic labelling, LC/LC, MS/MS
Williams et al. (2010a)	*M. burtonii*	23, 4		iTRAQ, LC/LC, MS/MS
Williams et al. (2010b)	*M. burtonii*	23, 4		iTRAQ, LC/LC, MS/MS
Burg et al. (2010)	*M. burtonii*	23, 4		LC/LC, MS/MS
Zheng et al. (2007)	*P. arcticus*	22, 4		LC/LC, MALDI-MS

and is adapted to growth in the cold and to pressure. The genome is a single 5.4 Mb circular chromosome that encodes 4,944 protein coding genes (Wang et al. 2008). It contains eight rRNA operons and 89 tRNAs. The organism appears well adapted to life in the deep sea, with numerous genes and gene clusters facilitating cold adaptation present in the genome.

Structural RNA modification is an important mechanism for maintaining tRNA flexibility at low temperature, and the *S. piezotolerans* genome contains more pseudouridylate synthase genes than any other genome to date (7 genes). A wide variety of carbon and energy utilization pathways and osmolyte transport and synthesis systems are present in the genome, as are the genes required for synthesis of eicosapentaenoic acid (EPA). EPA and branched chain fatty acid content have been demonstrated to be upregulated at low temperature in this organism (Wang et al. 2009). The genome also contains gene clusters for production of polar and lateral flagella; the lateral flagella are upregulated by exposure to low temperature and are essential for motility at 4°C (Wang et al. 2008). A single filamentous phage is also present in the genome. The phage was demonstrated to be active, and at low temperatures a putative single stranded DNA binding protein and several other key phage genes were significantly upregulated (Wang et al. 2007).

Pseudoalteromonas haloplanktis TAC125 was isolated from Antarctic coastal seawater. It has two circular chromosomes of 3.2 Mb and 0.63 Mb, which encode 3,488 protein coding genes (Medigue et al. 2005). All nine rRNA operons and 106 tRNA genes are present on the larger chromosome. Seventy two percent of the tRNA genes are located on the leading strand and this organization is thought to facilitate the organism's rapid growth at low temperature. Several genes with a role in cold adaptation are clustered together in approximate repeats such as those coding for Csps, *cspA*-paralogs, and putative calcium binding proteins, which may be involved in exopolysaccharide (EPS) production. Nineteen genes coding for RNA binding proteins and RNA chaperones are present. In order to cope with the increased dioxygen solubility at low temperatures *P. haloplanktis* possesses 12 putative dioxygenases along with other proteins capable of scavenging chemical groups that are damaged by reactive oxygen species (ROS).

Although not yet published, the genome of *Psychrobacter arcticus* 273–4 is complete. This aerobic heterotrophic Gammaproteobacterium was isolated from a Siberian permafrost core and is capable of growth at −10°C (Bakermans et al. 2006). The genome is a single circular chromosome 2.6 Mb long, which encodes 2,215 proteins, 4 rRNA operons, and 49 tRNAs.

Desulfotalea psychrophila LSv54 is a sulfate-reducing Deltaproteobacterium. It was isolated from permanently cold Arctic sediments and is capable of growth at −1.8°C. It has one circular chromosome 3.5 Mb long and two plasmids 121 and 14 kb long (Rabus et al. 2004). The genome contains 3,234 protein coding genes, 7 rRNA operons, and 64 tRNA genes. The capacity to produce selenocysteine, the 21st amino acid, is genomically encoded and nine genes which require selenocysteine are present. Five complete IS elements and three partial IS element fragments were identified, along with four phage-related integrases. Nine homologs of known *csps* and a further nine putative *csp* geneswere identified.

All characterized members of the genus *Colwellia* are psychrophilic isolates obtained from stably cold marine environments. *Colwellia psychrerythraea* 34H, the type species, was isolated from Arctic marine sediment and produces maximum cell yield when grown at −1°C. Its single circular chromosome of 5.37 Mb encodes 4,937 protein coding genes, 9 rRNA operons, and 88 tRNAs (Methe et al. 2005). Two phage genomes are integrated into the genome. To retain membrane fluidity at low temperature, several copies of genes involved in fatty acid and phospholipid biosynthesis are present in the genome including a putative operon related to polyketide-like polyunsaturated fatty acid synthases. Transport and production of the compatible solute glycine betaine is genomically encoded, and this compound may perform both osmoprotection and cryoprotection roles in the cell. EPS and extracellular enzyme production is prominent, with many copies of glycosyltransferases (17 proteins) and degradative proteolytic enzymes with a signal peptide (49 proteins) in the genome. Protection from ROS

is provided by a variety of genes encoding antioxidants including three copies of catalase. An unusual Csp appears to localize to the membrane based on the presence of three transmembrane-spanning regions and a further four Csps are found in the cytoplasm.

The genomes of three pathogens from psychrophilic fish are available: *Flavobacterium psychrophilum* JIP02/86, *Aeromonas salmonicida* subsp. *salmonicida* A449, and *Aliivibrio salmonicida* LF11238. *F. psychrophilum* JIP02/86 has one circular chromosome 2.86 Mb long containing 2,432 protein coding genes, 6 rRNA operons, and 49 tRNA genes, and a single plasmid pCP1 (3.4 kb), which encodes four proteins (Duchaud et al. 2007). There are 74 IS elements in the genome. Many genes for the synthesis, export, modification, and polymerization of EPS are found in a 70 kb region of the genome. Genome features that reflect the organism's cold adaptation include lipid desaturases (three proteins), RNA helicases (six proteins, including three which are DEAD/DEAH box RNA helicases), a Csp, three chaperones and numerous enzymes with the capacity to counteract the effects of ROS (30 proteins). *A. salmonicida* subsp. *salmonicida* A449 has one circular chromosome 4.7 Mb long, two large plasmids (pAsa4, 166.7 kb; pAsa5, 155 kb), and three small plasmids (pAsa1, 5.4 kb; pAsa2, 5.2 kb; pAsa3, 5.6 kb) encoding a total of 4,437 protein coding genes (Reith et al. 2008). There are nine rRNA operons and 110 tRNA genes. A total of 170 pseudogenes and 88 insertion sequences are present, along with several large genome inversions when compared with closely related bacteria. *A. salmonicida* lF11238 has two circular chromosomes (3.3 and 1.2 Mb) and four plasmids (pVSAL840, 83.5 kb; pVSAL320, 30.8 kb; pVSAL54, 5.4 kb; pVSAL43, 4.3 kb) (Hjerde et al. 2008). There are a total of 4,286 protein coding genes, 12 rRNA operons, and 107 tRNA. The genome has undergone a high degree of gene rearrangement, deletion, and acquisition, as demonstrated by the presence of 370 pseudo-/partial genes, 521 transposases, and 288 IS elements. However, identifying any role genome plasticity may have in the organism's cold adaptation as distinct from adaptations related to pathogenicity is very difficult.

Idiomarina loihiensis is a eurypsychrophilic Gammaproteobacterium isolated from partially oxygenated cold waters at the periphery of a deep-sea vent, Hawaii. Its genome is 2.8 Mb and encodes 2,640 predicted proteins (Hou et al. 2004). There are four rRNA operons and 56 tRNAs. Genomic features that may contribute to cold adaptation in this organism include a cluster of 32 genes related to EPS and capsular polysaccharide synthesis, along with a further cluster for sialic acid biosynthesis and sialyation of surface polysaccharides. In particular, the sialic acid synthetase contains a C-terminal antifreeze domain important for maintenance of enzyme function at low temperatures. Versatile signal transduction machinery allows *I. loihiensis* to sense changes in dissolved oxygen and other environmental parameters in order to regulate EPS production. There are also several diverged copies of fatty acid biosynthesis enzymes, which are important for maintenance of cell membrane fluidity under changing temperature and pressure.

Saccharophagus degradans 2–40 is a eurypsychrophile with a single circular chromosome 5.06 Mb long encoding 4,008 protein coding genes (Weiner et al. 2010). For a Gammaproteobacterium it contains atypically few rRNA operons (two copies) and tRNA genes (41 genes). This is the first single organism (rather than a consortium) that has been demonstrated to degrade cellulosic algae and higher plant material, and the genome organization and composition reflects this unusual capacity. Genes for the degradation of more than 10 complex polysaccharides including cellulose, agar, alginate, and chitin are present, as are 15 megaproteins (>2,000 aa long) each containing domains and motifs reported to bind calcium and mediate protein–protein interactions. The genome is significantly enriched in regulators of

EPS production/degradation and biofilm formation. While all of these features contribute to the organism's success in the cold marine environment, elucidating specific cold adaptations is difficult. Five integron/phage integrases, two integrase psuedogenes, and three IS elements were detected in the genome.

Rhodoferax ferrireducens is a eurypsychrophilic facultative anaerobe belonging to the Betaproteobacteria that possesses the novel ability to convert sugars into electricity. It may play an important role in carbon and metal cycling in sediments and was isolated from subsurface sediment at a depth of 18 feet. The genome is comprised of a circular chromosome 4.7 Mb long (4,451 coding sequences) and a single plasmid 257 kb long (319 coding sequences) (Risso et al. 2009). There are two rRNA operons and 45 tRNA genes. Over 70% of genes on the plasmid are hypothetical. No genes coding for Csps have been identified in the genome even though other Betaproteobacteria such as *Nitrobacter* and *Ralstonia* spp. do contain these proteins. This suggests that *R. ferrireducens* may possess alternative mechanisms for surviving at cold temperatures.

Exiguobacterium sibiricum 255–15 was isolated from 3-million-year-old Siberian permafrost and is capable of growth from −5°C to 39°C. It has one circular chromosome 3 Mb long and two plasmids pEXIG01 (4.9 kb) and pEXIG02 (1.8 kb), which encode a total of 3,054 proteins (Rodrigues et al. 2008). There are nine rRNA operons and 69 tRNA genes. It has the capacity to produce and degrade EPSs, which may be important as cryoprotectants. A number of genes that may be involved in thermal adaptation were identified including translation factors (e.g., chaperones, DEAD-box RNA helicase), DNA replication genes (GyrA, GyrB), genes for maintenance of membrane fluidity (desaturase, beta-ketoacyl carrier protein), and sigma factors.

Leuconostoc citreum KM20 is a member of the Lactobacillales that was isolated from the traditional Korean fermented food kimchi. The genome contains one circular chromosome 1.8 Mb long and four circular plasmids (pLCK1, 38713 bp; pLCK2, 31463 bp; pLCK3, 17971 bp; and pLCK4, 12,183 bp) (Kim et al. 2008). The G+C content is 39.0%, and there are 1,820 protein coding genes in total. Four rRNA operons are present, along with 69 tRNA genes, 5 IS3 insertion sequences, and 5 derivatives of IS30 insertion elements. Research has focused on this organism's ability to suppress the growth of food pathogens such as *Bacillus cereus* and *Listeria monocytogenes* rather than its adaptation to the cold.

Archaeal Genomes

Cenarchaeum symbiosum is the sole archaeal symbiont of the marine sponge *Axinella mexicana* (Preston et al. 1996). Although uncultivated, a full genome sequence of *C. symbiosum* A was obtained through fosmid library construction (Hallam et al. 2006) (*Table 6.6.3*). The single circular chromosome 2.0 Mb long encodes 2,066 predicted ORFs, and contains one rRNA operon, and 45 tRNA genes. Although there is an abundance of *C. symbiosum*-related sequences in marine metagenome data (Hallam et al. 2006), indicating the environmental significance of these members of the Crenarchaeota, little work has focused on low temperature adaptation of *C. symbiosum*.

Methanococcoides burtonii DSM 6242 and *Methanogenium frigidum* Ace−2 are two methanogenic archaea that were isolated from Ace Lake in the Vestfold Hills, Antarctica. Draft genomes of these two organisms were published in 2003 (Saunders et al. 2003). *M. frigidum* has the lowest known T_{opt} of all the methanogens (15°C) (Franzmann et al. 1997; Cavicchioli 2006),

and its draft genome assembly was 1.6 Mb long (estimated total genome size 2–2.5 Mb). A total of 1,815 protein coding regions were identified, including a Csp and five proteins common to *M. frigidum* and *M. burtonii* but not identified in any other species. One of these five had highest structural similarity to a "winged helix" DNA binding domain protein, suggesting that transcriptional regulation may be an important aspect of these organisms' psychrophily. The bulk amino acid composition of proteins from *M. frigidum* and *M. burtonii* was distinct from that of mesophilic and thermophilic archaea, with a roughly linear trend in Gln, Thr, and Leu content over the range of optimal growth temperatures (Saunders et al. 2003).

The subsequent completion of the *M. burtonii* genome in 2009 (Allen et al. 2009) revealed that this organism uses highly skewed amino acid content to facilitate its psychrophilic lifestyle whilst retaining codon usage in common with its close mesophilic relatives. In addition, greater selective pressure was observed on genes that are predicted to be efficiently expressed (Allen et al. 2009). The completed *M. burtonii* genome is a single chromosome 2.57 Mp long encoding 2,494 genes. There are three rRNA operons, each containing two 5S, one 23S and one 16S rRNA gene, and a total of 53 tRNA genes including tRNA-pyl, which codes for pyrrolysine. The capacity for dihydrouridine incorporation into tRNAs is genomically encoded, allowing enhanced tRNA flexibility at cold temperatures. *M. burtonii*'s genome appears to be extremely dynamic with 67 transposons, five transposase fragments, seven transposase-disrupted proteins, and several duplicated cassettes in the genome. A large number of signal transduction proteins (45 genes) are present, providing considerable adaptive potential and allowing *M. burtonii* to sense and respond to its environment. A full pathway for synthesis of unsaturated isoprenoid lipids is present and interestingly is identical to that of *M. jannaschii*, a hyperthermophilic relative. Four operon-like clusters of polysaccharide biosynthesis genes (containing 10, 11, 16, and 39 genes) are present. Based on a comparative analysis of psychrophilic and non-psychrophilic archaeal genomes, the Defense Mechanism COG category of proteins was statistically overrepresented in *M. burtonii* compared to other methanogens or total archaea. These proteins included the restriction-modification systems, which may be required to combat high levels of foreign DNA in Ace Lake, and six putative novel ABC transporters.

In summary, the completed psychrophilic genomes reveal a number of common traits involved in cold adaptation and survival. These include:

- Genome arrangement and content, including genome recombination through the presence of transposons, insertion sequences, and phage
- Capacity to modulate and maintain membrane fluidity via lipid unsaturation
- EPS production
- Altered thermodynamics of proteins and enzymes through amino acid skew or addition of antifreeze domains
- Presence of Csps, chaperones, RNA helicases
- tRNA modifications (e.g., pseudouridine, dihydrouridine)
- Osmolyte production and transport systems
- Mechanisms to cope with ROS
- Sensitive signal transduction systems

While many of these features are common to several or more organisms, no two species share the exact same set of adaptations suggesting there is a wide variety of ways to reach the same goal of growth at low temperature. Undoubtedly, as more psychrophilic genomes are completed further cold-adaptation strategies will be uncovered. It will then be possible to begin

to link specific genomic traits (and hence mechanisms of adaptation) to ecologically relevant selection pressures (e.g., subsurface versus sea-ice versus planktonic habitat; aerobic versus anaerobic; salinity; nutrient flux; temperature stability).

Functional Genomics of Psychrophilic Bacteria and Archaea

Transcriptomics and proteomics provide global views of RNA and protein levels in the cell, respectively (*Table 6.6.4*). By measuring RNA and protein abundance, the functional genomic methods provide a combined measure of both gene expression or protein synthesis, and mRNA or protein stability/turnover. By comparing RNA or protein abundances from the same organism under at least two test conditions, changes in abundance can be measured and related to the specific test parameters. For example, by comparing the growth of a psychrophile at a relatively low versus high temperature, inferences can be made about mechanisms of growth temperature adaptation from the quantitative changes in gene product abundance. In addition to global gene expression studies, studies of cellular composition (e.g., intracellular solutes, membrane lipid composition) and targeted studies of specific genes and proteins, have provided important functional information about cold adaptation. These functional approaches have often been led by inferences made from genomic analyses.

Membrane and Cell Wall

Low temperatures reduce membrane fluidity and permeability, and microorganisms respond by producing less saturated fatty acids to improve membrane fluidity (Russell 2008). While many microorganisms respond by modifying the types and proportions of membrane lipids, this can occur in different ways. Fatty acid desaturases reduce saturation of preexisting fatty acids and a desaturase is upregulated at low temperature in *E. sibiricum* (Rodrigues et al. 2008). In contrast, the Antarctic methanogen *M. burtonii* does not encode a desaturase but alters its expression of several lipid biosynthesis genes resulting in less saturated isoprenoid lipid precursors (Nichols et al. 2004). A similar observation has been made for the archaeon *H. lacusprofundi* (Gibson et al. 2005). *P. arcticus* appears to upregulate a fatty acid desaturase, a fatty acid synthase and a phosphatidylethanolamine synthase, thereby modifying existing lipids and regulating de novo synthesis pathways (Zheng et al. 2007).

Other lipid modifications can also improve membrane fluidity. *S. oneidensis* upregulates two lipid biosynthesis proteins, so2088 and so3179, which synthesize Lipid A modified by palmitoleate acylation (Gao et al. 2006). It is also likely that isoleucine and valine degradation at low temperature produces intermediates, which increase the proportion of anteiso-methyl-branched fatty acids in the membrane of *S. oneidensis* (Gao et al. 2006). Membranes with a higher proportion of anteiso-methyl-branched fatty acids are more fluid than those with a higher proportion of iso-methyl-branched fatty acids (Russell 2008).

P. arcticus achieves changes to its cell wall composition at low temperature by downregulating transcription of peptidoglycan biosynthesis genes including murein disaccharides, transglycosylases, and peptidoglycan transpeptidases while upregulating genes for peptidoglycan breakdown. Transcription of one peptidoglycan crosslinking DD-peptidase isozyme (*dac2*) was upregulated at low temperature while another (*dac1*) was downregulated, and it was

speculated that the suppression of peptidoglycan crosslinking may function to maintain cell wall elasticity at low temperatures (Bergholz et al. 2009). *E. sibiricum* similarly strengthens its cell wall at −2 to 10°C, upregulating the *murADEI* peptidoglycan biosynthesis and *dupABD* lysine biosynthesis operons (Rodrigues et al. 2008). In *M. burtonii*, numerous surface layer proteins were found to be more abundant at 4°C, indicating an extensive remodeling of the cell envelope in response to low temperature (Williams et al. 2010a). These include a large number of putative S-layer proteins containing domains that point to roles in protein–protein or protein–carbohydrate interactions. It was speculated that deployment of cell surface proteins that promote intercellular interactions may facilitate nutrient exchange under challenging environmental conditions, and/or improve the stability of the cell membrane (Williams et al. 2010a).

Membrane transporters and other membrane-bound proteins are upregulated at low temperatures in several bacterial species and in *M. burtonii* (Table 6.6.5). This is likely to reflect, at least in part, a compensation for lower transporter efficiency at low temperature; in the cold, membrane transport and diffusion are impeded by reduced membrane permeability and lower thermodynamic efficiency (Kurihara and Esaki 2008; Russell 2008). However, in some species the upregulation of a transporter may be a way of increasing the import or export of a specific compound (e.g., cryoprotectant or accommodating a shift in metabolism).

Table 6.6.5

Membrane-bound proteins upregulated at low temperature

Species	Gene/protein
B. psychrosaccharolyticus	ATP-binding ABC transporter, ABC transporter-associated protein (Seo et al. 2004)
E. sibiricum	BetT (choline-glycine-betaine transporter), carnitine transporter (Rodrigues et al. 2008)
M. burtonii	Mbur_0060 (YVTN/NHL protein), Mbur_0314 (cadherin), Mbur_2003 (Ig-like domain protein), Mbur_0714 (MxaI/MoxI-like protein), Mbur_0728, and Mbur_0729 (proteins with dockerin and cohesin domains), other putative S-layer proteins (DUF1608, etc.), glycine betaine ABC transporter, GspE-3 (type II secretion system protein), probable TolB related transporter, probable potassium, sodium and cation transporters, SufB ABC transporter (Goodchild et al. 2004a; Goodchild et al. 2005; Burg et al. 2010; Williams et al. 2010a, b)
P. arcticus	Psyc_1070 (periplasmic subunit for ABC sulfate transporter), Psyc_2041 (sulfate uptake transporter), Psyc_2033 to Psyc_2035 (ABC zinc transporter) (Bergholz et al. 2009)
P. cryohalolentis	AfuA and FecA (ferric iron transporters), LolD (lipoprotein transporter), TolC (efflux protein), DctP (TRAP-T dicarboxylate transporter), Uup (ABC transporter) (Kurihara and Esaki 2008; Bakermans et al. 2007)
S. livingstonensis	OmpA, OmpC (probable outer membrane porins) (Kawamoto et al. 2007)
S. oneidensis	LolE (lipoprotein releasing system transmembrane protein), LolA (outer membrane lipoprotein carrier protein), LolD (lipoprotein trasporter), LolB (unspecified outer membrane lipoprotein) (Gao et al. 2006)

In *P. cryohalolentis*, the upregulation of a putative acetate transporter and acetate kinase at low temperature may increase acetate transport and processing to accommodate energy and carbon demands (Bakermans et al. 2007). Upregulated ferric iron transporters may also relieve oxidative stress and allow increased production of iron-dependent enzymes to counter decreased rates of enzyme activity (Bakermans et al. 2007). The upregulation of an ABC zinc transporter and downregulation of Fe^{2+} uptake transporters in *P. arcticus* also appears to be an adaptative strategy for oxidative stress (Bergholz et al. 2009).

In *E. sibiricum*, the upregulation of choline, glycine, betaine, and carnitine transporters at −2–10°C compared to 28°C is a possible osmoregulatory response triggered by water flow from the cell at low temperatures (Rodrigues et al. 2008). A similar type of response has been observed for a glycine betaine ABC transporter at 4°C in *M. burtonii* (Williams et al. 2009a).

DNA Modulating and Translational Proteins

At low temperatures, many microbial species upregulate DNA-modulating and translational proteins to compensate for reduced efficiency of transcription, translation, and DNA replication (*Tables 6.6.6* and *6.6.7*). The most prominent class of upregulated proteins from this category are ribosomal proteins, which is likely to reflect a need to compensate for reduced translational efficiency at low temperatures. RNA helicases also appear to be generally important for cold adaptation and are likely to facilitate the unwinding of secondary structures in nucleic acids. For example, a DEAD-box RNA helicase, *csdA* (Psyc_1082), is upregulated in *P. arcticus* and is important for low temperature growth and possesses a highly disordered C-terminal extension (Bergholz et al. 2009), similar to that observed in *M. burtonii* (Lim et al. 2000).

Csps are a family of nucleic acid binding proteins, which share a Cold Shock Domain (CSD), and are frequently associated with cold shock responses in bacteria (Gao et al. 2006). CspA is thought to moderate RNA secondary structure by chaperoning unwound RNA (Jiang et al. 1997), and may also act as a transcriptional inducer and antiterminator (Kurihara and Esaki 2008). Csps are upregulated in a broad range of bacteria including *P. cryohalolentis*, *P. arcticus*, *S. oneidensis*, *S. livingstonensis* and *Arthrobacter globiformus* following cold shock

Table 6.6.6

Ribosomal proteins upregulated at low temperature

Species	Ribosomal protein
B. psychrosaccharolyticus	S30P, L7/L12, L10, S6 (Seo et al. 2004)
E. sibiricum	L25, L7AE (Rodrigues et al. 2008)
M. burtonii	L37E, L24E, L22P, L24P, S4E, L5P, L21E, L3P, L23P, L19E, L30P, L15P, S13P, S4P, S11P, S19E, L15E, L7Ae, S7P, S12P, L1P, L10E, L12P, S8E, L18E, L13P, S9P (Goodchild et al. 2005; Burg et al. 2010; Williams et al. 2010a)
P. arcticus	S3, S4, S6, S15, L2, L7/L12, L15, L28 (Zheng et al. 2007)
P. cryohalolentis	S2, L25 (Bakermans et al. 2007)

Table 6.6.7

Non-ribosomal transcription and translation proteins upregulated at low temperature

Species	Gene/protein
A. globiformus	CspA, CspB-like protein (Berger et al. 1996)
B. psychrosaccharolyticus	HU-like DNA binding protein, putative elongation factor (Seo et al. 2004)
E. sibiricum	GyrAB (gyrase A/B), NusA (transcription terminator/antiterminator), RpoN (RNA polymerase sigma 70), numerous RNA helicases, IF-1 and IF-2 (transalation initiation factors), RbfA (ribosome-binding factor A), EF-Ts (elongation factor), CspABCD homologs (Qiu et al. 2006; Rodrigues et al. 2008)
M. burtonii	CheY-like DNA binding protein, TATA-box binding protein (TBP), three TRAM-domain proteins (Mbur_0304, Mbur_0604, Mbur_1445), DEAD-box RNA helicases (Mbur_1950, Mbur_0245), RNase J-like protein (Mbur_2398), aRadC (RecA family recombinase; Mbur_2095), RadA (DNA repair and recombinase) (Goodchild et al. 2004a; Burg et al. 2010; Williams et al. 2010a)
P. arcticus	gene 14 (tRNA synthetase), gene 1195 (EF-Tu elongation factor), DNA-directed RNA polymerase subunit, CspA homolog, rbfA (ribosomal binding factor), NusA and NusB (transcription terminator/antiterminators), IF-2 (translation initiation factor) (Zheng et al. 2007; Bergholz et al. 2009)
P. cryohalolentis	CspA, EF-Ts and EF-Tu (elongation factors), NusA (transcription terminator/antiterminator) (Bakermans et al. 2007)
S. livingstonensis	CspA, RpoA (RNA polymerase subunit), GreA (transcriptional regulator/elongation factor), TufB (elongation factor), Efp (translation elongation/initiation factor), lysU (tRNA synthase) (Kawamoto et al. 2007)
S. oneidensis	so1648 (CspA-like protein), TopB (topoisomerase), Rbn (ribonuclease), three HU-like DNA binding proteins, EF-Tu (elongation factor), yfiA-2 (ribosomal subunit interface protein), NusA (transcription terminator/antiterminator), IF-1 and IF-2 (translation initiation factors), TufB (elongation factor) (Gao et al. 2006)

and/or during low-temperature growth (Berger et al. 1996; Gao et al. 2006; Kawamoto et al. 2007; Bakermans et al. 2007; Bergholz et al. 2009). Enhanced Csp synthesis is not exclusively associated with cold shock/growth, and Csps appear to play diverse cellular roles. In *S. oneidensis*, only one of three Csps appears to play a particular role in low temperature growth (Gao et al. 2006). Only few archaea possess *csp* genes, and the function of a Csp protein from *M. frigidum* and a protein with a CSD-fold (but little sequence identity to Csp proteins) from *M. burtonii*, have been examined (Giaquinto et al. 2007). In addition, small proteins each composed of a single TRAM domain (unique to archaea) were found to be upregulated at low temperature in *M. burtonii*, and proposed to serve as RNA chaperones in an analogous manner to Csp proteins (Williams et al. 2010a).

Modification of nucleosides (e.g., methylation) can stabilize tRNA, and as a result, the extent of tRNA modification in archaea and bacteria tends to be much higher in

hyperthermophiles (Dalluge et al. 1997; Noon et al. 2003). On the other hand, dihydrouridine is a specific modified nucleoside that can enhance tRNA flexibility. Consistent with this, relative to hyperthermophilic archaea, tRNA in *M. burtonii* is characterized by an overall low extent of modification, but a high proportion of dihydrouridine per tRNA molecule (Noon et al. 2003).

In *S. oneidensis*, a topoisomerase (TopB), a ribonuclease (Rbn), and three DNA binding proteins (with identity to *Escherichia coli* HU family DNA binding proteins) are upregulated by the cold, and may function to minimize DNA and RNA secondary structure (Gao et al. 2006). A HU family DNA-binding protein is also upregulated at 4°C in *B. psychrosaccharolyticus* (Seo et al. 2004). In *M. burtonii* numerous proteins with relatively clear functional annotations and others with nucleic acid binding domains but with less confident predictions of cellular functions have been found to be upregulated at low temperature (Saunders et al. 2005; Williams et al. 2010a).

Global regulation studies have illustrated the complexity of molecular responses for some cellular processes, particularly those involving numerous individual gene products, such as the transcription and translation machinery. In *P. arcticus*, while proteins which promote tRNA-ribosome binding and translational accuracy (tRNA synthetase and EF-Tu) are upregulated at 4°C, a number of ribosomal proteins (S3, S4, S6, S15, L2, L7/L12, L15, and L28) are simultaneously downregulated (Zheng et al. 2007; Kurihara and Esaki 2008). This has been suggested to reflect a strategy of conserving energy by minimizing ribosomal count while simultaneously ensuring extant ribosomes are fully utilized. However, in a different *Psychrobacter* species, *P. cryohalolentis*, ribosomal proteins S2 and L25 and two elongation factors, EF-Ts and the EF-Tu-like TypA, were found to be upregulated at −4°C (Bakermans et al. 2007). The differences in response between the two *Psychrobacter* species may in part reflect the 8°C temperature difference used in the two studies.

In *M. burtonii*, proteins involved in translation initiation rather than elongation are upregulated at low temperature, and it has been speculated that this may be to ensure that the translation machinery is ready to process mRNA before inhibitory secondary structures form that would otherwise stall polypeptide synthesis (Williams et al. 2010a). At the transcriptional level, the basal transcription machinery is less influenced by temperature than a host of bacterial-like regulatory proteins, indicating that transcriptional regulation is mainly facilitated by these types of transcriptional regulators (Williams et al. 2010a). Consistent with this, a specific cold responsive mechanism of gene regulation that involves a long 5′-untranslated region has been identified as a feature of an RNA helicase gene in *M. burtonii* and a number of genes from several bacteria (Lim et al. 2000).

In *E. sibiricum*, GyrAB gyrase is upregulated at −2°C and may counteract increased DNA supercoiling that could occur at low temperature (Rodrigues et al. 2008). In *M. burtonii*, an archaeal RecA family recombinase (aRadC) was reported to be upregulated at 4°C and may function to rescue collapsed DNA replication forks as a consequence of increased duplex DNA stability at low temperature (Williams et al. 2010a). In contrast, PCNA (DNA sliding clamp protein) and XPB (catalyze ATP-dependent local DNA strand opening) are upregulated at 23°C (high temperature) and have been proposed to effect nucleotide excision repair in response to heat and oxidative stress that may occur at this elevated growth temperature (Williams et al. 2010a). Interestingly, transposases are not only highly represented in the genomes of some psychrophiles (e.g., *M. burtonii*; Allen et al. 2009), but appear to be expressed (Goodchild et al. 2004b), indicating that they are active and genome rearrangement may be occuring.

Chaperonins and Proteolysis

Protein misfolding is a major cellular challenge at both high and low temperature, and proteins involved in chaperoning, refolding and turnover of nascent, and mature proteins are involved in microbial adaptive responses (Table 6.6.8). Following cold shock, chaperonin *groES, groEL, dnaK, dnaJ, htpGd*, and *hslU* and protease *lon, aprE, so3942*, and *so4162* genes are upregulated in *S. oneidensis*. With the exception of *aprE*, all these genes are also upregulated by heat shock (Gao et al. 2006). Similarly, chaperonins Hsp10 and Hsp60 were upregulated at 4°C in *P. arcticus* (Zheng et al. 2007), and Hsp10 is upregulated in *B. psychrosaccharolyticus* at the same temperature (Seo et al. 2004). Hsp70 is upregulated at 4°C in *E. sibiricum* (Qiu et al. 2006). It is noteworthy that cold shock or heat shock conditions confer an inherently greater challenge to cells than steady-state growth, that is, stress caused by a sudden change often invokes a more pronounced and transient response until the cell adapts to growth (if possible) at the new temperature. As a result, molecular responses to these types of conditions should not be equated to an adaptive response to growth at relatively low or high growth temperatures.

Peptidyl-prolyl *cis-trans* isomerases (PPIases) have been reported to be upregulated at low temperature in *S. oneidensis, S. livingstonensis*, and *Shewanella* sp. SIB1 (Kurihara and Esaki 2008; Suzuki et al. 2004), *E. sibiricum* (Qiu et al. 2006), and *M. burtonii* (Goodchild et al. 2004a; Goodchild et al. 2005; Williams et al. 2010a). This observation in bacteria and archaea highlights the importance of the functions that the PPIases perform to optimize protein folding at low temperature, which includes their ability to isomerize proline imide bonds, and possibly refold proteins.

In *M. burtonii*, several chaperones are upregulated at 23°C (compared to 4°C) indicating that they are likely to play a more important role in rescuing protein function under conditions of high temperature stress (Goodchild et al. 2004b, 2005; Williams et al. 2010a; Burg et al. 2010). In contrast, an atypical J-domain (type III) protein, which may bind specific protein

Table 6.6.8

Chaperonins and proteolysis proteins upregulated at low temperature

Species	Gene/protein
B. psychrosaccharolyticus	HSP10 (heat shock chaperonin) (Seo et al. 2004)
E. sibiricum	Pnp (polyribonucleotide nucleotidyltransferase/RNase) (Rodrigues et al. 2008), HSP70, peptidyl-prolyl *cis-trans* isomerase (Qiu et al. 2006)
M. burtonii	Peptidyl-prolyl *cis-trans* isomerase (cyclophilin-type and FKBP-type), Mbur_1212 (possible DnaK recruiter) (Goodchild et al. 2004a; Goodchild et al. 2005; Burg et al. 2010; Williams et al. 2010a)
P. arcticus	HSP10, HSP60 (heat shock chaperonins) (Zheng et al. 2007), 5 RNases, 12 peptidases, ClpB homolog (chaperone) (Bergholz et al. 2009)
S. livingstonensis	Peptidyl-prolyl *cis-trans* isomerase (Kawamoto et al. 2007)
S. oneidensis	GroES, GroEL, DnaK, DnaJ, HtpGd and HslU (chaperones), Ion, AprE, so3942 and so4162 (proteases), tatA, tatB and tatC (Sec-independent translocases), peptidyl-prolyl *cis-trans* isomerase (Suzuki et al. 2004; Gao et al. 2006)

substrates and recruit these to DnaK was upregulated at 4°C and may therefore play a specific role as part of a low temperature chaperone system (Burg et al. 2010). In *Shewanella* sp. Ac10, DnaK has been reported to be upregulated at low temperature (Yoshimune et al. 2005).

In *P. arcticus*, 12 peptidases and 5 RNases were upregulated at low temperature, although two RNases were downregulated (Bergholz et al. 2009). RNases probably serve to maintain the turnover of biosynthesis precursors at low temperature, with some proteolysis enzymes possibly serving the same role. In *M. burtonii* the α-subunit of the proteasome and a number of secreted proteins that were predicted to have proteolytic function were upregulated at 4°C, indicating that degradation and subsequent recycling of proteins, and/or post-translational processing of secreted proteins may be important for cold adaptation of this archaeon (Williams et al. 2010a).

In *S. oneidensis*, Sec-encoding genes were either downregulated or unaffected following cold shock, while genes encoding Sec-independent translocases *tatA*, *tatB*, and *tatC* were upregulated more than 3.4-fold, indicating a shift from Sec-mediated to Sec-independent protein translocation (Gao et al. 2006). Conversely, in the archaeon *M. burtonii* a Tat system is not present and secretion appears to be primarily mediated by the Sec pathway (Saunders et al. 2006). The abundance of numerous secreted proteins in *M. burtonii* were found to be upregulated at 4°C, being either released on the external side of the membrane or anchored via a C-terminal membrane anchor (Williams et al. 2010a). The proteins are likely to form part of the protective glycoprotein and protein S-layer.

Metabolic Proteins

Metabolic responses to low temperature are dependent on the specific physiology of individual psychrophiles (*Table 6.6.9*). *M. burtonii* is a methylotrophic methanogen capable of growth with trimethylamine (TMA) and methanol as sole sources of carbon. The abundance of methanogenic and biosynthetic proteins has been found to be greatly affected by both substrate (TMA versus methanol) and growth temperature (4 versus 23°C) (Williams et al. 2010b). The strong influence of substrate on abundance of substrate-specific methanogenesis enzymes, and higher protein abundance at 23°C consistent with higher growth rate, indicates that these core metabolic enzymes do not play a central role in cold adaptation per se.

However, despite this overall metabolic response in *M. burtonii*, it has been noted that while the membrane-bound proton pump subunit $F_{420}H_2$ dehydrogenase is upregulated at 4°C, ATP synthesis and several oxidative methylotrophic genes are downregulated (Goodchild et al. 2004b, 2005; Kurihara and Esaki 2008). This has been interpreted as a simultaneous downshift in biosynthesis and a switch from a sodium to proton motive force, which is thermodynamically economical at low temperature. A similar downshift and switch, also characterized by decreased ATP synthesis, was proposed for *E. sibiricum*, to exploit higher oxygen solubility at low temperature and switch from substrate level to oxidative phosphorylation and a proton motive force (Rodrigues et al. 2008).

In *S. oneidensis*, a large number of energy metabolism genes are downregulated during cold shock to 8°C (Gao et al. 2006). However, a large pyruvate synthesis operon (so2486–so2489) and genes from pathways for other fermentative end products (formate, acetyl-CoA, lactate, and aceto-lactate) are upregulated. This has been interpreted as *S. oneidensis* preferentially utilizing fermentative end products upon cold shock, although it is not clear why this may be advantageous (Gao et al. 2006).

Table 6.6.9

Metabolic proteins differentially regulated at low temperature

Species	Upregulated	Downregulated
B. psychrosaccharolyticus	Five glycolytic and four other metabolic proteins (Seo et al. 2004)	
E. sibiricum	glpA (glycerol 3-phosphate dehydrogenase), glpKF (glycerol degradation), D-galactose catabolism genes, PTS glucose transport genes, Exig_1739 (alpha-amylase), PflD (pyruvate formate lyase), histidine, serine, aerginine, and lysine synthesis genes (Rodrigues et al. 2008)	Exig_2537 (alpha-amylase), numerous exopolysaccharide synthesis genes, ATPase synthase, cyoCBAE (cytochrome synthesis), PorA (pyruvate ferredoxin oxidoreductase alpha subunit) (Rodrigues et al. 2008)
M. burtonii	ketol-acid reductoisomerase, Mbur_0686 (possible RimK-like amino acid ligase), L-threonine O-3-phosphate decarboxylase, Mbur_1269 (6-pyrovoyl tetrahydrobiopterin synthase) (Burg et al. 2010; Williams et al. 2010a, b)	Methanogenesis (methyltransferases; oxidative and reductive methylotrophy proteins) and ATP synthesis proteins, pyrophosphate proton pump (HppA), acetyl-CoA decarbonylase/synthase complex and pyruvate synthase complex subunits, ThiC and Thi4 thiamine biosynthesis proteins, dihydrodipicolinate synthase, Mbur_2001 (2-amino-3,7-dideoxy-D-threo-hept-6-ulosonate synthase) (Goodchild et al. 2004a; Goodchild et al. 2005; Burg et al. 2010; Williams et al. 2010a, b)
P. arcticus	TrpG, TrpD (tryptophan synthesis), Psyc_2024–2027 and Psyc_2028–2031 (ATP synthesis), relA (amino acid biosynthesis regulator), acs (acetyl co-A sythetase), Psyc_0728 (choline dehydrogenase) (Bergholz et al. 2009)	Branched-chain amino acid, arginine, and lysine biosynthesis genes, Atk (acetate kinase), Pta (phosphotransacetylase), Psyc_1301 (betatine-carnitine-choline type transporter), Psych_0729 (betaine aldehyde dehydrogenase), numerous TCA cycle and glycoxylate shunt genes, numerous amino acid synthesis genes excluding tryptophan synthesis, GcvH and GcvP (glycine cleavage), Psyc_0826 (betatine-carnitine-choline type transporter) (Bergholz et al. 2009)
S. oneidensis	Pyruvate synthesis operon so2486-so2489, pathways for other fermentative end products (formate, acetyl co-A, lactate and aceto-lactate) (Gao et al. 2006)	Several energy metabolism genes (Gao et al. 2006)

In *P. cryohalolentis*, two glyoxylate cycle enzymes (malate dehydrogenase *mdh* and isocitrate lyase *aceA*) and acetate kinase *ackA* were upregulated at −4°C (Bakermans et al. 2007). As the cells were grown with acetate as the sole carbon source, and an acetate membrane transport protein DctP was also upregulated (see *Table 6.6.5*), it was suggested the changes reflect an increase in cellular energy production rather than compensation for lower enzymatic activity at low temperature (Bakermans et al. 2007). It was also postulated that the glyoxylate cycle may also be upregulated to produce intermediate products needed in other stress response pathways (Bakermans et al. 2007). In *P. arcticus*, two genes from one acetate activation pathway (acetate kinase *atk* and phosphotransacetylase *pta*) were downregulated at low temperature while acetyl coenzyme A synthetase *acs* from an alternate pathway is upregulated. This implies a switch to the alternate pathway as a response to cold temperature (Bergholz et al. 2009). In *P. arcticus*, a large number of energy and metabolism genes including NADH dehydrogenase, ATP synthase, sodium-translocating NADH-ubiquinone oxidoreductase, tricarboxylic acid cycle, and glyoxylate shunt genes were also downregulated at low temperature, perhaps reflective of a low temperature regulated stringent response (Bergholz et al. 2009) (*Tables 6.6.9* and *6.6.10*).

In *E. sibiricum* the expression of two α-amylase genes was found to be inversely regulated by growth temperature, and it was proposed that this may reflect the thermal properties of the individual enzymes (Rodrigues et al. 2008).

Future Prospects

Functional genomic studies of a few psychrophiles indicate that growth temperature causes relatively few quantitative changes in gene expression; for example, based on transcriptome analysis, *E. sibiricum* gene expression is largely unchanged within the growth temperature range 4°C–28°C, with differential gene expression mostly occurring at growth temperature extremes

Table 6.6.10

Miscellaneous proteins upregulated at low temperature

Species	Gene/protein
E. sibiricum	Proline dehydrogenase, PspA (Qiu et al. 2006; Rodrigues et al. 2008)
M. burtonii	ParA partitioning protein, Mbur_2028 (exopolysaccharide synthesis), Mbur_0356 (chemotaxic protein), CheW (chemotaxic protein), Mbur_0104 and Mbur_0346–0347 (flagellins) (Goodchild et al. 2004a; Goodchild et al. 2005; Burg et al. 2010; Williams et al. 2010a, b)
P. arcticus	ahpC, hsp33, isc operon encoded by Psyc_1477–1482, Psyc_1043 and Psyc_1950 (peptide methionine sulfate reductases), peroxide-resistant aconitase A (Bergholz et al. 2009)
P. cryohalolentis	OsmC (organic hydorperoxide detoxifier), cheA (chemotaxic protein histidine kinase) (Bakermans et al. 2007)
S. livingstonensis	FlgE and FlgL (hook-related flagellum proteins), FtsZ (septum formation protein) (Kawamoto et al. 2007)
S. oneidensis	so0584, so1056, so3282 and so4053 (methyl-accepting chemotaxic proteins), so2125, so2318, so3202 (chemotaxic proteins) (Gao et al. 2006)

(i.e., at −2.5°C and 39°C) (Rodrigues et al. 2008). A similarly small number of changes (in this case in the proteome) were observed for *Desulfobacterium autotrophicum*; a marine sulfate-reducing bacterium described as being psychrotolerant (Rabus et al. 2002). In contrast, extensive changes in differential abundance have been observed for global levels of proteins and mRNA in a range of other psychrophiles (see section Functional Genomics of Psychrophilic Bacteria and Archaea above). To better understand the molecular mechanisms of adaptation in psychrophiles it will be valuable to extend functional genomic studies to a range of other psychrophiles that represent broader phylogenetic diversity and ecotypes. Performing studies using a range of relevant growth substrates (and varying growth temperature) will also help to identify genes that are core to a growth temperature response of an individual organism (e.g., Williams et al. 2010a, b). Similarly, determining quantitative changes in different subcellular fractions (e.g., cytosolic versus membrane versus secreted) and adopting robust statistical methods for evaluating quantitative changes in gene product abundance will help to clarify the role that specific proteins play in the cell (e.g., Williams et al. 2010a, b; Burg et al. 2010; Ting et al. 2009). Moreover, it will be useful to assess changes that occur in gene product abundance across a range of temperatures that span the growth temperature range of an individual psychrophile, rather than limiting quantitative assessments to a binary comparison of two growth temperatures. These types of studies will provide important insight into the capacity of psychrophiles to regulate their response to growth temperature and help to clarify core psychrophilic versus organism specific mechanisms of cold adaptation.

It has been well established that cultivation approaches typically recover only a small, skewed fraction of the total cells present in many environmental samples. Moreover, molecular ecological surveys of PCR amplified rRNA genes do not allow inferences about cell physiology or biological capacity. Random shotgun sequencing of DNA extracted from entire environmental samples (metagenomics) provides information about which types of microorganisms are present and what their functional capacities are likely to be. More than 30 microbial communities from diverse polar and permanently cold environments are currently at various stages of sequencing. Metagenome sequencing efforts include Antarctic lakes, polar ocean waters, permafrost, and glacial ice. These data sets are likely to reveal an entirely new level of understanding about psychrophilic microbial communities and the microbial processes the microorganisms are driving (Cavicchioli 2007; Murray and Grzymski 2007). The metagenome data will also greatly enhance metafunctional studies, for example, providing the DNA sequence baseline for protein identifications from mass spectrometry data when performing metaproteomics. To date, no metatranscriptomic or metaproteomic studies have been published for cold adapted microbial communities. However, this will change in the near future with studies, such as those from an Antarctic lake, which incorporate comprehensive genome coverage and a high level of proteomic coverage for unique psychrophilic microorganisms (R. Cavicchioli et al. unpublished data).

Integrating meta/genomics and meta/functional genomics with meteorological, geological, chemical, and physical data will produce a considerably more comprehensive understanding of how psychrophiles have evolved and how they have transformed and presently interact with permanently cold environments. In this regard, as an environmental parameter, low temperature has been shaping the genomes of psychrophilic microorganisms since life first emerged on Earth. In fact, based on the stability of macromolecules and a range of other pertinent factors, there has been speculation that life may have evolved in low temperature environments (Bada and Lazcano 2002; Price 2009). Therefore, understanding the adaptations that allow

psychrophilic microorganisms to successfully compete in their environment is not just an exercise in comparative genomics, physiology, and biochemistry but a quest for understanding the fundamentals of life.

Cross-References

- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
- 6.2 Taxonomy of Psychrophiles
- 6.3 Diversity of Psychrophilic Bacteria from Sea Ice - and Glacial Ice Communities
- 6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens
- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats
- 6.7 Psychrophilic Enzymes: Cool Responses to Chilly Problems
- 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)
- 9.2 Physiology
- 9.3 Biochemistry

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6.7 Psychrophilic Enzymes: Cool Responses to Chilly Problems

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_6.7,

Introduction

Most of the biotopes on Earth are permanently exposed to low temperatures. This includes the Antarctic continent, the Arctic ice floe, the permafrost, the mountain and glacier regions, and the deep-sea waters, the latter covering 70% of the planet surface. If a psychrophile is defined as an organism living permanently at temperatures close to the freezing point of water, in thermal equilibrium with the medium, this definition encompasses a large range of species from Bacteria, Archaea, and Eukaryotes. This aspect underlines that psychrophiles are numerous, taxonomically diverse, and have a widespread distribution. In these organisms, low temperatures are essential for sustained cell metabolism. Some psychrophilic bacteria grown at 4°C have doubling times close to that of *Escherichia coli* at 37°C. Such deep adaptation of course requires a vast array of metabolic and structural adjustments at nearly all organization levels of the cell, which begins to be understood thanks to the availability of genome sequences and of proteomic approaches. Overviews on these various aspects have been recently published (D'Amico et al. 2006a; Gerday and Glansdorff 2007; Margesin et al. 2008; Casanueva et al. 2010).

This chapter focuses on protein structure and mainly on enzyme function at low temperatures. As a general picture, psychrophilic enzymes are all faced to a main constraint, to be active at low temperatures, but the ways to reach this goal are quite diverse. Previous reviews can also be consulted for a complete coverage of this topic (Smalas et al. 2000; Feller and Gerday 2003; Siddiqui and Cavicchioli 2006).

Biocatalysis in the Cold: A Thermodynamic Challenge

The activity of enzymes is strongly dependent on the surrounding temperature. The catalytic constant k_{cat} corresponds to the maximum number of substrate molecules converted to product per active site per unit of time, and the temperature dependence of the catalytic rate constant is given by the relation:

$$k_{cat} = \kappa \frac{k_B T}{h} e^{-\Delta G^{\#}/RT} \qquad (6.7.1)$$

In this equation, κ is the transmission coefficient generally close to 1, k_B is the Bolzmann constant (1.38×10^{-23} J K^{-1}), h the Planck constant (6.63×10^{-34} J s), R the universal gas constant (8.31 J K^{-1} $mole^{-1}$), and $\Delta G^{\#}$ the free energy of activation or the variation of the Gibbs energy between the activated enzyme-substrate complex ES^{*} and the ground state ES (see *Fig. 6.7.8*). Accordingly, the activity k_{cat} is exponentially dependent on the temperature. As a rule of thumb, for a biochemical reaction catalyzed by an enzyme from a mesophile (a bacterium or a warm-blooded vertebrate), a drop in temperature from 37°C to 0°C results in a 20–80 times lower activity. This is the main factor preventing the growth of non-adapted organisms at low temperatures.

The effect of temperature on the activity of psychrophilic and mesophilic enzymes is illustrated in *Fig. 6.7.1*. Equation 6.7.1 is only valid for the exponential rise of activity with temperature on the left limb of the curves. This figure reveals at least three basic features of cold adaptation. (1) In order to compensate for the slow reaction rates at low temperatures, psychrophiles synthesize enzymes having an up to tenfold higher specific activity in this temperature range. This is in fact the main physiological adaptation to cold at the enzyme level. (2) The temperature for apparent maximal activity for cold-active enzymes is shifted

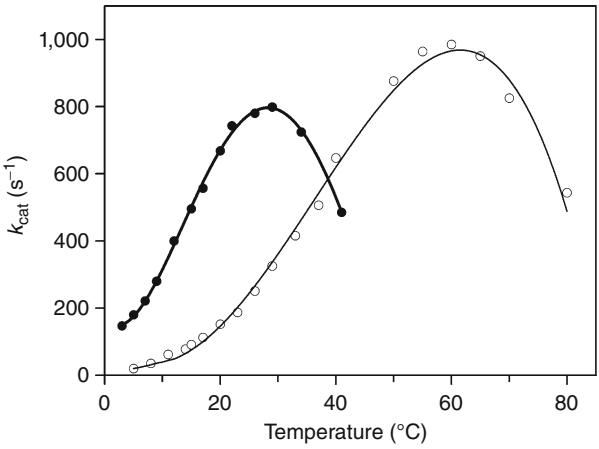

Fig. 6.7.1

Temperature dependence of the activity. The activity of psychrophilic (*filled symbols, heavy line*) and mesophilic (*open symbols*) enzymes recorded at various temperatures illustrates the main properties of cold-active enzymes (see text for details)

toward low temperatures, reflecting the weak stability of these proteins and their unfolding and inactivation at moderate temperatures. (3) Finally, the adaptation to cold is not perfect. It can be seen in *Fig. 6.7.1* that the specific activity of the psychrophilic enzymes at low temperatures, although very high, remains generally lower than that of the mesophilic enzymes at 37°C.

"Flexibility" and "Corresponding States" Hypotheses

Such activity curves have suggested relationships between the activity of the enzyme, the flexibility of the protein, and its stability. Indeed, the high activity at low temperatures seems to arise from an increased flexibility of the protein structure, especially at temperatures that strongly slow down molecular motions, but the consequence of this improved mobility of the protein structure is of course a weak stability. Fluorescence quenching of extremophilic enzymes was used to probe this "flexibility" hypothesis (*Fig. 6.7.2*). It was found that the structure of psychrophilic proteins has an improved propensity to be penetrated by a small quencher molecule, when compared to mesophilic and thermophilic proteins, and therefore revealing a less compact conformation undergoing frequent micro-unfolding events (D'Amico et al. 2003b; Collins et al. 2003; Georlette et al. 2003). The "flexibility" hypothesis has received further support by the quantification of macromolecular dynamics in the whole protein content of psychrophilic, mesophilic, thermophilic, and hyperthermophilic bacteria by neutron scattering (Tehei et al. 2004). This unique tool to study thermal atomic motions has indeed revealed that the resilience (equivalent to macromolecular rigidity in term of a force constant) increases with physiological temperatures. Furthermore, it was also shown that the atomic fluctuation amplitudes (equivalent to macromolecular flexibility) were similar for each microorganism at its physiological temperature. This is in full agreement with

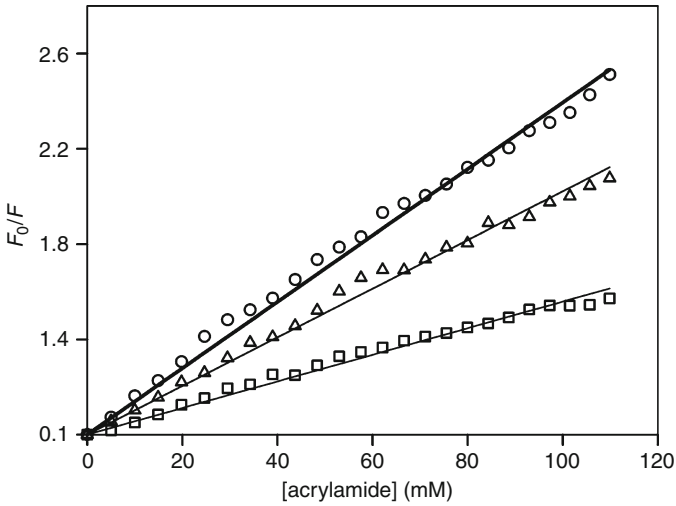

Fig. 6.7.2

Permeability of the protein structure. Fluorescence quenching experiments on psychrophilic (*circles, heavy line*), mesophilic (*triangles*), and heat-stable (*squares*) enzymes. The steep slope recorded for the psychrophilic enzyme indicates that its structure is easily penetrated by a small quencher molecule (acrylamide), resulting in a larger attenuation of the intrinsic fluorescence (F_0/F). This graph shows a clear correlation between this permeability index and the stability of the proteins. Adapted from (D'Amico et al. 2003b)

Somero's "corresponding state" concept (Somero 1995) postulating that enzyme homologues exhibit comparable flexibilities to perform catalysis at their physiologically relevant temperatures.

However, the "flexibility" hypothesis has been challenged from an evolutionary point of view. As a matter of fact, directed evolution (Wintrode and Arnold 2000) and protein engineering (Bae and Phillips 2006) of enzymes have demonstrated that activity and stability are not physically linked in protein. Accordingly, it has been proposed that the low stability of cold-active enzymes is the result of a genetic drift related to the lack of selective pressure for stable proteins (Wintrode and Arnold 2000). Nevertheless, several lines of evidences indicate that the situation is more subtle. For instance, in multi-domain psychrophilic enzymes containing a catalytic and a non-catalytic domain, the catalytic domain is always heat-labile (*Fig. 6.7.3*) whereas the non-catalytic domain can be as stable as mesophilic proteins (Lonhienne et al. 2001; Claverie et al. 2003; Suzuki et al. 2005). It is therefore unlikely that a genetic drift only affects the catalytic domain without modifying other regions of the protein. Furthermore, several directed evolution experiments have shown that when libraries of randomly mutated enzymes are only screened for improved activity at low temperatures without any other constraints, the best candidates invariably display the canonical properties of psychrophilic enzymes (see D'Amico et al. 2002a for discussion) whereas random mutations improving both activity and stability are rare (Giver et al. 1998; Cherry et al. 1999). It follows that improvement of activity at low temperatures associated with loss of stability appears to be the most frequent and accessible event. In conclusion, the current view suggests that the strong evolutionary pressure on psychrophilic enzymes to increase their activity at low temperatures

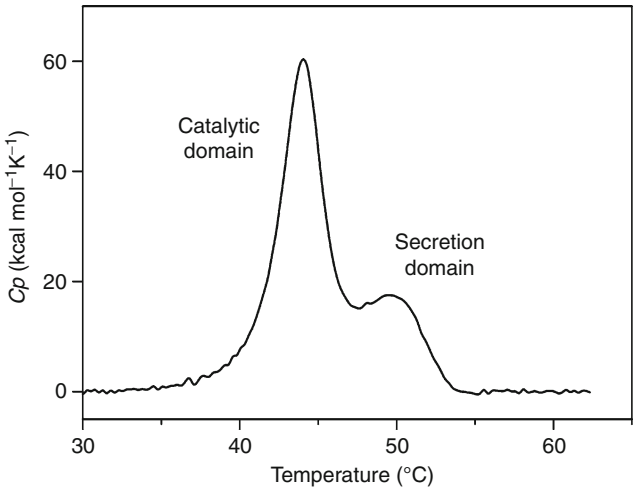

Fig. 6.7.3

Stability of domains in the α-amylase precursor. The precursor is composed by a catalytic domain and a secretion helper. In this thermogram, the top of the transitions corresponds to the melting points and the area under the transitions is roughly proportional to the domain sizes. Assuming that the low stability of psychrophilic enzymes is simply the result of a genetic drift (lack of selection for stable proteins), it is surprising that the more stable non-catalytic domain has not been subjected to the same extent to this drift. Adapted from (Claverie et al. 2003)

can be accommodated for by the lack of selection for stability and represents the simplest adaptive strategy for enzyme catalysis in the cold.

Flexibility and Structural Adaptations at the Active Site

Psychrophilic enzymes all share at least one property: a heat-labile activity, irrespective of the protein structural stability. Furthermore, the active site appears to be the most heat-labile structural element of these proteins (Collins et al. 2003; D'Amico et al. 2003b; Georlette et al. 2003). *Figure 6.7.4* illustrates this significant difference between the stability of the activity and the stability of the structure. The lower panel shows the stability of the structure as recorded by fluorescence. As expected, the structure of the cold-active enzyme is less stable than the mesophilic one. In the upper panel, the activity is recorded under the same experimental conditions and it can be seen that the mesophilic enzyme is inactivated when the protein unfolds. By contrast, activity of the cold-active enzyme is lost before the protein unfolds. This means that the active site is even more heat-labile than the whole protein structure. It was also shown that the active site of the psychrophilic α-amylase is the first structural element that unfolds in transverse urea gradient gel electrophoresis (Siddiqui et al. 2005). All these aspects point to a very unstable and flexible active site and illustrate a central concept in cold adaptation: localized increases in flexibility at the active site are responsible for the high but heat-labile activity (Fields and Somero 1998), whereas other regions of the enzyme might or might not be characterized by low stability when not involved in catalysis (Chiuri et al. 2009).

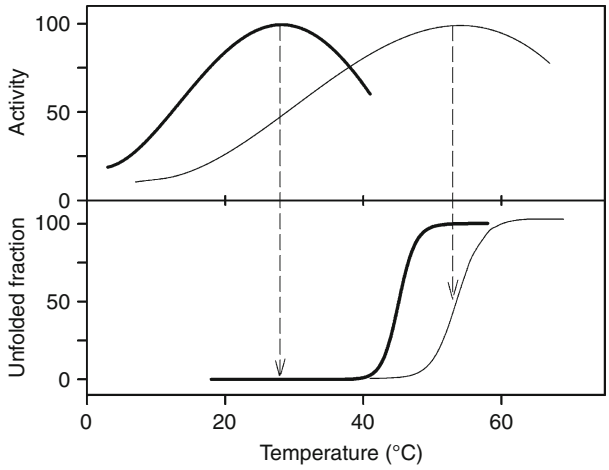

Fig. 6.7.4

Inactivation and unfolding of psychrophilic enzymes. The activity of psychrophilic enzymes (upper panel, *heavy line*) is inactivated by temperature before unfolding of the protein structure (lower panel, *heavy line*) illustrating the pronounced heat-lability of the active site. By contrast, inactivation of mesophilic (*thin lines*) or thermophilic enzymes closely corresponds to the loss of the protein conformation. Adapted from (D'Amico et al. 2003b)

Crystal structures of psychrophilic enzymes were of course of prime importance to investigate the properties of these heat-labile and cold-active catalytic centers. The first basic observation is that all side chains involved in the catalytic mechanism are strictly conserved. Indeed, comparison of the first X-ray structure of a psychrophilic enzyme, the cold-active α-amylase (Aghajari et al. 1998a, b), and of its closest structural homologue from pig both in complex with acarbose, a pseudosaccharide inhibitor mimicking the transition state intermediate (Aghajari et al. 2002; Qian et al. 1994), has shown that all 24 residues forming the catalytic cleft are strictly conserved in the cold-active α-amylase (*Fig. 6.7.5*). This outstanding example of active site identity demonstrates that the specific properties of psychrophilic enzymes can be reached without any amino acid substitution in the reaction center. As a consequence, changes occurring elsewhere in the molecule are responsible for the optimization of the catalytic parameters.

Nevertheless, significant structural adjustments at the active site of psychrophilic enzymes have been frequently reported. In many cases, a larger opening of the catalytic cleft is observed and achieved by various ways, including replacement of bulky side chains for smaller groups, distinct conformation of the loops bordering the active site, or small deletions in these loops, as illustrated by a cold-active citrate synthase (Russell et al. 1998). In the case of a Ca^{2+}, Zn^{2+}-protease from a psychrophilic *Pseudomonas* species, an additional bound Ca^{2+} ion pull the backbone forming the entrance of the site and markedly increases its accessibility when compared with the mesophilic homologue (Aghajari et al. 2003). As a result of such a better accessibility, cold-active enzymes can accommodate substrates at lower energy cost, as far as the conformational changes are concerned, and therefore reduce the activation energy required for the formation of the enzyme-substrate complex. The larger active site may also facilitate easier release and exit of products and thus may alleviate the effect of a rate limiting step on the

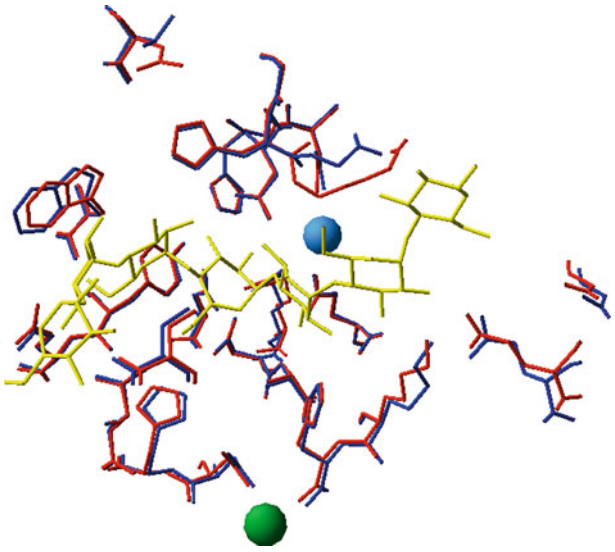

Fig. 6.7.5

Structure of the active site. Superimposition of the active site residues in psychrophilic (*blue*) and mesophilic (*red*) α-amylases. The chloride and calcium ions are shown as blue and green spheres, respectively. The 24 residues performing direct or water-mediated interactions with the substrate analog derived from acarbose (*yellow*) are identical and superimpose perfectly within the resolution of the structures, demonstrating a structural identity in these psychrophilic and mesophilic enzymes

reaction rate. It was also shown that an opening of the active site takes place upon binding of substrate or product in a cold-active xylanase whereas similar large scale movements are not observed in mesophilic or thermophilic structural homologues (De Vos et al. 2006). This can be tentatively related to higher active site mobility in the psychrophilic enzyme.

In addition, differences in electrostatic potentials in and around the active site of psychrophilic enzymes appear to be a crucial parameter for activity at low temperatures. Electrostatic surface potentials generated by charged and polar groups are an essential component of the catalytic mechanism at various stages: as the potential extends out into the medium, a substrate can be oriented and attracted before any contact between enzyme and substrate occurs. Interestingly, the cold-active citrate synthase (Russell et al. 1998), malate dehydrogenase (Kim et al. 1999), uracyl-DNA glycosylase (Leiros et al. 2003), and trypsin (Smalas et al. 2000; Gorfe et al. 2000; Brandsdal et al. 2001) are characterized by marked differences in electrostatic potentials near the active site region compared to their mesophilic or thermophilic counterparts that may facilitate interaction with ligand. In all cases, the differences were caused by discrete substitutions in non-conserved charged residues resulting in local electrostatic potential differing in both sign and magnitude.

Finally, two last examples illustrate the unsuspected diversity of strategies used to improve the activity in psychrophilic enzymes. With few exceptions, β-galactosidases are homotetrameric enzymes bearing four active sites. However, the crystal structure of a cold-active β-galactosidase revealed that it is a homohexamer, therefore possessing six active sites

certainly contributing to improve the activity at low temperatures (Skalova et al. 2005). Cellulases are microbial enzymes displaying a modular organization made of a globular catalytic domain connected by a linker to a cellulose-binding domain. Psychrophilic cellulases were found to possess unusually long linkers about five times longer than in mesophilic cellulases (Garsoux et al. 2004; Violot et al. 2005). The long linker adopts a large number of conformations, and considering the cellulose-binding domain anchored to the cellulose fibers and a rotation of the extended molecule around this axis, it was calculated that the catalytic domain has a 40-fold higher accessible surface area of substrate when compared with a mesophilic cellulase possessing a much shorter linker. Here also, increasing the available surface of the insoluble substrate to the catalytic domain should improve the activity of this enzyme at low temperatures.

Active Site Dynamics

The heat-labile activity of psychrophilic enzymes suggests that the dynamics of the functional side chains at the active site is improved in order to contribute to cold-activity and the above-mentioned structural adaptations seem to favor a better accessibility to the substrate and release of the product (Tsigos et al. 1998; Smalas et al. 2000). This active site flexibility of cold-active enzymes in solution is also well demonstrated by the psychrophilic α-amylase (D'Amico et al. 2006b). In this specific case, the above-mentioned structural identity of the catalytic cleft with its mesophilic homologue from pig precludes the involvement of adaptive mutations within the active site in the analysis of the results. As shown in *Table 6.7.1*, both the psychrophilic and mesophilic α-amylases degrade large macromolecular polysaccharides made

Table 6.7.1

Relative activity of the psychrophilic (AHA) and the mesophilic (PPA) α-amylases on macromolecular polysaccharides and on maltooligasaccharides. Adapted from D'Amico et al., 2006b

Substrate	Relative activity (%)	
	AHA	PPA
Macromolecular substrates		
Starch	100	100
Amylopectin	96	68
Amylose	324	214
Dextrin	108	95
Glycogen	74	59
Short oligosaccharides		
Maltotetraose G4	17	22
Maltopentose G5	69	145
Maltohexaose G6	94	147
Maltoheptaose G7	119	155
Maltooligosaccharides (G4–G10 mix)	64	101

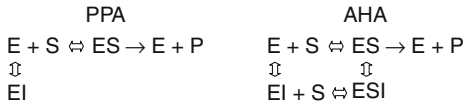

Fig. 6.7.6

Inhibition models of α-amylases. Reaction pathways for the competitive inhibition of starch hydrolysis by maltose for the mesophilic α-amylase PPA and of the mixed type inhibition for the psychrophilic α-amylase AHA. Under identical experimental conditions, the cold-active enzyme forms the ternary complex ESI (D'Amico et al. 2006b)

of glucose units linked by α-1,4 bonds. These substrates have a complex structure and are generally branched. Taking the natural substrate, starch, as the reference, it can be seen that the psychrophilic enzyme is more active on all these large substrates. Being more flexible, the active site can accommodate easily these macromolecular polysaccharides. Considering the small substrates, the reverse situation is observed. Both enzymes are active on short oligosaccharides of at least four glucose units, but in this case, the psychrophilic α-amylase is less active on all these small substrates. Apparently, the flexible active site accommodates less efficiently these short oligosaccharides.

The inhibition patterns provide additional insights into the specific properties of psychrophilic active sites (*Fig. 6.7.6*). Both the mesophilic and the psychrophilic α-amylases are inhibited by maltose, the end product of starch hydrolysis. In the case of the mesophilic enzyme, the enzyme can bind either the substrate (in a productive mode) or the inhibitor, but not both. By contrast, the cold-active enzyme can also bind either the substrate or the inhibitor but also both, forming the ternary complex ESI, once again suggesting a more accessible and flexible active site.

Adaptive Drift and Adaptive Optimization of Substrate Affinity

As a consequence of the improved active site dynamics in cold-active enzymes, substrates bind less firmly in the binding site (if no point mutations have occurred) giving rise to higher K_m values. An example is given in *Table 6.7.2* showing that the psychrophilic α-amylase is more active on its macromolecular substrates whereas the K_m values are up to 30-fold larger, i.e., the affinity for the substrates is up to 30-fold lower. Ideally, a functional adaptation to cold would mean optimizing both k_{cat} and K_m. However, a survey of the available data on psychrophilic enzymes (Xu et al. 2003) showed that optimization of the k_{cat}/K_m ratio is far from a general rule but on the contrary that the majority of cold-active enzymes improve the k_{cat} value at the expense of K_m, therefore leading to suboptimal values of the k_{cat}/K_m ratio, as also shown in *Table 6.7.2*. There is in fact an evolutionary pressure on K_m to increase in order to maximize the overall reaction rate. Such adaptive drift of K_m has been well illustrated by the lactate dehydrogenases from Antarctic fish (Fields and Somero 1998) and by the psychrophilic α-amylase (D'Amico et al. 2001) because both enzymes display rigorously identical substrate binding site and active site architecture when compared with their mesophilic homologues. In both cases, temperature-adaptive increases in k_{cat} occur concomitantly with increases in K_m in cold-active enzymes. As already mentioned, such identity of the sites also implies that adjustments of the kinetic parameters are obtained by structural changes occurring distantly from the

Table 6.7.2

Kinetic parameters for the hydrolysis of polysaccharides at 25°C by the psychrophilic (AHA) and the mesophilic (PPA) α-amylases. Adapted from D'Amico et al., 2006b

Substrate	AHA			PPA		
	k_{cat} s^{-1}	K_m mg l^{-1}	k_{cat}/K_m $s^{-1}mg^{-1}$ l	k_{cat} s^{-1}	K_m mg l^{-1}	k_{cat}/K_m $s^{-1}mg^{-1}$ l
Starch	663	155	4.3	327	41	8.0
Amylopectin	636	258	2.5	222	53	4.2
Amylose	2,148	178	12.1	700	36	19.4
Dextrin	716	586	1.2	311	61	5.1
Glycogen	491	1,344	0.3	193	46	4.2

reaction center. This aspect has received strong experimental support (D'Amico et al. 2003a) as discussed latter in this chapter.

Several enzymes, especially in some cold-adapted fish, counteract this adaptive drift of K_m in order to maintain or to improve the substrate binding affinity by amino acid substitutions within the active site (Smalas et al. 2000). The first reason for these enzymes to react against the drift is obvious when considering the regulatory function associated with K_m, especially for intracellular enzymes. The second reason is related to the temperature dependence of weak interactions. Substrate binding is an especially temperature-sensitive step because both the binding geometry and interactions between binding site and ligand are governed by weak interactions having sometimes opposite temperature dependencies. Hydrophobic interactions form endothermically and are weakened by a decrease in temperature. By contrast, interactions of electrostatic nature (ion pairs, hydrogen bounds, Van der Waals interactions) form exothermically and are stabilized at low temperatures. Therefore low temperatures do not only reduce the enzyme activity (k_{cat}), but can also severely alter the substrate binding mode according to the type of interaction involved.

The chitobiase from an Antarctic bacteria nicely illustrates both aspects, as well as the extent of the kinetic optimization that can be reached during cold adaptation of enzymes (Lonhienne et al. 2001). *Figure 6.7.7* shows that the k_{cat} of the cold-active chitobiase is eight times higher than that of a mesophilic chitobiase at 5°C. However, the K_m for the substrate is 25 times lower at this temperature, and as a result, the k_{cat}/K_m for the cold-active enzyme is nearly 200 times greater at low temperature. Because the cell-bound bacterial chitobiase has to access its substrate in the extracellular medium, the physiological advantage of a high affinity for the substrate is clear. In addition, the cross-shaped plot of K_m shows that the K_m of each enzyme tends to minimal and optimal values in the range of the corresponding environmental temperatures, reflecting the fine tuning of this parameter reached in the course of thermal adaptation. In the case of the mesophilic chitobiase, the 3D-structure indicates that two tryptophan residues are the main substrate binding ligands and perform hydrophobic interactions with the substrate. This can be related to the decrease of K_m with temperature, according to the above-mentioned thermal dependence of hydrophobic interactions. Interestingly, the two tryptophan residues are not found in the cold-active chitobiase but are replaced by polar residues that are able to perform stronger interactions as the temperature is decreased.

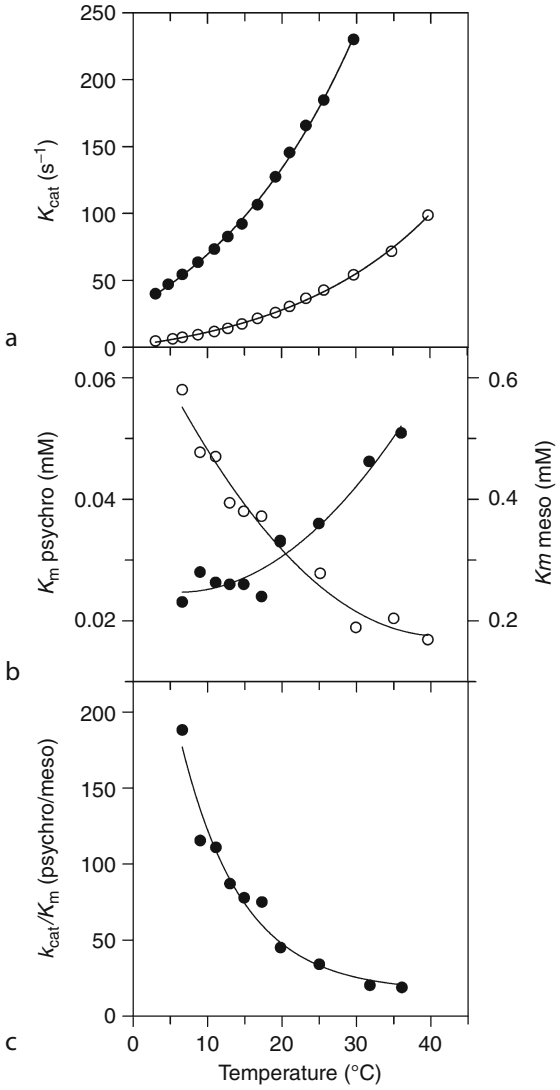

Fig. 6.7.7

Kinetic optimization in a cold-active chitobiase. Temperature dependence of the kinetic parameters for psychrophilic (*closed symbols*) and mesophilic (*open symbols*) chitobiases. Data for (a) the catalytic rate constant k_{cat}; (b) the Michaelis parameter K_m, note the different scales used; and (c) the relative catalytic efficiency k_{cat}/K_m (psychrophile/mesophile). The cold-adapted chitobiase is characterized by a higher activity, an optimal K_m value at low temperatures, and a 200 times higher catalytic efficiency at 7°C. Adapted from (Lonhienne et al. 2001)

Energetics of Activity at Low Temperatures

Referring to Eq. 6.7.1, the high activity of cold-adapted enzymes corresponds to a decrease of the free energy of activation $\Delta G^{\#}$. Two strategies have been highlighted to reduce the height of this energy barrier. *Figure 6.7.8* illustrates the first strategy where an evolutionary pressure increases K_m in order to maximize the reaction rate. According to the transition state theory, when the enzyme encounters its substrate, the enzyme-substrate complex ES falls into an energy pit. For the reaction to proceed, an activated state $ES^{\#}$ has to be reached, which eventually breaks down into the enzyme and the product. The height of the energy barrier between the ground state ES and the transition state $ES^{\#}$ is defined as the free energy of activation $\Delta G^{\#}$: the lower this barrier, the higher the activity as reflected in Eq. 6.7.1. In the case of cold-active enzymes displaying a weak affinity for the substrate, the energy pit for the ES complex is less deep (dashed in *Fig. 6.7.8*). It follows that the magnitude of the energy barrier is reduced and therefore the activity is increased. This thermodynamic link between affinity and activity is valid for most enzymes (extremophilic or not) under saturating substrate concentrations and this link appears to be involved in the improvement of activity at low temperatures in numerous cold-active enzymes (Fields and Somero 1998; Xu et al. 2003).

The second and more general strategy involves the temperature dependence of the reaction catalyzed by cold-active enzymes. *Table 6.7.3* reports the enthalpic and entropic contributions to the free energy of activation in extremophilic α-amylases. The free energy of activation $\Delta G^{\#}$ is calculated from Eq. 6.7.1 using the k_{cat} value at a given temperature and the enthalpy of activation $\Delta H^{\#}$ is obtained by recording the temperature dependence of the activity (Lonhienne et al. 2000). Finally, the entropic contribution $T\Delta S^{\#}$ is deduced from the Gibbs–Helmholtz equation:

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \tag{6.7.2}$$

The enthalpy of activation $\Delta H^{\#}$ depicts the temperature dependence of the activity: the lower this value, the lower the variation of activity with temperature. The low value found for almost all psychrophilic enzymes demonstrates that their reaction rate is less reduced than for

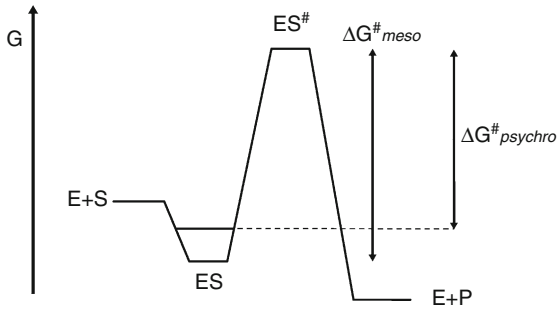

Fig. 6.7.8

Optimization of activity by decreasing substrate affinity in psychrophilic enzymes. Reaction profile for an enzyme-catalyzed reaction with Gibbs energy changes under saturating substrate concentration. Weak substrate binding (*dashed line*) decreases the activation energy ($\Delta G^{\#}$*psychro*) and thereby increases the reaction rate (see text for details)

Table 6.7.3

Activation parameters of the hydrolytic reaction of α-amylases at 10°C. Adapted from (D'Amico et al. 2003b)

	k_{cat} s^{-1}	$\Delta G^{\#}$ kcal mol^{-1}	$\Delta H^{\#}$ kcal mol^{-1}	$T\Delta S^{\#}$ kcal mol^{-1}
Psychrophile	294	13.3	8.3	−5.5
Mesophile	97	14.0	11.1	−2.9
Thermophile	14	15.0	16.8	1.8

other enzymes when the temperature is lowered. Accordingly, the decrease of the activation enthalpy in the enzymatic reaction of psychrophilic enzymes can be considered as the main adaptive character to low temperatures. This decrease is structurally achieved by a decrease in the number of enthalpy-driven interactions that have to be broken during the activation steps. These interactions also contribute to the stability of the protein folded conformation, and, as a corollary, the structural domain of the enzyme bearing the active site should be more flexible. It is interesting to note that such a macroscopic interpretation of the low activation enthalpy in cold-active enzymes fits with the experimental observation of a markedly heat-labile activity illustrated in *Fig. 6.7.4*. *Table 6.7.3* shows that the entropic contribution $T\Delta S^{\#}$ for the cold-active enzyme is larger and negative. This has been interpreted as a large reduction of the apparent disorder between the ground state with its relatively loose conformation and the well organized and compact transition state (Lonhienne et al. 2000). The heat-labile activity of cold-active enzymes suggests a macroscopic interpretation for this thermodynamic parameter. As a consequence of active site flexibility, the enzyme-substrate complex ES occupies a broader distribution of conformational states translated into increased entropy of this state, compared to that of the mesophilic or thermophilic homologues. This assumption has received strong experimental support by using microcalorimetry to compare the stabilities of free extremophilic enzymes with the same enzymes trapped in the transition state conformation by a non-hydrolysable substrate analog (D'Amico et al. 2003b). The larger increase in stability for the psychrophilic enzyme in the transition state conformation demonstrated larger conformational changes between the free and bound states when compared to mesophilic and thermophilic homologues. Furthermore, a broader distribution of the ground state ES should be accompanied by a weaker substrate binding strength, as indeed observed for numerous psychrophilic enzymes.

Conformational Stability of Extremophilic Proteins

Considering the numerous insights for strong relationships between activity and stability in psychrophilic enzymes, the conformational stability of these proteins has been intensively investigated in comparison with mesophilic and thermophilic counterparts. *Figure 6.7.9* displays the calorimetric records of heat-induced unfolding for psychrophilic, mesophilic, and thermophilic proteins. These enzymes clearly show distinct stability patterns that evolve from a simple profile in the unstable psychrophilic proteins to a more complex profile in very stable thermophilic counterparts. The unfolding of the cold-adapted enzymes occurs at lower

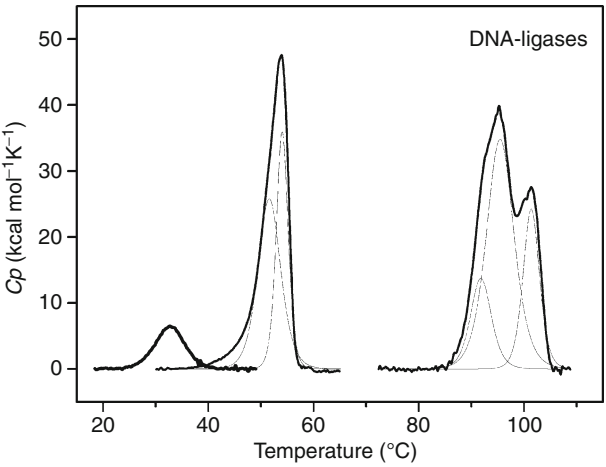

Fig. 6.7.9

Thermal unfolding of extremophilic enzymes. Thermograms of DNA-ligases recorded by differential scanning microcalorimetry showing, from left to right, psychrophilic (*heavy line*), mesophilic, and thermophilic proteins. The cold-active enzyme is characterized by a lower T_m (top of the transition) and ΔH_{cal} (area under the transition), by a sharp and cooperative transition, and by the lack of stability domains (indicated by *thin lines* in stable proteins). Adapted from (Georlette et al. 2003)

temperatures as indicated by the temperature of half-denaturation T_m, given by the top of the transition. This property, known for decades, has been highlighted by various techniques. By contrast, the energetics of structure stability was essentially revealed by microcalorimetry (D'Amico et al. 2001; Collins et al. 2003; Georlette et al. 2003). The calorimetric enthalpy ΔH_{cal} (area under the curves in *Fig. 6.7.9*), corresponding to the total amount of heat absorbed during unfolding, reflects the enthalpy of disruption of bonds involved in maintaining the compact structure and is markedly lower for the psychrophilic enzymes. In addition, there is a clear trend for increasing ΔH_{cal} values in the order psychrophile $<$ mesophile $<$ thermophile. The transition for the psychrophilic enzymes is sharp and symmetric whereas other enzymes are characterized by a flattening of the thermograms. This indicates a pronounced cooperativity during unfolding of the psychrophilic enzymes: the structure is stabilized by fewer weak interactions and disruption of some of these interactions strongly influences the whole molecular edifice and promotes its unfolding. The psychrophilic enzymes unfold according to an all-or-none process, revealing a uniformly low stability of the architecture. By contrast, all other homologous enzymes display two to three transitions (indicated by deconvolution of the heat capacity function in *Fig. 6.7.9*). Therefore, the conformation of these mesophilic and thermophilic enzymes contains structural blocks or units of distinct stability that unfold independently. Finally, the unfolding of the psychrophilic proteins is frequently more reversible than that of other homologous enzymes that are irreversibly unfolded after heating. The weak hydrophobicity of the core clusters in cold-adapted enzymes and the low T_m at which hydrophobic interactions are restrained certainly account for this reversible character because, unlike mesophilic proteins, aggregation does not occur or occurs to a lower extent.

As a practical and useful consequence of the unfolding reversibility, it has been possible to calculate accurately the conformational stability of a psychrophilic α-amylase over a broad range of temperatures (Feller et al. 1999). The comparison of these data with those of other proteins reveals some unsuspected properties of cold-adapted proteins. The thermodynamic stability of a protein that unfolds reversibly according to a two-state mechanism

$$N \rightleftharpoons U \tag{6.7.3}$$

is described by its stability curve, i.e., the free energy of unfolding as a function of temperature (*Fig. 6.7.10*). By definition, this stability is nil at T_m (equilibrium constant $K = [U]/[N] = 1$ and $\Delta G = -RT\ln K$). At temperatures below T_m, the stability increases, as expected, but perhaps surprisingly for the non-specialist, the stability reaches a maximum close to room temperature then it decreases at lower temperatures (*Fig. 6.7.10*). In fact, this function predicts a temperature of cold unfolding, which is generally not observed because it occurs below 0°C. Nevertheless, cold unfolding has been well demonstrated under specific conditions (Privalov 1990). Increasing the stability of a protein is essentially obtained by lifting the curve toward higher free energy values (Kumar and Nussinov 2004). As far as extremophiles are concerned, one of the most puzzling observations of the last decade is that most proteins obey this pattern, i.e., whatever the microbial source, the maximal stability of their proteins is clustered around room temperature (for more details see Kumar and Nussinov 2004). Accordingly, the environmental temperatures for mesophiles and (hyper)thermophiles lie on the right limb of the bell-shaped stability curve and, obviously, the thermal dissipative force is used to promote molecular

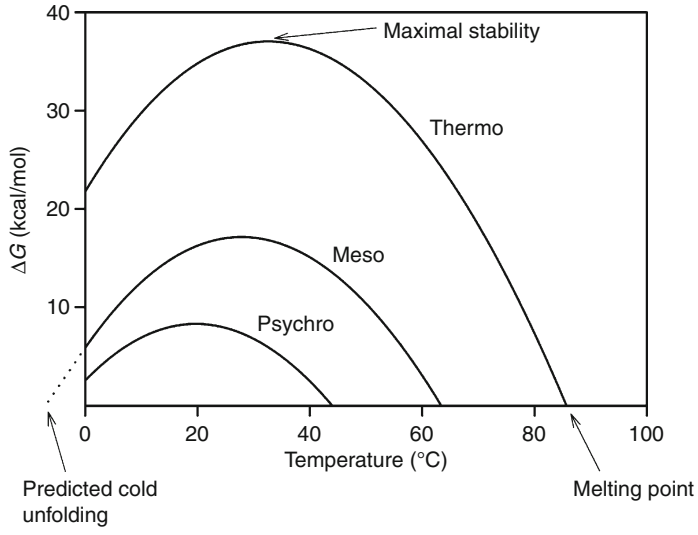

Fig. 6.7.10

Representative stability curves of homologous extremophilic proteins. The energy required to disrupt the native state (i.e., the conformational stability) is plotted as a function of temperature. At the melting point, this energy = 0 and in addition, the curves also predict cold unfolding and a maximal stability close to room temperature. A high stability in thermophiles is reached by lifting the curve toward higher free energy values, whereas the low stability in psychrophiles corresponds to a collapse of the bell-shaped stability curve. Adapted from (D'Amico et al. 2003b)

motions in these molecules. By contrast, the environmental temperatures for psychrophiles lie on the left limb of the stability curve. It follows that molecular motions in proteins at low temperatures are gained from the factors ultimately leading to cold unfolding (Feller et al. 1999), i.e., the hydration of polar and nonpolar groups (Makhatadze and Privalov 1995). The origin of flexibility in psychrophilic enzymes at low temperatures is therefore drastically different from mesophilic and thermophilic proteins, the latter taking advantage of the conformational entropy rise with temperature to gain in mobility.

A surprising consequence of the free energy function for the psychrophilic protein shown in *Fig. 6.7.10* is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas it was intuitively expected that cold-active proteins should also be cold stable. This protein is in fact both heat and cold labile. Assuming constant properties of the solvent below 0°C (i.e., no freezing) and the absence of protective effects from cellular components, this α-amylase should unfold at −10°C. Therefore, cold denaturation of some key enzymes in psychrophiles can be an additional, though unsuspected factor fixing the lower limit of life at low temperatures. It has also been shown that the psychrophilic α-amylase has reached a state close to the lowest possible stability of the native state (D'Amico et al. 2001). If psychrophilic enzymes have indeed gained in flexibility at the expense of stability in the course of evolution, this implies that the actual native state precludes further adaptation toward a more mobile structure. This aspect can account for the imperfect adaptation of the catalytic function in some psychrophilic enzymes, mentioned at the beginning of this chapter and illustrated in *Fig. 6.7.1*.

Structural Basis of Low Stability

The number of X-ray crystal structures from psychrophilic enzymes has increased dramatically, demonstrating the growing interest for these peculiar proteins. However, the interpretation of these structural data is frequently difficult for two main reasons. First, the structural adaptations are extremely discrete and can easily escape the analysis. Second, these structural adaptations are very diverse, reflecting the complexity of factors involved in the stability of a macromolecule at the atomic level. For instance, it was found that all structural factors currently known to stabilize the protein molecule could be attenuated in strength and number in the structure of cold-active enzymes (Smalas et al. 2000; Russell 2000; Gianese et al. 2002). An exhaustive description of all these factors is beyond the scope of this chapter and only the essential features are summarized below. Two review articles can be consulted for a comprehensive discussion of this topic (Smalas et al. 2000; Siddiqui and Cavicchioli 2006).

The observable parameters related to protein stability include structural factors and mainly weak interactions between atoms of the protein structure. In psychrophilic proteins, this involves the clustering of glycine residues (providing local mobility), the disappearance of proline residues in loops (enhancing chain flexibility between secondary structures), a reduction in arginine residues which are capable of forming multiple salt bridges and H-bonds, as well as a lower number of ion pairs, aromatic interactions or H-bonds, compared to mesophilic enzymes. The size and relative hydrophobicity of nonpolar residue clusters forming the protein core are frequently smaller, lowering the compactness of the protein interior by weakening the hydrophobic effect on folding. The N and C-caps of α-helices are also altered (weakening the charge-dipole interaction) and loose or relaxed protein extremities appear to be preferential sites for unzipping. The binding of stabilizing ions, such as calcium, can be extremely weak, with binding constants

differing from mesophiles by several orders of magnitude. Insertions and deletions are sometimes responsible for specific properties such as the acquisition of extra-surface charges (insertion) or the weakening of subunit interactions (deletion).

Calculation of the solvent accessible area showed that some psychrophilic enzymes expose a higher proportion of nonpolar residues to the surrounding medium (Aghajari et al. 1998b; Russell et al. 1998). This is an entropy-driven destabilizing factor caused by the reorganization of water molecules around exposed hydrophobic side chains. Calculations of the electrostatic potential revealed in some instances an excess of negative charges at the surface of the protein and, indeed, the pI of cold-active enzymes is frequently more acidic than that of their mesophilic or thermophilic homologues. This has been related to improved interactions with the solvent, which could be of prime importance in the acquisition of flexibility near 0° (Feller et al. 1999). Besides the balance of charges, the number of salt bridges covering the protein surface is also reduced. There is a clear correlation between surface ion pairs and temperature adaptation, since these weak interactions significantly increase in number from psychrophiles to mesophiles, to thermophiles and hyperthermophiles, the latter showing arginine-mediated multiple ion pairs and interconnected salt bridge networks (Yip et al. 1995; Vetriani et al. 1998). Such an altered pattern of electrostatic interactions is thought to improve the dynamics or the "breathing" of the external shell of cold-active enzymes.

However, each enzyme adopts its own strategy by using one or a combination of these altered structural factors in order to improve the local or global mobility of the protein edifice. Comparative structural analyses of psychrophilic, mesophilic, and thermophilic enzymes indicate that each protein family displays different structural strategy to adapt to temperature. However, some common trends are observed: the number of ion pairs, the side-chain contribution to the exposed surface, and the apolar fraction of the buried surface show a consistent decrease with decreasing optimal temperatures (Gianese et al. 2002; Bell et al. 2002; Bae and Phillips 2004; Mandrich et al. 2004). As a result of the great diversity of factors involved in protein stability, the bias in the amino acid composition observed in individual psychrophilic protein (low proline or arginine content, etc.) is not found when analyzing the mean amino acid composition of the whole genome. On the contrary, the available genomic data have produced ambiguous results (Gerday and Glansdorff 2007; Margesin et al. 2008) and it is currently difficult to correlate the reported trends in genomic amino acid composition with adaptations to low temperatures or with species-specific differences.

Activity–Stability Relationships: Experimental Insights

In order to check the validity of the proposed relationships between the activity and the stability in cold-active enzymes, a psychrophilic α-amylase has been used as a model because the identical architecture of its active site, when compared with a close mesophilic homologue, indicates that structural adaptations affecting the active site properties occur outside from the catalytic cavity. Accordingly, the crystal structure (Aghajari et al. 1998a, b) has been closely inspected to identify structural factors involved in its weak stability, such as those described in the previous section. On this basis, 17 mutants of this enzyme were constructed, each of them bearing an engineered residue forming a weak interaction found in mesophilic α-amylases but absent in the cold-active α-amylase, or a combination of up to six stabilizing structural factors (D'Amico et al. 2001, 2002b, 2003a). As illustrated in *Fig. 6.7.11*, it was found that single amino acid side-chain substitutions can significantly modify the melting point T_m and the

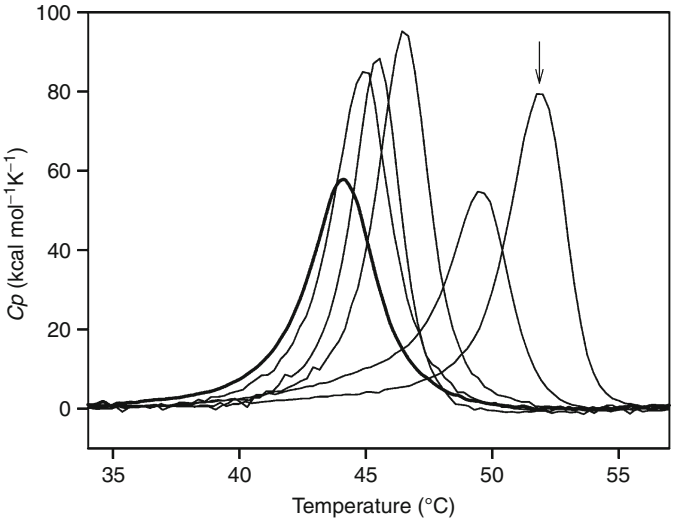

Fig. 6.7.11

Engineering mesophilic-like stability in mutants of the psychrophilic α-amylase. Structure-stabilizing interactions have been introduced in the heat-labile enzyme (*heavy line*). As shown by the microcalorimetric thermograms, the resulting mutants (*thin lines*) display increased melting points (top of the transitions) and calorimetric enthalpies (area below the curves). The most stable mutant (*arrow*) bears six additional interactions. Adapted from (D'Amico et al. 2001, 2003a)

calorimetric enthalpy ΔH_{cal} but also the cooperativity and reversibility of unfolding as well as the thermal inactivation rate constant. Therefore, these mutants of the psychrophilic α-amylase consistently approximate and reproduce the unfolding patterns of the heat-stable enzymes depicted in *Fig. 6.7.9*.

However, in the context of catalysis at low temperatures, the most significant observation was that these mutations tend to decrease both k_{cat} and K_m. As shown in *Fig. 6.7.12*, stabilizing the cold-active α-amylase tends to decrease the k_{cat} values and concomitantly the K_m values of the mutant enzymes, revealing the high correlation between both kinetic parameters (illustrated in *Fig. 6.7.8*). In fact, in addition to an engineered mesophilic-like stability, the multiple-mutant bearing six stabilizing structural factors also displays an engineered mesophilic-like activity in terms of alterations in k_{cat} and K_m values and even in thermodynamic parameters of activation (D'Amico et al. 2003a). Considering the various available data on the psychrophilic α-amylase, it can be concluded that the improved molecular motions of the side chains forming the active site (motions responsible for the high activity, the low affinity and heat-lability) originate from the lack of structure-stabilizing interactions in the vicinity or even far from the active site. This is another strong indication that structural flexibility is an essential feature related to catalysis at low temperatures in psychrophilic enzymes.

Psychrophilic Enzymes in Folding Funnels

The various properties of psychrophilic enzymes that have been presented in this chapter can be integrated in a model based on folding funnels (Dinner et al. 2000; Schultz 2000) to describe

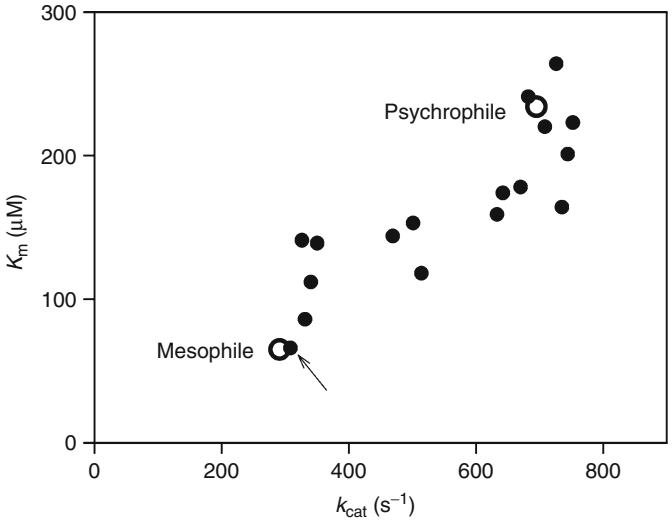

Fig. 6.7.12

Engineering mesophilic-like activity in mutants of the psychrophilic α-amylase. This plot of the kinetic parameters for the stabilized mutants (*filled symbols*) shows that the general trend is to decrease the activity and to increase the affinity for the substrate of the wild-type psychrophilic enzyme (*open symbol*). The most stable mutant bearing six additional interactions (*arrow*) displays kinetic parameters nearly identical to those of the mesophilic homologue (*open symbol*). Adapted from (D'Amico et al. 2001, 2003a)

the activity–stability relationships in extremophilic enzymes. *Figure 6.7.13* depicts the energy landscapes of psychrophilic and thermophilic enzymes. The top of the funnel is occupied by the unfolded state and having a high free energy (considering the spontaneous folding reaction), whereas the bottom of the funnel is occupied by the stable (low free energy) native state. The height of the funnel, i.e., the free energy of folding, also corresponding to the conformational stability, has been fixed here in a 1–5 ratio according to the stability curves shown in *Fig. 6.7.10*. The upper edge of the funnels is occupied by the unfolded state in random coil conformations but it should be noted that psychrophilic enzymes tend to have a lower proline content than mesophilic and thermophilic enzymes, a lower number of disulfide bonds and a higher occurrence of glycine clusters (Russell 2000; Smalas et al. 2000; Gianese et al. 2002; Siddiqui and Cavicchioli 2006). Accordingly, the edge of the funnel for the psychrophilic protein is slightly larger (broader distribution of the unfolded state) and is located at a higher energy level. When the polypeptide is allowed to fold, the free energy level decreases, as well as the conformational ensemble. However, thermophilic proteins pass through intermediate states corresponding to local minima of energy. These minima are responsible for the ruggedness of the funnel slopes and for the reduced cooperativity of the folding-unfolding reaction, as demonstrated by heat-induced unfolding (*Fig. 6.7.9*). By contrast, the structural elements of psychrophilic proteins generally unfold cooperatively without intermediates, as a result of fewer stabilizing interactions and stability domains (Feller et al. 1999; D'Amico et al. 2001; Georlette et al. 2003) and therefore the funnel slopes are steep and smooth. The bottom of the funnel depicts the stability of the native state ensemble.

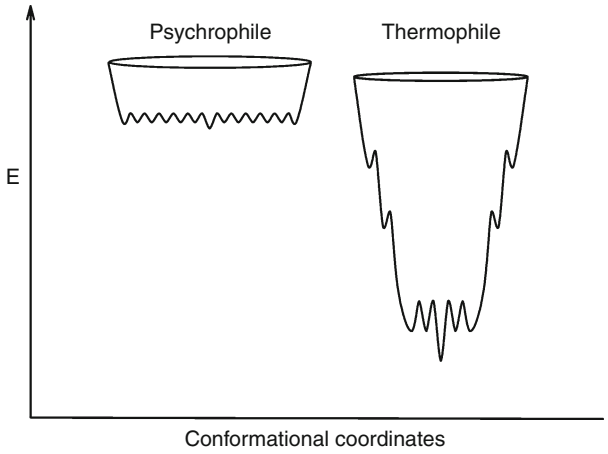

Fig. 6.7.13

Folding funnel model for extremophilic enzymes. In these schematic energy landscapes, the free energy of folding (E) is depicted as a function of the conformational diversity. The height of the funnels is deduced from the determination of the conformational stabilities. The top of the funnels is occupied by the unfolded states in the numerous random coil conformations, whereas the bottom of the funnels corresponds to native and catalytically active conformations. The ruggedness of the bottom depicts the energy barriers for interconversion, or structural fluctuations of the native state (D'Amico et al. 2003b)

The bottom for a very stable and rigid thermophilic protein can be depicted as a single global minimum or as having only a few minima with high energy barriers between them, whereas the bottom for an unstable and flexible psychrophilic protein is rugged and depicts a large population of conformers with low energy barriers to flip between them. Rigidity of the native state is therefore a direct function of the energy barrier height (Tsai et al. 1999; Kumar et al. 2000) and is drawn here according to the results of fluorescence quenching (*Fig. 6.7.2*) and neutron scattering experiments (Tehei et al. 2004). In this context, the activity–stability relationships in these extremophilic enzymes depend on the bottom properties. Indeed, it has been argued that upon substrate binding to the association-competent sub-population, the equilibrium between all conformers is shifted toward this sub-population, leading to the active conformational ensemble (Tsai et al. 1999; Kumar et al. 2000; Ma et al. 2000; Benkovic et al. 2008). In the case of the rugged bottom of psychrophilic enzymes, this equilibrium shift only requires a modest free energy change (low energy barriers), a low enthalpy change for interconversion of the conformations, but is accompanied by a large entropy change for fluctuations between the wide conformer ensemble. The converse picture holds for thermophilic enzymes, in agreement with the activation parameters shown in *Table 6.7.3* and with the proposed macroscopic interpretation. Such energy landscapes integrate nearly all biochemical and biophysical data currently available for extremophilic enzymes but they will certainly be refined by future investigations of other series of homologous proteins from psychrophiles, mesophiles and thermophiles. This model has nevertheless received support from independent studies (Bjelic et al. 2008; Xie et al. 2009).

Acknowledgments

Research at the author's laboratory was supported by the European Union, the Région wallonne (Belgium), the Fonds National de la Recherche Scientifique (Belgium), and the University of Liège. The facilities offered by the Institut Polaire Français are also acknowledged.

Cross-References

- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
- 6.2 Taxonomy of Psychrophiles
- 6.3 Diversity of Psychrophilic Bacteria from Sea Ice - and Glacial Ice Communities
- 6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens
- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats
- 6.6 Genetics, Genomics, Evolution

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Part 7

Extremophiles: Xerophiles

7.1 Microbiology of Volcanic Environments

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_7.1,

Introduction

Volcanism may be defined as "the manifestation at the surface of a planet or satellite of internal thermal processes through the emission at the surface of solid, liquid, or gaseous products" (Francis 1993). At any given time there are active volcanoes in some location on the Earth (Schminke 2004). Environments resulting from volcanic activity are diverse, from acidic hot springs to deep-ocean basaltic habitats and volcanic soils.

It is not possible to do justice to the microbiological investigation of all these environments in a single chapter. As thermophilic microorganisms are covered by separate chapters they will not be discussed here. In this chapter focus is given to rocky volcanic environments generated by eruptions and the soils they produce. In terms of land area, volcanic rocks and soil dominate the products of volcanism. For example in India alone, the volcanically formed Deccan Traps, an area of basalt, cover over one million square kilometers. Volcanic rocks are often xeric environments that can be considered extreme from the perspective of microbial growth and survival. In this chapter we will focus on work on the microbial diversity of basaltic glass and obsidian (silica-rich glass) in Iceland (*Figs. 7.1.1* and *7.1.2*). Although not xeric, we also briefly discuss the microbial diversity of oceanic volcanic habitats in order to make some comparisons with microorganisms in the xeric environments of terrestrial volcanic rocks.

As volcanic environments are widely distributed on Earth, it is of geomicrobiological importance to understand the diversity and characteristics of microbial life that they harbor. Volcanic environments might be used as model systems to understand more general patterns of the diversity and distribution of prokaryotes through time. Such investigations improve our understanding of how the geochemistry and microenvironmental conditions in different rocks can influence microbial diversity that persists on the surface and in the subsurface of the Earth.

More specifically, volcanic environments are important in a number of Earth system processes: (1) Rock weathering contributes significantly to the carbonate-silicate cycle (Dessert et al. 2001, 2003), in which carbon dioxide is consumed in reactions with rock silicates. An important task is to elucidate the potential role of organisms in contributing to rock weathering, for which a chemical role has been well-established (e.g., Gíslason and Eugster 1987; Oelkers and Gíslason 2001; Stefánsson and Gíslason 2001). (2) Volcanic rocks weather to release nutrients into the biosphere and they produce some of the most fertile soils in the world. Understanding the mechanisms by which nutrients are released from volcanic rocks into the biosphere and their accessibility to the biota requires that we understand which organisms inhabit volcanic rocks (Dahlgren et al. 1993). Other ancillary reasons include the use of glasses as nuclear storage materials (Petit 1992; Magonthier et al. 1992). Investigating the biotic alteration of natural glasses, such as volcanic glass, will allow for better assessments of the expected longevity of these storage materials.

Understanding the way in which life can take advantage of post-volcanic environments has relevance for assessing the possibility of life on other planets. Many of the perturbations that occur to ecosystems on the Earth, such as fire and storm damage, cannot convincingly be described as universal phenomena since they depend on planetary atmospheric conditions. Fire and storm damage, for instance, depend upon combustible matter and sufficient oxygen in the atmosphere to support fire in the former case (Agee 1993; del Pino et al. 2007) and atmospheric conditions suitable for sufficiently sized storms to threaten surface ecosystems in the latter (Tester et al. 2003). Similar arguments can be advanced for disease and ocean turnover, for instance. On any planet that has not completely cooled down, volcanism would be expected to occur. As plate tectonics, which is one mechanism for generating volcanic activity, might be

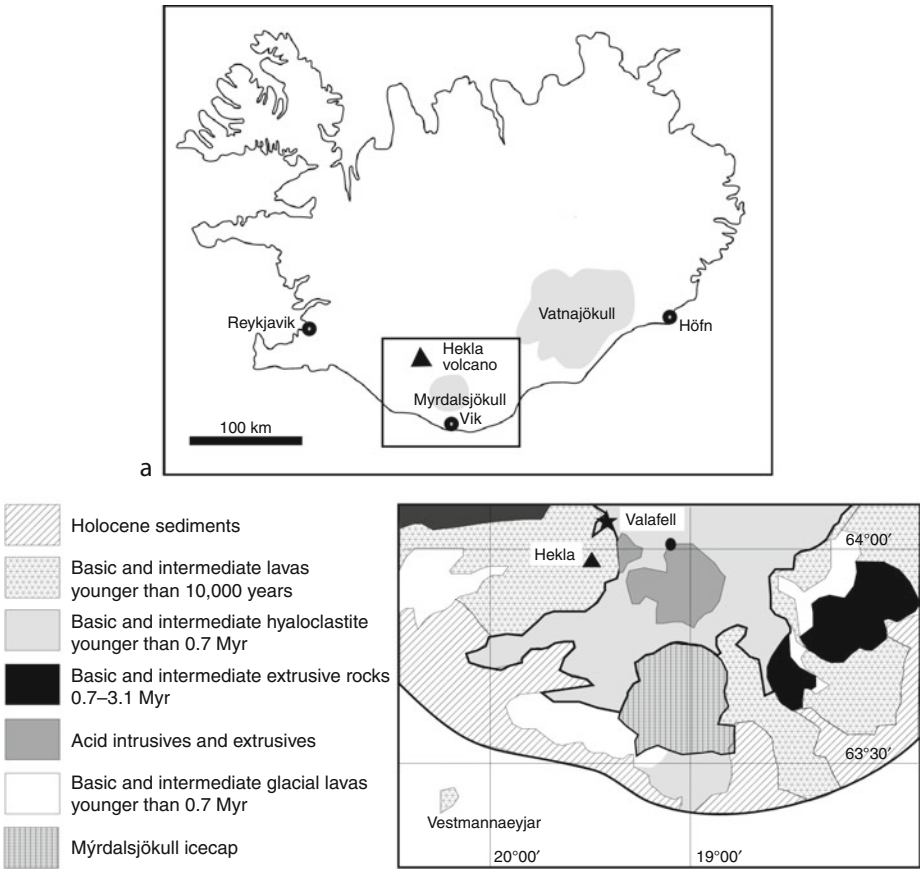

Fig. 7.1.1

Map showing location of sites where basaltic glass and obsidian were collected in Iceland (a) against major geological formations (b). The *star* is basaltic glass, the *circle* obsidian

required to create conditions suitable for life (van Thienen et al. 2007), then it may be the case that volcanism is inextricably linked to the phenomenon of life. Thus, understanding the geomicrobiology of volcanic environments has astrobiological significance in assessing the habitats available for life on volcanically active planets.

The Geological Context

Igneous petrology is a vast area of research, but from a geomicrobiological perspective, several points can be made about volcanism that are essential to the discussion in this chapter. Rocks are split into different groups depending upon their silica and alkali content (in this context alkali means the concentration of Na and K). This convention, established by the International Union of Geological Sciences (IUGS), is shown in *Fig. 7.1.3* (Le Bas et al. 1992). Igneous rocks are split into 15 fields. These categories are established, among other factors, by the melting temperature of the magma. Rocks of high silica content are formed at lower

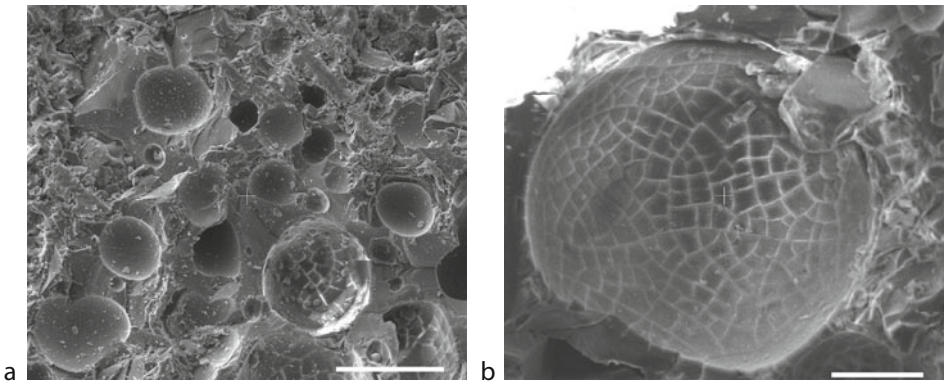

Fig. 7.1.2

Scanning Electron Microscopy images of interior of basaltic glass showing typical vesicular nature of surface volcanic rocks (a; scale bar 100 μm), and in the case of basaltic glass, formation of palagonite layer on surface of vesicles visible as a layer of cracked material (b; scale bar 50 μm)

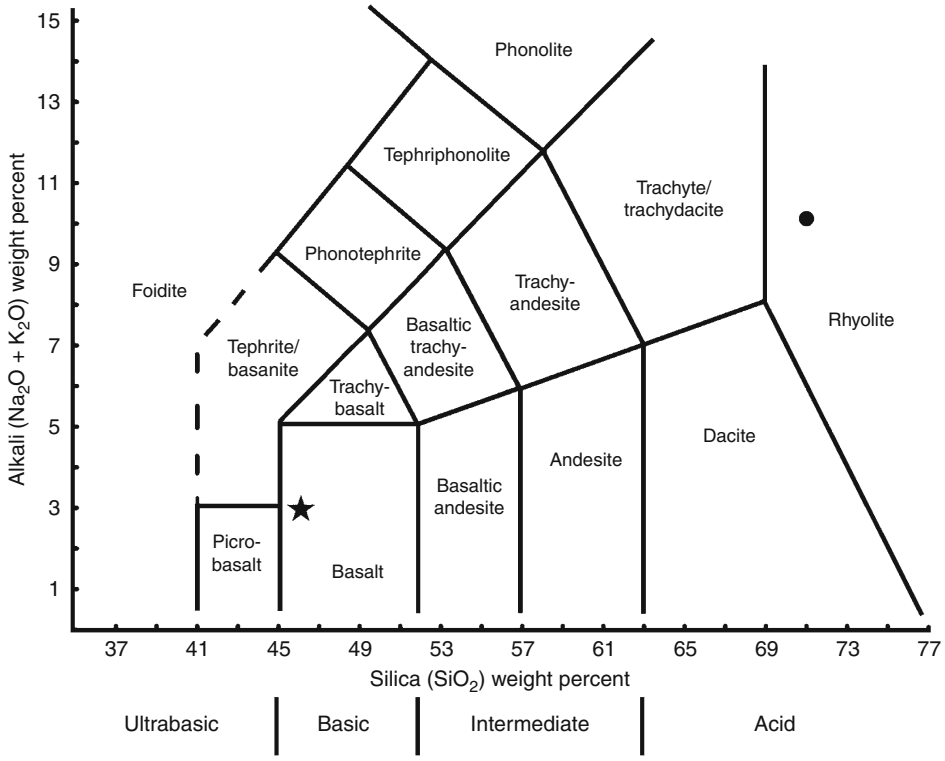

Fig. 7.1.3

Total Alkali-Silica (TAS) diagram of volcanic rocks. The composition of the basaltic glass described in this chapter is shown with a *star*, the composition of the obsidian with a *circle*

temperatures (typically about 700°C) than basaltic and ultramafic rocks, which are formed at higher melting temperatures (between about 1,000–1,500°C). High silica rocks (also referred to as "acidic" rocks) generally have a higher content of orthoclase (a K-containing mineral) and quartz and a lower content of pyroxenes, olivines, and plagioclase (predominantly Mg, Fe, and Ca containing minerals) compared to mafic and ultramafic rocks (referred to as "basic"). Not all igneous rocks can be classified on the TAS (Total Alkali-Silica) diagram and there are variants on this diagram, for example the QAPF (Quartz, Feldspar, Plagioclase, Feldspathoid) double triangle. However, the TAS diagram is useful to categorize most igneous rocks.

Another factor that is not shown on the TAS diagram is the glassiness of the material. Volcanic lava that comes into contact with ice or water is rapidly quenched before crystals can form, resulting in a homogeneous glass, in contrast to other rocks that are formed subaerially and cool slowly, forming crystalline materials. Biologically, this may be an important difference from two perspectives. Firstly, glasses will present to a biota a material with a more homogenous and mixed composition, whereas in crystalline materials bioessential elements will be localized to particular crystals, meaning that less of the total number of bioessential elements required can be found in one location at the microscale. Secondly, basaltic glass, in contrast to crystalline basalt, has the distinctive feature of weathering to palagonite, a clay-like substance (Thorseth et al. 1991; Stroncik and Schminke 2001) which tends to form within vesicles and fractures within the material and may have implications for the enclosed biota, as will be discussed later.

The Microbial Diversity of Volcanic Rocks

Volcanic rocks can harbor remarkable microbial diversity. The extent to which rocks can be colonized, particularly in their interior space, is dependent upon their porosity. One means by which porosity is generated is by outgassing when the rocks reach the surface of the Earth and cool. However, even non-porous rocks will be colonized on their surface.

A recent study of volcanic rocks in Iceland illustrates the microbial diversity that these rocks can harbor. Obsidian, which is a glassy silica-rich rock, is found in several locations in Iceland, including areas near Hekla volcano (*Fig. 7.1.1*). The microbial diversity of these rocks was investigated by culture-independent methods (Herrera et al. 2008, 2009). In the obsidian, forty-seven bacterial sequences were affiliated to six divisions. The most dominant division was the Actinobacteria (19%). The second most abundant, accounting for 14% of the total sequences, was the Acidobacteria. Ten percent of the total sequences were related to the Verrucomicrobia. The Proteobacteria sequences, representing 9% of the total sequences, fell into the α-subdivision. Many of these sequences were related to organisms or sequences previously obtained in rock environments. For example, two Actinobacteria showed closest similarity with an uncultured actinobacterium 16S rDNA clone isolated from an endolithic community in the Rocky Mountains (Walker and Pace 2007). Four other 16S rDNA sequences also showed closest similarity to an uncultured α-proteobacterium 16S rDNA clone isolated from cryptoendolithic communities present in the Dry Valleys of Antarctica (de la Torre et al. 2003), another α-proteobacterium sequence was found to be similar to an uncultured bacterium identified from a deglaciated soil (Nemergut et al. 2007). One sequence was most similar to *Geodermatophilus* sp. isolated from rocks, monument surfaces, and dried soils (Eppard et al. 1996). The remaining 33 sequences were identified as "unclassified" by RDP II database analysis. These sequences showed similarity with only a few sequences from unidentified bacterial clones or *Chloroflexi* related clones and they showed less than 85% similarity with known 16S rDNA sequences from isolated bacteria in GenBank. These data show that volcanic

rocks harbor previously uncharacterised phylotypes. This is consistent with the presence of unclassified phylotypes in organic-poor volcanic rocks in Hawaii (Gomez-Alvarez et al. 2007).

Investigations were also carried out with basaltic glass, which is the less silica-rich counterpart to obsidian. The community within the material was dominated by phylotypes belonging to Actinobacteria (30%); Proteobacteria (26%), which fell into the α, β, and γ subdivisions; and Bacteroidetes (11%). Seven of the sequences (6%) were related to phototrophs. Thirty-one of the clones had a closest sequence match to organisms previously described in cold soil and/or endolithic environments (12 from Antarctic soils represented by three Bacteroidetes, one Acidobacteria, five Actinobacteria, one α- and one β-proteobacteria, and one unclassified), 7 from Antarctic endoliths [all α-proteobacteria], 5 from glaciers and deglaciated soils [three Bacteroidetes, one α- and one β-proteobacteria], 3 from the Atacama desert [all Actinobacteria], and four from other endolithic environments [two Actinobacteria and one α- and one β-proteobacteria] (Cockell et al. 2009a).

Among the clones, Actinobacteria and Proteobacteria were found in both obsidian and basaltic glass and have previously been shown to be abundant in endolithic habitats (de la Torre et al. 2003; Walker and Pace 2007). They are important groups in soils. Bacteroidetes (formally Cytophaga-Flexibacter-Bacteroidetes) were present and have previously been associated with soil crusts and soils (Shivaji et al. 2004; Nagy et al. 2005; Gundlapally and Garcia-Pichel 2006), although they are also found in freshwater (Wu et al. 2007), marine (O'Sullivan et al. 2004; Murray and Grzymski 2007), and microbial mat (Abed et al. 2007) environments, and have been associated with the degradation of a wide range of organic compounds.

In Hawaiian volcanic materials, Acidobacteria, Actinobacteria, and α-Proteobacteria were found (Gomez-Alvarez et al. 2007). We also observed Firmicutes, Verrucomicrobia, and Bacteroidetes as prominent taxa. All of these phyla were observed in a variety of bare and vegetated volcanic soils associated with the Mount St Helen's eruption in 1980 (Ibekwe et al. 2007).

Which Organisms are Active?

Some of the DNA sequences observed in volcanic rocks probably belong to inactive organisms that have become incorporated in the vesicular rocky material and become entrained by wind, water, and brought in on the surface of mineral grains. For example, we have cultured hyperthermophiles from within the volcanic rocks (unpublished data). The provenance of the organisms is likely to be hot springs in Iceland. However, both Bacteroidetes and Actinobacteria isolates can be cultured from basaltic glass using crushed basalt glass (and its indigenous carbon sources) as the growth substrate (Cockell et al. 2009b), showing that there exists a core population of these organisms within the rocks that represent the metabolically active components. These organisms exhibit resistance to desiccation and temperature fluctuations, and some have resistance to heavy metals, attributes that would be expected from volcanic rock-dwelling organisms. The addition of a small amount of carbon in the form of yeast extract yielded many other Actinobacteria and *Bacillus* spp. (Cockell et al. 2009b). The ease with which Actinobacteria can be cultured from the rocks is consistent with their ubiquity in clone libraries from a number of volcanic rock environments. In addition to having known desiccation resistance, many of them form spores, improving their likelihood of dispersal from one rock to another. Yet another characteritic of many of the Actinobacteria is their filamentous growth habit which is likely to improve their ability to invade and move throughout the rock interstices, particularly between connected vesicles within volcanic rocks (Cockell et al. 2009b).

All of the 16S rRNA sequences obtained in Iceland and most reported from other volcanic rocks are associated with heterotrophic taxa (Herrera et al. 2008; Cockell et al. 2009a; Kelly et al. 2010). None clearly matched previously recognized chemolithotrophic taxa. These results suggest that the majority of the microbial population that is active within volcanic rocks must depend on carbon input, whether in precipitation, in the form of other dead and decaying organisms, or carbon produced by phototrophs and other autotrophs.

Effects of Geology on the Microbial Diversity

These observations can be better understood when the chemical weathering characteristics of these rocks are considered. Although weathering of the obsidian occurs, it is much slower than the weathering of basaltic glass (Wolff-Boenisch et al. 2004, 2006). The higher silica concentration impedes the degradation of the material since Si–O bonds are much more difficult to break than other bonds, and the silica will tend to retard leaching of cations from the rock. Thus, the habitat is more long-lived. In contrast, the basaltic glass is comprised of vesiculated glass which weathers to the clay-like material, palagonite, which forms rinds on the glass (Cockell et al. 2009a) and may provide a more readily accessible source of nutrients. This would be consistent with observations of microbial abundance. Cockell et al. (2009a) observed high cell numbers in basalt glass samples with abundances of $\sim 10^7$ cells/gram. By contrast, Herrera et al. (2008), using FISH (Fluorescent In Situ Hybridisation), found that cells were highly localized to altered regions in the obsidian glass and subsequent attempts to gather cell number data (unpublished) find total cell numbers less than 10^4 cells/gram. Because the cells are so localized within the obsidian, whole rock cell numbers were unreliable, but nevertheless the results show that cell numbers are generally less in the obsidian than the basaltic glass samples. This would be consistent with a more oligotrophic environment within the obsidian.

Pioneers in the Post-Volcanic Environment

Phototrophs are already known to be some of the first colonists of post-volcanic environments, colonizing the surface of new volcanic substrates and soils (Carson and Brown 1978; Adamo and Violante 1991; Adamo et al. 1993; Fermani et al. 2007). However, volcanic rocks are generally opaque and considered poor substrates for the establishment of phototroph populations within the substrate. Phototrophic colonization of hydrothermal deposits in volcanic geothermal regions (Gross et al. 1998; Gaylarde et al. 2006; Walker and Pace 2007; Walker et al. 2005) has previously been reported and in these cases the geothermal substrates formed around hot springs, which are often iron poor and silica rich, probably provide favorable light penetration for phototrophs.

Phototroph sequences can be found in all Icelandic volcanic rocks (*Fig. 7.1.4*). For example, in the obsidian, three sequences (Genbank accession numbers AM773380, AM773352, AM773416) showed a closest similarity (99%) with the cyanobacterium *Chamaesiphon* sp. 16S rDNA (AY170472) (Turner 1997) and 98% similarity with an uncultured cyanobacterium 16S rDNA clone (DQ514063) isolated from a deglaciated soil (Nemergut et al. 2007). Three other sequences (AM773393, AM773369 and AM773349) showed closest similarity (99%) with an uncultured chlorophyte 16S rDNA clone (EF522360) isolated from an endolithic community inhabiting the Rocky Mountains (Walker and Pace 2007). Their closest similarity (90%) to a cultured organism was *Koliella* sp. (AF278747) (Katana et al. 2001).

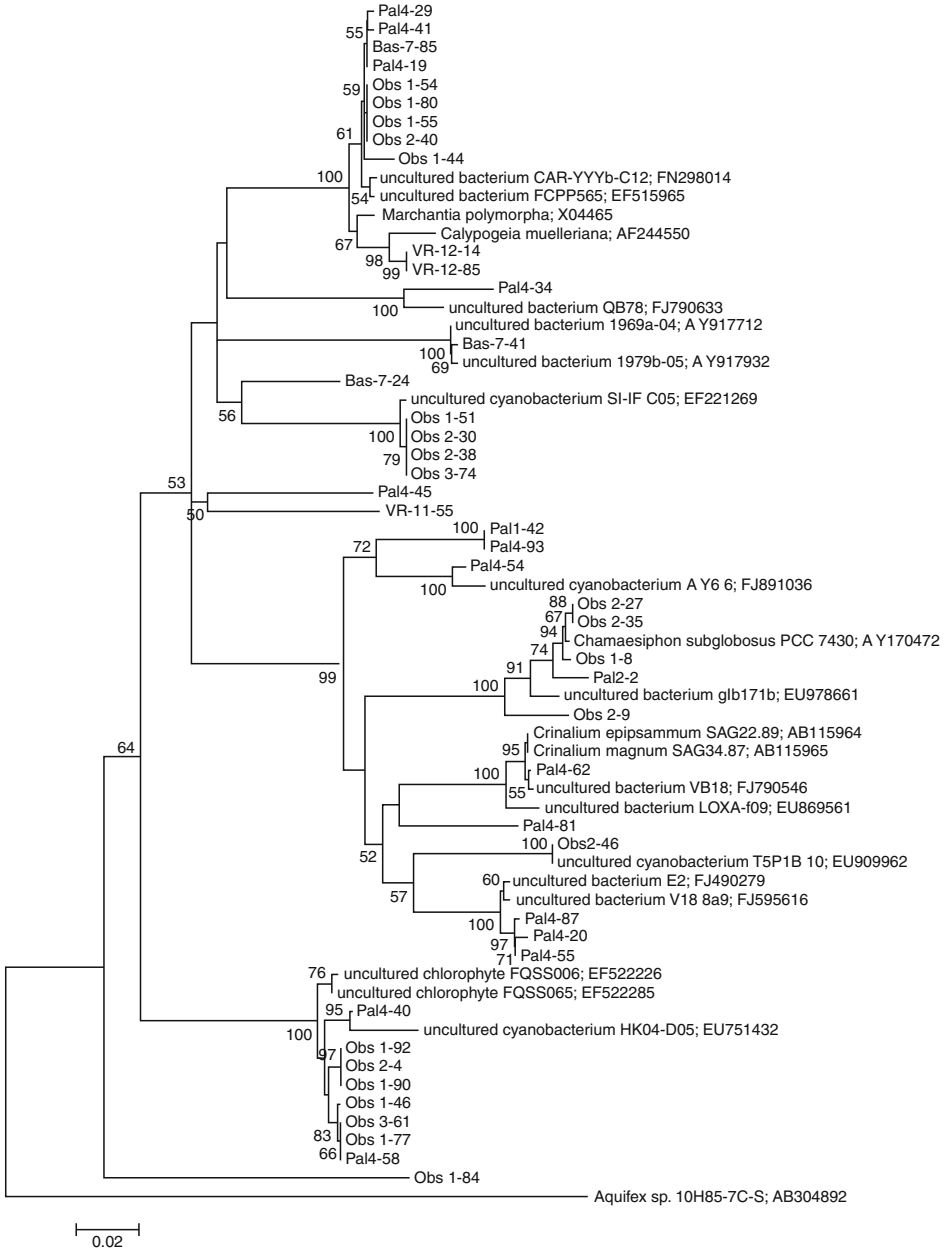

Fig. 7.1.4

Neighbor-Joining phylogenetic tree of *Cyanobacteria*. Clones from hyaloclastite (basaltic glass; Pal) and obsidian (Obs) are shown. Also included are phototrophs from crystalline volcanic rocks discussed by Kelly et al. (2010): crystalline basalt (Bas) and crystalline rhyolite (Rhy)

Molecular analyses of the basaltic glass revealed the presence of phototrophs. 16S rDNA cyanobacteria-related and plastid sequences were identified. Two sequences (EU621975, FJ360644) showed a closest similarity (99%) to *Klebsormidium* sp. (AF393600) (Turmel et al. 2002). One sequence (EU621980) had a closest similarity (97%) to *Stichococcus* sp. (AF278751) (Katana et al. 2001). One sequence (EU621967) had a closest similarity (96%) to an uncultured eubacterium (AJ292689) found in soil (Nogales et al. 2001). Its closest similarity (86%) to a cultured organism was *Closteriopsis* sp. (Y17632) (Ustinova et al. 2001). Three sequences (FJ360671, FJ360653, FJ360664) showed a closest similarity (96–97%) to an uncultured cyanobacterium found under quartz substrates in the Atacama Desert, Chile (FJ890990). Their closest similarity (95–96%) to a cultured organism was *Acaryochloris* sp. (AM710387) (Jezberova 2006).

In the obsidian, the phototrophs, which include algal and cyanobacterial components, can be observed to form layers of growth within the glass, which can be seen by SEM (*Fig. 7.1.5*). They can also be observed using FISH in association with presumptive heterotrophs (Herrera et al. 2008). The endolithic phototrophs are found under glassy layers on the obsidian.

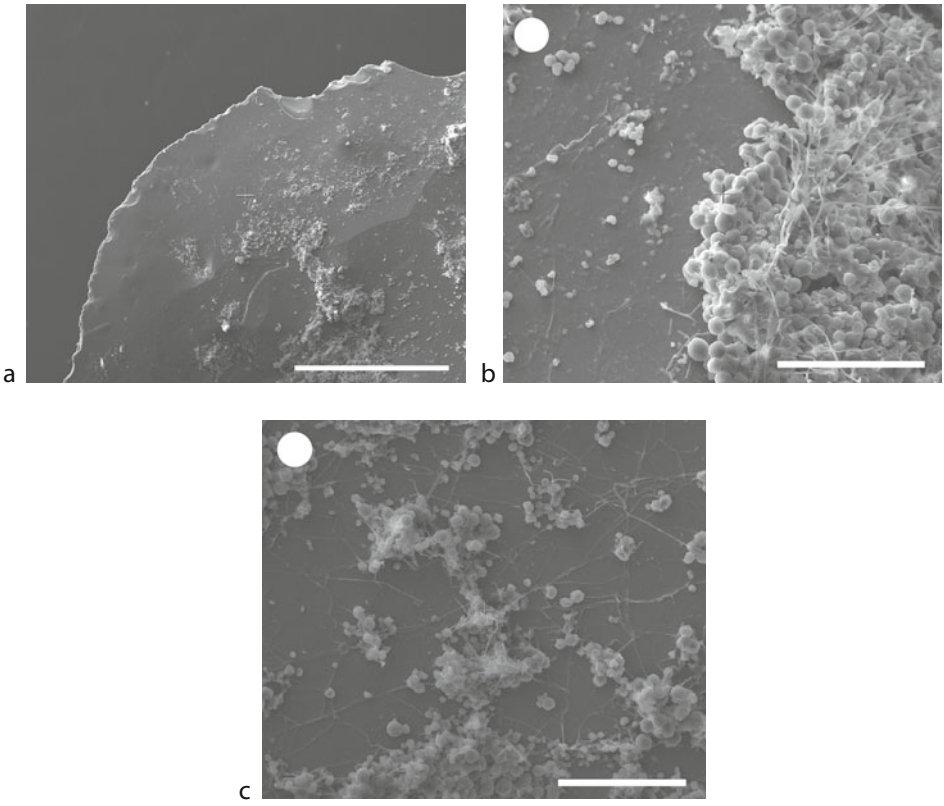

Fig. 7.1.5

Phototrophs inhabiting obsidian visualized with secondary scanning electron microscopy (SEM). (a) Obsidian shard covered by phototroph biofilm (scale bar 500 μm). (b) Magnified biofilm showing filamentous organisms (presumptive heterotrophs) associated with phototrophs (scale bar 100 μm). (c) Filamentous organisms growing across glass linked to biofilm comprised of small coccoid phototrophs (scale bar 100 μm)

The endolithic growth of the phototroph population bears similarities to phototrophic endolithic communities in sedimentary rocks such as sandstones and limestones (Friedmann 1980; Friedmann 1982; Saiz-Jimenez et al. 1990; Büdel and Wessels 1991; Bell 1993; Weber et al. 1996; Büdel et al. 2004; Omelon et al. 2006); however, the obsidian phototrophic endoliths are not widespread and do not form coherent layers throughout the rock substrate. Instead they form localized layers within the rock where vesicles and exfoliation of the glass allow them to penetrate the substrate and grow under sheet-like layers of glass. Filamentous organisms (presumptive heterotrophs) are associated with these biofilms of phototrophs (Fig. 7.1.5).

These observations are in contrast to the basaltic glass where phototrophs are observed in the 16S rDNA library, but they are not observed to form macroscopically visible layers on or within the material. The presence of viable phototrophs can be confirmed and tested for both rock types by simple culturing experiments. Pieces (1 cm^3) of the obsidian and basaltic glass were incubated at room temperature (21°C) in 5 ml of BG-11 medium to culture phototrophs. After incubation for one month, the phototrophic microorganisms growing in each sample were characterized by light microscopy using a Leica DMRP microscope. After one month incubation microscopic observations revealed the presence of filamentous and coccoid phototrophs, whose morphology resembled *Anabaena* and *Chlorella* (Herrera et al. 2009).

How long do the organisms take to colonize the rocks? We investigated this by placing four microscope slides (2.54 × 7.62 cm) in each of the obsidian and basaltic glass locations in July 2007 and collecting them in July 2008. The slides were placed on exposed rock surfaces. After collection, the slides were transferred to 50 mL tubes with 30 mL BG11 in the field and left to incubate under a natural light/dark cycle at 21°C. After one month all of the tubes showed visible growth of phototrophs. All cultured organisms from the slides were coccoid. They were exclusively found in two morphotypes. One morphotype was a coccoid alga and corresponded in size to large cells observed in the endolithic habitat by SEM. The second morphotype was smaller, lighter green organisms which resemble coccoid cyanobacteria and also corresponded in size to the small cells observed in the endolithic habitat by SEM. Intriguingly, we did not observe filamentous forms, which dominate the phototroph sequences in the clone library and occur in cultures from the rocks. These data show that coccoid organisms are the dominant early aerial colonists of the volcanic rocks, which might reflect more efficient aerial transport than filamentous organisms.

The data yield insights into the way in which volcanic rocks, once they have cooled, can be colonized and a carbon cycle established by phototrophs. In the case of silica-rich volcanic materials, the long weathering times allow for phototroph populations to become established within the material in analogy to previously described endolithic communities in sedimentary rocks. The potentially important role that phototrophs could play in providing carbon is suggested by the dominance of carbon-requiring heterotrophs in the 16S rDNA clone libraries from volcanic rocks that have been examined so far.

These data are consistent with findings elsewhere. Ibekwe et al. (2007) detected cyanobacteria in lupine-vegetated soils associated with Mount St. Helen's and Gomez-Alvarez et al. (2007) detected them in vegetated volcanic soils in Hawaii. In contrast to our data, neither of these authors detected cyanobacteria in unvegetated materials. This may have been caused by small clone library size.

In addition to phototrophs, chemolithotrophs are important in some volcanic rocks, and potentially as a source of primary, non-phototrophic productivity. Although we did not detect chemolithotrophs by 16S rRNA analysis, they are known to colonize volcanic materials. Within many newly formed volcanic rock communities are lithotrophs that oxidize CO and H_2

(King 2003; Dunfield and King 2005; Nanba et al. 2004) consistent with the oligotrophic characteristics expected in lava flows. The work by Dunfield and King (2005) shows that the diversity of CO-oxidisers (based on amplification of *cox*L genes) is low in a recent (42 years old) lava flow in Hawaii, but lava between 108–300 years old had similar diversity. The availability of CO and H_2 may benefit leguminous symbionts, accelerating colonization of new volcanic substrates by plants (King 2003). In active volcanic environments where CO and sulfur gas (e.g., H_2S, SO_2) emissions are sufficient, then chemolithotrophic primary production in newly formed volcanic rocks may provide organic carbon for heterotrophs in addition to allochthonous organic carbon input.

Protection from Extremes

The interior of volcanic rocks protects the microorganisms from a number of environmental extremes and this may partly account for the high diversity they can harbor. One extreme is UV radiation. Measurements of light penetration through the obsidian and basaltic glass (Herrera et al. 2008; Cockell et al. 2009b) showed that UV radiation was hardly measurable with standard spectrophotometric equipment. The high iron content of volcanic materials probably accounts for the high UV absorbance of the material as iron is known to be an efficient absorber of short wavelength UV radiation (Pierson et al. 1993). In addition, the vesiculated rock substrate scatters the UV radiation.

Absorption and scattering of UV radiation also occurs concomitantly with absorption and scattering of light in the photosynthetically active region (PAR). This will establish the lowest depth at which phototrophs can be sustained. In both of these substrates the depth of minimum light required for photosynthesis is less than 250 μm (Cockell et al. 2009b; Herrera et al. 2009). These measurements were made with solid volcanic rock and the presence of vesicles and fractures is likely to extend the depth at which phototrophs can persist.

Another advantage to be gained by living within rocks is an altered temperature regimen. Observations of temperature changes were made in the previously described obsidian outcrop in Iceland from June 11–16, 2008. The temperatures 10 cm deep within the rock outcrop only exceeded 20°C on one of the measurement days. The mean temperature over the measurement period was 12.1°C. Under two centimeters of rock temperatures exceeded 30°C for the three days. Temperatures on the surface of nearby soils exceeded 40°C for three of the five days (Herrera et al. 2008).

The high temperatures can be accounted for by the dark colored volcanic soil which effectively absorbs solar radiation. These data show that the habitat deep within volcanic rock can provide some protection against heating experienced on the surface. However, for phototrophs, which must be near the surface of the rock to be active, the data show that dark volcanic rocks and soils, even in the relatively cool environment of Iceland, might achieve temperatures within the range of thermophily and potentially exert stress on mesophilic phototrophs. Nevertheless, temperatures within the rock are generally higher than the corresponding air temperatures during summer (Herrera et al. 2008) and so in some cases, when air temperatures are very low, such as during spring and autumn, it is likely that microorganisms within the rocks would gain advantage from the warming of the dark volcanic materials.

Another advantage obtained by microorganisms within volcanic rocks will be water availability. Except for the interior, Iceland is not an extreme xeric environment. For example, at Haell (64°03.904′N, 20°14.471′W), the weather station recorded a total of 1,435 mm of

precipitation in 2007 and 1,170 mm in 2008. Most of this falls in March and April, and August to February. However, the surface of rocks is rapidly desiccated by wind. Porous volcanic rocks will tend to trap water, extending the period of liquid water availability within the rock substrate and potentially extending the potential period of growth compared to the rock surface after the input of precipitation.

Volcanic Soils

Volcanic rocks ultimately weather through to soils called andosols, which form an important part of the terrestrial volcanic environment. Andosols exist in a myriad of states such as extreme pH in soils surrounding geothermal pools, hot and cold desert soils, as well as humid andosols (Oskarsson et al. 2004; Parfitt & Kimble 1989). The microbial diversity of andosols encompasses many extremophilic microorganisms which are discussed, at length, both in this book and throughout the scientific literature. A key area of research with andosols is understanding how microorganisms are responsible for converting volcanic rocks into fertile soils.

The risks endured by many people living near volcanoes are acceptable owing to the high agricultural fertility of andosols. However, fertile andosols are the end result of many years of microbial and floral amelioration. Young andosols are limited in micro and macronutrients and organics, which are required to permit a good crop harvest. Andosols generally possess a good soil structure, water retention, and porosity; yet these soils are prone to erosion (Arnalds 2004; Oskarsson et al. 2004). They are generally high in Fe and Al. These metals, in addition to high concentrations of humic acids, give andosols the characteristic of high phosphate (P) retention (Lukito et al. 1998). This retention of biologically available P combined with the soil precipitation of phosphorus critically impedes the potential for microbial and floral growth on younger andosols (Kimble et al. 2000).

Initial colonization of andosols is a key stage in promoting further microbial and floral growth. These initial colonists must be able to survive and grow in soils where there is little biologically available P. It is not currently known what the initial microbial colonists are for many andosols as it depends on the chemical composition of each individual soil. Richardson (2001) showed that microorganisms were key to the release of organic and inorganic P, making it available for plant adsorption in young andosols. Even young andosols, although hostile, are capable of sustaining highly diverse microbial communities; Nüsslein and Tiedje (1998) investigated a 200 year old andosol in Hawaii with high diversity. They showed that although there was high diversity, the soil biomass was dominated by *Pseudomonas*, *Rhizobium-Agrobacterium*, and *Rhodospirillium* species.

As discussed, not all andosols are subject to the same nutrient limitations. If phosphate limitations are overcome or not present to begin with, there are always other limits that andosols have on soil fertility. Some of the initial floral species to inhabit Mount St Helens' soils are *Lupinus* spp. and have been proposed to provide support for nitrogen-fixing cyanobacteria (Halvorson et al. 1992). This bacterial nitrogen fixation not only in turn supports *Lupinus* spp. growth but they, through decomposition of plant and microorganisms, provide a supply of bioavailable N and C to the soils (Halvorson et al. 1991).

It is clear that flora and microorganisms are closely entwined in the amelioration of andosols. The simultaneous occurrence of flora with the microbes reduces the likelihood of soil erosion. As initial transformation and floral stabilization of andosols occurs, the potential for further colonists and biodiversity increases (Hopkins et al. 2007).

Deep-Ocean Volcanic Environments

Deep-ocean volcanic environments are obviously not xeric, but some comparisons with the terrestrial volcanic rock environment are valuable. During the rifting that occurs at plate boundaries in the oceans, basaltic glass is produced. The rate of crustal production may be well over a billion tonnes a year (Bach and Edwards 2003), implying large annual production of basaltic glass. Insofar as basaltic glass contains many major cations required for growth, including Ca, Mg, K, Na, and reduced Fe, then it would be expected to be a source of material for microbial growth (Staudigel et al. 1995, 1998; Thorseth et al. 2001; Santelli et al. 2008).

The extent to which basaltic glass is used by a biota in situ is unclear. There is evidence for the deep-ocean biological alteration of glass. In investigations of 0–30 Myr old basalts from the Australian Antarctic Discordance Thorseth et al. (2003) recorded the presence of Mn-rich encrustations which they suggested could be linked to Mn-yielding reactions. Partially fossilized endolithic microbes were associated with rims of altered glasses. Basaltic glass from the Knipovich Ridge, Arctic, shows pervasive colonization of fractures, including stalk, coccoid, rod, and filamentous forms. Organisms were found to be associated with the alteration product in the fractures and they were often covered in Fe- and Al-rich precipitates (Thorseth et al. 2001).

In laboratory experiments, a natural population of organisms from seawater was shown to enhance release of Ca into sediments produced from basaltic glass during weathering compared to the parent glass, but to reduce Mg concentrations relative to the abiotic control (Staudigel et al. 1998).

In seafloor and subsurface basaltic glass tubular and granular alteration textures in samples to 500 m depth in the crust have been correlated to the presence of nucleic acid and carbon isotopic signatures of microbial activity suggesting a biological role for their formation (e.g., Torsvik et al. 1998; Staudigel et al. 2006). These studies on basalts on the seafloor and in the subsurface do not clearly resolve whether the organisms are causing alteration textures as an indirect by-product of metabolic activity or whether they require elements from the glass to grow, although recent evidence suggests that some organisms might access basaltic glass for specific mineral crystal inclusions (Walton 2008).

The organisms that might be involved in weathering glass have been the subject of several investigations. Templeton et al. (2005) showed the presence of Mn-oxidizing bacteria in basalts from Loihi Seamount, although autotrophic Mn-oxidisers were not recovered, which suggests Mn oxidation is a secondary process occurring in heterotrophic organisms (Tebo et al. 2005). Diverse neutrophilic Fe-oxidizing bacteria have been isolated from oceanic environments (Edwards et al. 2003), although it is not clear to what extent they might be accessing iron from basaltic glass or from the surrounding sea water. Culture-independent methods have not revealed clades of previously known iron-oxidizing bacteria, but culturing methods have shown their presence, although many of these organisms are most closely related to previously characterized heterotrophs (Edwards et al. 2003). The iron oxide minerals found associated with deep-ocean basalts have been shown to be similar to minerals produced by iron-oxidizing bacteria in culture (Daughney et al. 2004).

In the study of the microbial community of the basaltic glasses of the Knipovich Ridge, Arctic, Thorseth et al. (2001) identified heterotrophs and some chemolithotrophs including phylotypes belonging to the ε-Proteobacteria and closely matching with sulfur-oxidisers. Although stalk-like deposits similar to those produced by *Gallionella* spp. were seen, this genus was not identified in the DGGE analysis. Iron-reducing organisms were cultured from Arctic Ridge seafloor basaltic glasses by Lysnes et al. (2004) and these authors suggested that this

is evidence for an iron-cycle within seafloor basalts. They cultured a diversity of other organisms belonging to the Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, and Crenarchaeota of unknown physiology. Phylogenetic analysis of seafloor basaltic glasses around Hawaii show high diversity (Santelli et al. 2008; Orcutt et al. 2009) which is hypothesized to be linked to the chemolithotrophic use of basaltic glass alteration products (Santelli et al. 2008).

Importantly, none of the sequences obtained in Icelandic terrestrial volcanic rocks (at least those available) have a close match to those in the deep-ocean environment (Cockell et al. 2009a). Although the potential geochemical nutrient and energy availability from terrestrial and deep-ocean basaltic rocks are similar because of similar composition, the Icelandic terrestrial environment is very different from deep-ocean environments. It is exposed to freshwater in the form of snowmelt, acidic rainwater, and large temperature fluctuations. Almost certainly, one of the primary factors influencing which organisms can survive within terrestrial volcanic rocks is periodic desiccation, which is not encountered in the deep ocean environment and will select for vegetative desiccation resistant organisms or spore-forming organisms such as Actinobacteria and Firmicutes.

Conclusion

Volcanic environments harbor remarkable and as yet uncharacterised diversity. In the terrestrial environment volcanic rocks and soils are often xeric. An investigation of the microbiota yields insights into the key phyla that are adapted to these environments and the sources of energy that sustain these communities in newly formed volcanic environments. Insofar as volcanic rocks weather to soils, release nutrients into the biosphere and play a major role in climate regulation, understanding their microbiology constitutes an important task in environmental microbiology.

Acknowledgments

We thank the Leverhulme Trust (project number F/00 269/N) and the Royal Society for support for the work on microorganisms in volcanic environments.

Cross-References

- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
- 6.5 Ecological Distribution of Microorganisms in Terrestial, Psychrophilic Habitats
- 12.1 Actinobacteria of the Extremobiosphere

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Part 8

Extremophiles: Organic Solvent Tolerant Microorganisms

8.1 Discovery and Taxonomy of Organic Solvent Tolerant Microorganisms

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Discovery and Taxonomy of Organic Solvent-Tolerant Microorganisms

Discovery of Organic Solvent-Tolerant Microorganisms

The discovery of new microbes is necessary for the development of novel fields of microorganism research. In general, organic synthesis reactions differ from biochemical reactions in a reactive environment since the former occur in organic solvent solutions. On the other hand, biochemical reactions occur chiefly in aqueous solutions. Many biochemical reactions are inhibited by organic solvents. The scope of biochemical reactions would therefore be extended if they became possible in organic solvents. Biochemical reactions can occur in organic solvents if specific microbes develop tolerance to them. Organic solvent-tolerant bacteria are, therefore, thought to be useful microorganisms for investigating biochemical reactions in organic solvents. New microbial reactions may be found with the discovery of novel microorganisms.

Solvent-tolerant microorganisms have numerous potential commercial applications in industrial biotransformation processes that involve the use of organic substrates with low solubility in water. When such compounds are used as substrates, large quantities of water are required, and water consumption is a major cost factor in the fermentation industry. The development of solvent-tolerant microorganisms or microbial catalysts for use in bioreactors might solve this problem. Also, from the academic viewpoint, such microorganisms are an interesting tool for the elucidation of the biological mechanisms of solvent tolerance. Therefore, a microorganism that can grow in organic solvents is needed.

Most organic solvents are biotoxic and inhibit the growth of microorganisms even at low concentrations, that is, less than 1% in culture media. No microbe able to grow at organic solvent concentrations of more than 2–3% has been reported. However, our group attempted to isolate a new microbe that can grow in an organic solvent environment.

Toluene is a highly toxic solvent that kills most microorganisms at a concentration of 0.1%. It has therefore been used for many years to sterilize microbial cultures and maintain solutions in a sterile condition. Furthermore, because toluene is effective in extracting lipids from cell membranes, it has been used as an unmasking agent in the assay of various enzymes.

It has been reported that some microorganisms, such as *Pseudomonas putida* (Worsey and Williams 1975), *P. aeruginosa* (Kitagawa 1956), a *Pseudomonas* sp. (Claus and Walker 1964), an *Achromobacter* sp.(Worsey and Williams 1975), and a *Nocardia* sp.(Raymond et al. 1967), can assimilate toluene. However, these bacteria tolerated toluene concentrations of less than 0.3%. Microorganisms that can grow in the presence of more than 0.3% toluene have not been found previously.

From 800 soil samples collected from oil plants, oil wells, sludge, and tar sands in Japan and other countries, we isolated a toluene-tolerant bacterium that grew well in a nutrient medium containing toluene. To isolate toluene-tolerant microorganisms, a small amount of soil was suspended in sterile water, and 0.2 ml of this suspension was transferred to test tubes containing 5 ml of a nutrient medium comprising 0.1% glucose, 0.25% yeast extract, and 0.5% polypeptone, pH 7.0. Toluene was then added to a final concentration of 30% (v/v), the test tubes were plugged with butyl-rubber, and the cultures were incubated at 37°C for 1 week in a test-tube shaker. As a result of screening, one bacterial strain that grew well in the toluene-containing medium was isolated from a soil sample collected in Kyushu, southern Japan. Subsequent characterization of this bacterium showed that it is aerobic, Gram-negative, and rod-shaped (0.8–1.0 × 2.0–4.0 μm) and has four polar flagella. The G and C content of its DNA

is 62.5%, it reacts positively in the catalase and oxidase tests, and is oxidative in the oxidative-fermentative (O-F) test. Based on these and other morphologic and biochemical characteristics of the strain determined using the standard methods, the isolate was identified as a strain of *P. putida*, which differs from other strains only its resistance to toluene. Other strain in this genus, including *P. putida* IFO 3738 and *P. putida* IAM 1506, failed to grow in culture medium containing 30% toluene. This newly discovered strain was designated *P. putida* IH-2000 (Inoue and Horikoshi 1989).

We submitted a paper describing the toluene-tolerant *P. putida* IH-2000 to the journal *Nature*, and the referee was initially skeptical. After responding to numerous questions from the referee and providing a detailed account, the paper was finally accepted for publication.

Characteristics of *P. putida* IH-2000

The effects of various metal ions on the stabilization of the toluene tolerance of *P. putida* IH-2000 were investigated. Growth was determined after incubation for 48 h at 30°C in Luria-Bertani (LB) medium containing metal ions (Cu^{2+}, Rb^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Al^{3+}, Sn^{2+}, Pb^{2+}, V^{2+}, Mo^{7+}, W^{6+}, Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+}) at a concentration of 2 or 5 mM and 30% toluene per culture. Among the ions examined, Mg^{2+} and Ca^{2+} were the most effective in stabilizing toluene tolerance. The cell yield was increased by about twofold and threefold when

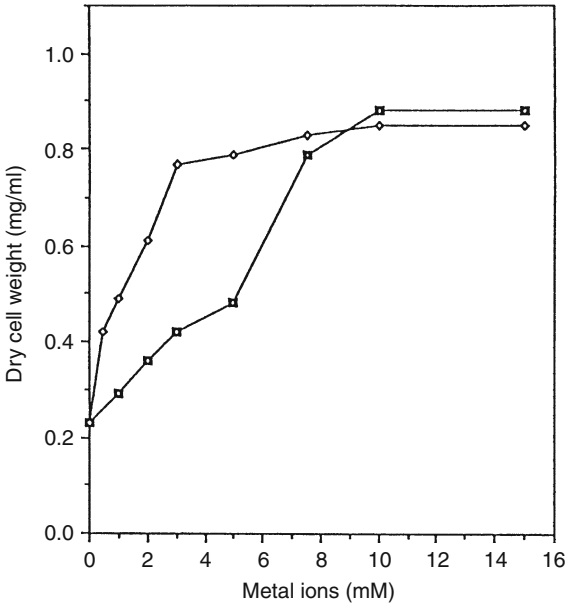

Fig. 8.1.1

Effects of metal ion concentration on cell growth. Growth was determined after incubation for 48 h at 30°C in LB medium containing $MgSO_4 \cdot 7H_2O$ or $Ca(NO_3)_2 \cdot 4H_2O$ and 30% (vol/vol) toluene. Cell dry weight was calculated on the basis of the predetermined correlation of 0.6 mg (dry weight)/ml as equivalent to 1 OD_{660} unit. Symbols: □, Mg^{2+}ions; ◇, Ca^{2+}ions

Mg^{2+} and Ca^{2+}, respectively, were added to the LB medium containing toluene, compared with the control without metal ions. Other metal ions inhibited the growth of *P. putida* IH-2000 at a concentration of 2 mM and prevented growth at 5 mM.

Figure 8.1.1 shows the effects of different concentrations of Mg^{2+} and Ca^{2+} ions on the growth of strain IH-2000. Growth increased in the presence of Mg^{2+} concentrations greater than 2 mM and in the presence of C^{2+} concentrations greater than 0.5 mM Ca. The optimal concentration of Mg^{2+} was greater than 7.5 mM, and that of Ca^{2+} was 3.0 mM. The addition of Ca^{2+} was more effective than Mg^{2+} for stable solvent tolerance (Inoue and Horikoshi 1991). With different solvents, such as styrene, *p*-xylene, and cyclohexane, these metal ions were as effective as with toluene for stabilizing solvent tolerance. The growth of strain IH-2000 was inhibited by the addition of different solvents to medium without metal ions. These observations suggest that metal ions may improve solvent tolerance in living cells. It is interesting that only Mg^{2+} and Ca^{2+} among the metal ions examined improved solvent tolerance.

Figure 8.1.2 shows the growth curve of strain IH-2000 in modified LB medium with and without solvent. The doubling time for growth was 1.22 h and 0.77 h in the presence of 30% toluene and in the absence of the solvent, respectively. After 24 h, the LB medium with toluene added contained 2×10^9 cells/ml. Furthermore, single cells of this strain

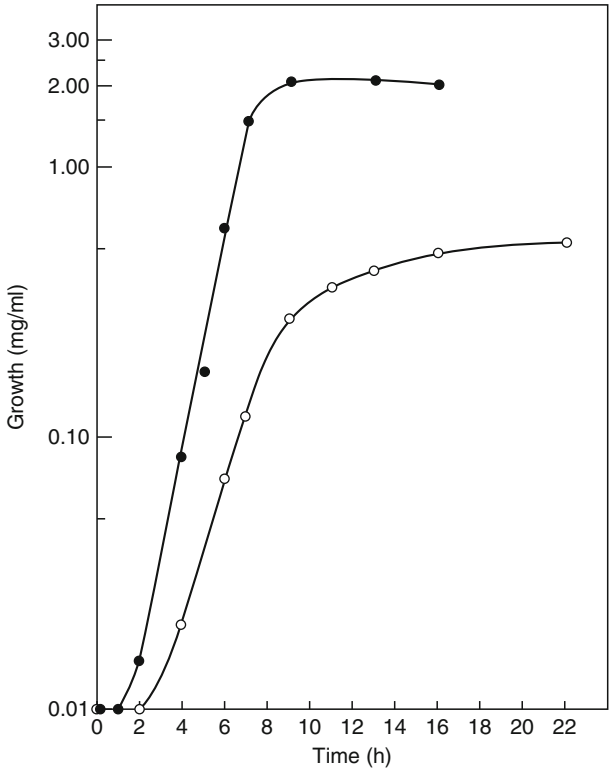

Fig. 8.1.2
Growth curve of strain IH-2000 in LB medium containing 30% toluene

formed colonies on solid nutrient medium overlaid with toluene, which remained viable for several weeks. This indicates that solvent tolerance is a stable phenotypic property of strain IH-2000 (Inoue and Horikoshi 1989).

To investigate the influence of different concentrations of toluene on the growth of strain IH-2000, this strain was incubated in a test-tube shaker for 24 h at 30°C in modified LB medium supplemented with toluene at concentrations ranging from 0.1% to 200% (v/v). The strain has a high tolerance for toluene and grew without significant evidence of inhibition over a wide range of toluene concentrations (*Fig. 8.1.3*). The colonies that grew on nutrient agar plates overlaid with toluene remained viable for several weeks.

CS basal medium, consisting of 1 g of NH_4NO_4, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.2 g of KCl, pH 7.2, was used to test for the utilization of hydrocarbons. Cultures were generally grown in test tubes containing 5 ml of medium with shaking at 30°C. Cell growth was determined by measuring the optical density at 660 nm (OD_{660}). Each hydrocarbon examined, that is, toluene, *p*-xylene, cyclohexane, hexane, and octane, was added to CS basal medium at a final concentration of 0.5% (v/v), and the cultures were incubated at 30°C for 48 h. Strain IH-2000 could not grow in the CS medium containing hydrocarbon and could not utilize hydrocarbons as an energy source.

The tolerance of strain IH-2000 was tested in liquid cultures containing 50%, 5%, and 2.5% of each solvent. This strain tolerated other toxic solvents in addition to toluene, including saturated and unsaturated aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers. Interestingly, this strain was not tolerant to benzene, fluorobenzene, nitrobenzene, propanol, butanol, diethylether, propyleneoxide, chloroform, and ethylacetate. This strain could not grow in media containing 50% ketone solvent, but exhibited growth at acetone and cyclohexanone concentrations of 2.5%. *Table 8.1.1* summarizes the solvent

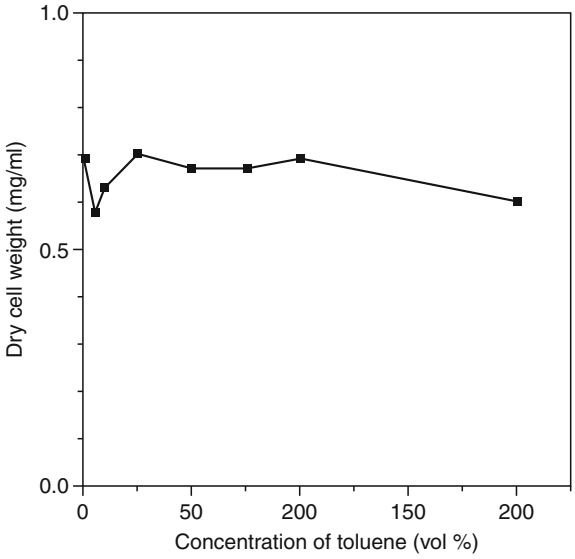

Fig. 8.1.3

Effects of toluene concentration on the growth of strain IH-2000

Table 8.1.1

Solvent tolerance of strain IH-2000

Inhibition of growth and solvent in group	Growth at solvent concn. (vol/vol)		
	50.0%	5.0%	2.5%
No inhibition Hydrocarbon (pentane, hexane, heptane, octane, dodecane, 2-pentane, 2-hexane, 1,3-pentadiene, 1,7-octadiene styrene, toluene, p-xylene, chlorobenzene) Alcohols (heptanol, octylalcohol, decylalcohol) Ethers (butylether, hexylether, benzylether)	+	+	+
Slight inhibition Alcohols (methanol) Ethers (butylvinylether) Ketones (2-heptone) Miscellaneous (acetal, dimethlformamide, dimethyl, sulfoxide)	−	± or +	+
Moderate inhibition Alcohols (ethanol) Ketones (acetone, cyclohexanone)	−	−	± or +
Inhibition Hydrocarbons (benzene, fluorobenzene, nitrobenzene) Alcohols (propanol, butanol) Ethers (diethylether, propylene oxide) Ketones (methylethylketone, 2-pentanone, 2-hexanone) Miscellaneous (chloroform, ethylacetate, acetonitrile)	−	−	−

Symbols, +; growth (>0.3 mg [dry weight]/ml) ; ±, slight growth; −, no growth.

tolerance of strain IH-2000. The results indicate that solvent tolerance is a stable phenotypic property of *P. putida* strain IH-2000 (Inoue et al. 1991). We believe that certain unique cell surface properties of strain IH-2000 permit growth under such harsh conditions. It will be interesting to compare the cell surface components of this strain with those of other strains.

Strain IH-2000 has considerable potential for application in bioreactors. It is possible that it can be altered for use in bioreactors by genetic engineering, such as by transformation with plasmids that confer degradation or biotransformation properties. At present, we are investigating the mechanisms of solvent tolerance from the viewpoint of the cell surface components and the genetic mechanism of solvent tolerance.

Cross-References

- 8.3. Molecular Responses to Solvent Stress:Strategies for Living in Unpalatable Substrates
- 8.4. Genetics Evolution and Applications

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8.2 Diversity and Ecology of Organic Solvent Tolerant Microorganisms

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_8.2,

Index of Solvent Toxicity to Microorganisms

The results of our investigation of solvent tolerance clarified that the growth of *Pseudomonas putida* strain IH-2000 differed depending on the organic solvent added to the culture medium. To determine how organic solvents affect the growth of microorganisms, the structural correlation between growth and the type of organic solvent was examined.

The physical basis of solvent toxicity is still poorly understood, and no physical parameter has been identified by which the relative toxicity of different solvents can be determined. Consequently, the solvent tolerance of microorganisms has not yet been correlated with organic solvent type. To investigate the correlation between the solvent tolerance of microorganisms and solvent toxicity, we isolated solvent-sensitive mutants using the replica-plating method in conjunction with penicillin selection after culture with 1-methyl-3-nitro-1-nitrosoguanidine (NTG). First, we constructed a Leu and Trp auxotroph from strain IH-2000 after two NTG treatments. We then isolated three toluene-sensitive mutants from 3,000 colonies of mutant cells. Remarkably, one of these (IH-2124T) was sensitive to toluene but resistant to xylene and cyclohexane.

We subsequently isolated one xylene-sensitive mutant (IH-2124TX) from 2,000 colonies of mutant cells derived from the toluene-sensitive strain. This strain was sensitive to both toluene and xylene, but resistant to cyclohexane. A cyclohexane-sensitive mutant (IH-2124TXC) that remained hexane resistant was isolated from strain IH-2124TX. Each of these mutants retained the Leu and Trp auxotrophic markers. The toluene-sensitive mutant, for which the frequency of back-mutation was less than 10^{-9}, was very stable. The xylene-sensitive and cyclohexane-sensitive mutants exhibited frequencies of back-mutation of 10^{-7} and 10^{-8}, respectively. In addition to toluene, these strains tolerated other toxic solvents including aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers.

By examining the partition efficient of an organic solvent, we can predict accurately the tolerance of microorganisms to other solvents in the polarity scale. It was found that the effects of solvents on the growth of toluene-tolerant strain IH-2000 and its toluene-tolerant mutants were quantitatively correlated by the parameter log P (Corwin and Anderson 1967; Rekker and de Kort 1979), where P is the partition coefficient of solvent dissolved in an equimolar mixture of octanol and water. Log P is also used as a quantitative index of solvent polarity in quantitative structure-activity relationship analysis (Hansch and Fujita 1964; Harnish et al. 1983). The correlations between log P values of solvents and the growth limits of strain IH-2000 and its three mutants are given in *Table 8.2.1*, together with those of other bacteria and yeasts. It is evident that although strain IH-2000 grows in solvents with log P values $\geq$2.4, such as heptanol (log P = 2.4), toluene (log P = 2.8), and *n*-octane (log P = 4.9), it does not grow in fuorobenzene, benzene, and butanol with log P values of 2.3, 2.1, and 0.8, respectively. Therefore, it appears that a critical point is reached between log P values of 2.4 and 2.3, at which the solvent is sufficiently polar to prevent growth. The concept of such a tolerance limit can be extended to predict the growth of other microbial species in solvent-saturated environments (Inoue and Horikoshi 1989).

These observations indicate that the nature of the interaction between the solvent and the cell surface which limits growth is at least partly determined by solvent polarity. The unusually high tolerance of strain IH-2000 to toluene and other solvents probably reflects the presence of cell surface components that are unique to the strain. In addition to mutant strains of *P. putida*, they can order other microorganisms in a similar hierarchy of solvent tolerance, in which growth in a solvent of a given polarity indicates tolerance to solvents of lower polarity.

Table 8.2.1

Solvent tolerance of bacteria and mutants

Solvent	Log P	IH-2000	IH-2124T	IH-2124TX	IH-2124TXC	*Escherichia. Coli* IFO 3806	*Pseudomonas. putida* IFO 3738	*P.fluorescens* IFO 3507	*Achromobacter delicatlus IFO 3058*	*Agrobacterium tumefaciens* IFO 3058	*Alcaligenes faecalis* JCM 1474	*Aeromonas hydrophila* JCM 1027	*B.Subtilis* AHU 1219	*Corynebacterium glutamicum* JCM 1318	*S. uvarum* ATCC 26602
Dodecane	7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Decane	6.0	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Nonane	5.5	+	+	+	+	+	+	+	+	+	+	+	+	−	−
n-Hexylether	5.1	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Octane	4.9	+	+	+	+	+	+	+	+	−	+	+	+	−	−
Isooctane	4.8	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Cyclooctane	4.5	+	+	+	+	+	+	+	+	−	+	+	−	−	−
Diphenylether	4.2	+	+	+	+	+	+	+	+	−	−	−	−	−	−
n-Hexane	3.9	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Propylbenzene	3.8	+	+	+	−	+	+	+	−	−	−	−	−	−	−
o-Dichlorobenzene	3.6	+	+	+	−	−	+	+	−	−	−	−	−	−	−
Cyclohexane	3.4	+	+	+	−	−	+	+	−	−	−	−	−	−	−
Ethylbenzene	3.3	+	+	−	−	−	+	−	−	−	−	−	−	−	−
p-Xylene	3.1	+	+	−	−	−	+	−	−	−	−	−	−	−	−

Table 8.2.1 (Continued)

Solvent	Log P	IH-2000	IH-2124T	IH-2124TX	IH-2124TXC	*Escherichia. Coli* IFO 3806	*Pseudomonas. putida* IFO 3738	*P.fluorescens* IFO 3507	*Achromobacter delicatlus* IFO 3058	*Agrobacterium tumefaciens* IFO 3058	*Alcaligenes faecalis* JCM 1474	*Aeromonas hydrophila* JCM 1027	*B.Subtilis* AHU 1219	*Corynebacterium glutamicum* JCM 1318	*S. uvarum* ATCC 26602
Styrene	2.9	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Toluene	2.8	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Heptanol	2.4	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Benzene	2.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Limiting log P value for growth		2.4	2.9	3.4	3.9	3.8	3.1	3.4	3.9	4.8	4.5	4.5	4.9	7.0	7.0

+, growth; –, no growth

Diversity of Solvent-Tolerant Microorganisms Based on the Solvent Parameter log P

In previous systems, a direct correlation was observed between the solvent tolerance of microbes and the solvent parameter log P. It therefore appears that log P can serve as a quantitative index of the inherent ability of a solvent to limit the growth of microbes in solvent environment.

It was shown that the relative toxicities of different solvents are determined by their polarity. Limitations on the growth of microorganisms exerted by high concentrations of various organic solvents were shown to be correlated with the solvent parameter log P. This indicates that the solvent tolerance of microorganisms is determined by the log P value at water-saturated solvent concentrations.

To investigate the solvent-tolerant properties of bacteria, it is useful to elucidate the mechanisms conferring solvent tolerance. This section describes the solvent-tolerant limits of bacteria in terms of the log P parameter of solvents and compares the solvent tolerance of Gram-negative and Gram-positive bacteria and yeasts.

Type strains of Gram-negative bacteria belong to the genera *Pseudomonas*, *Escherichia*, *Aeromonas*, *Alteromonas*, *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Serratia*, *Proteus*, *Klebsiella*, *Flavobacterium*, and *Chromobacterium*, while Gram-positive bacteria belong to the genera *Bacillus*, *Micrococcus*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium*, *Rhodococcus*, *Leuconostoc*, and *Lactobacillus* (Inoue and Horikoshi 1991a). Yeasts belonging to the genera *Endomycopsis*, *Saccharomycodes*, *Saccharomycopsis*, *Pichia*, *Hansenula*, *Shizosaccharomyces*, *Leucosporidium*, *Bellera*, *Sporidiobolus*, *Candida*, *Kloekera*, *Rhodotorula*, *and Torulopsis* were investigated by our group. The growth of bacteria type strains was examined in modified LB medium consisting of tryptone 10 g (Difco), yeast extract 5 g (Difco), NaCl 5 g, glucose 1 g, and $MgSO_4 \cdot 7H_2O$ 10 mM in 1 L of deionized water, pH 7. Yeast type strains were cultured in a malt extract medium consisting of malt extract 20 g (Difco), glucose 20 g, peptone 1 g (Difco) in 1 L of deionized water, pH 6.2.

The solvent tolerance of the Gram-positive and Gram-negative bacteria and yeast type strains was examined on agar plates overlaid with different solvents. Cells of each strain were streaked on the agar plate medium, directly overlaid with one solvent, and incubated at 37°C for bacteria or 30°C for yeast. The growth was based on the formation of colonies on the agar plates after 48-h incubation. The limiting log P values for growth were defined as the critical point at which the solvent was sufficiently polar to prevent the growth of the strains. Different solvents with log P values ranging from 2.1 (benzene) to 7.0 (dodecane) were examined. The solvent-tolerance limits of the Gram-negative and Gram-positive bacterial type strains and yeasts are shown in *Tables 8.2.2*, *8.2.3*, and *8.2.4*, respectively.

As shown in *Table 8.2.2*, the limiting log P value for the growth of the genera *Pseudomonas* varied from 3.2 to 3.4. It could grow in such solvents as *p*-xylene (log P = 3.1) and cyclohexane (log P = 3.4), but not in solvents with low log P values, such as styrene (log P = 2.9) and toluene (log P = 2.8). *P. putida* IFO 3738, *P. chlororaphis* IFO 3904, and *P. syringae* IFO 3310 had the greatest solvent tolerance among all *Pseudomonas* species investigated. Depending on the strain, *Escherichia coli* could grow in the presence of solvents with log P values greater than 3.4 or 3.8 (cyclohexane and hexane) but not in more polar solvents. Other microorganisms examined had similar characteristic limiting log P values for growth, as shown in *Table 8.2.2*. These bacteria could grow in solvents with greater than the growth-limiting log P value but not in more polar solvents with low log P.

Table 8.2.2

Solvent tolerance of Gram-negative bacteria

Type strains			Limiting log P values for growth
Pseudomonas aeruginosa	I FO	3924	3.4
Pseudomonas putida	I FO	3738	3.1
Escherichia coli	I FO	3806	3.8
Escherichia coli	I FO	3366	3.4
Aeromonas hydrophila	JCM	1027	4.5
Aeromonas hydrophila	I FO	3820	4.2
Alteromonas putrefaciens	I FO	3908	4.2
Achromobacter delicatulus	IAM	1433	3.9
Agrobacterium tumefaciens	I FO	3058	4.8
Alcaligenes faecalis	JCM	1474	4.5
Serratia marcescens	I FO	3406	3.4
Proteus vulgaris	I OF	3167	4.2
Proteus mirabilis	I FO	3849	3.8
Klebsiella pneumonia	I FO	3317	3.4
Flavobacterium lutescens	I FO	3084	4.0
Flavobacterium suaveolens	I FO	3752	5.1
Chromobacterium chocolatum	I FO	3758	7.0

Table 8.2.3

Solvent tolerance of Gram-positive bacteria

Type strains			Limiting log P values for growth
Bacillus subtilis	AHU	1390	4.5
Bacillus subtilis	I FO	3009	5.1
Bacillus circulans	I FO	3329	7.0
Micrococcus luteus	I FO	3333	4.8
Micrococcus roseus	I FO	3764	4.8
Staphylococcus epidermidis	I FO	3762	4.8
Staphylococcus faecalis	I FO	3826	5.1
Corynebacterium glutamicum	JCM	1318	7.0
Corynebacterium flavescens	IAM	1614	6.0
Brevibacterium ammoniagenes	I FO	12072	7.0
Brevibacterium roseum	ATCC	13825	6.0
Rhodococcus erythropolis	I FO	12320	6.0
Rhodococcus eque	I FO	3730	7.0
Leuconostoc mesenteroides subsp. *dextran*	I FO	3425	5.1

Table 8.2.4

Solvent tolerance of yeasts

Type strains			Limiting log P values for growth
Endomycopsis tibliglr	N I	7404	5.1
Saccharomycodes ludwigii	I FO	0798	5.1
Sacchromyces cerevisiae	I FO	0213	5.1
Sacchromyces uvaram	ATCC	26602	7.0
Sacchromycopsis lipolitica	I FO	0746	4.2
Pichia membranaefaciens	I FO	0989	5.5
Pichia farinose	I FO	1003	4.8
Hansenula americana	I FO	1368	3.9
Shizosaccharomyces octosporas	IAM	4842	4.8
Leucosporidium nivalis	I FO	1922	5.5
Bellera alba	I FO	1192	6.0
Sporidiobolus johnsonii	I FO	6903	4.8
Candida sake	I FO	0435	7.0
Candida tropicalis	I FO	0589	3.9
Candida utilis	I FO	0619	5.1
Kloekera africana	I FO	1155	4.9
Kloekera corticis	I FO	0633	5.1
Rhodotorula aurantiaca	I FO	0951	5.1
Rhodotorula glutimis var. *daireesis*	I FO	0415	4.8
Torulopsis famata	N I	7550	3.9

As shown in *Table 8.2.3*, the limiting log P value for the growth of *Bacillus* species ranged from 4.5 to 7.0. They could grow in the presence of cycloctane and dodecane but not in solvents with lower log P values, such as trichlorobenzene (log P = 4.3) and hexane (log P = 3.9). *Bacillus subtilis* AHU 1390 had the highest solvent tolerance among the *Bacillus* species examined. *Table 8.2.3* also shows that other Gram-positive bacteria have characteristic limiting log P values for growth.

Gram-negative bacteria showed greater solvent tolerance than Gram-positive ones. Among all the microorganisms examined, the *Pseudomonas* group showed the greatest solvent tolerance. On the other hand, the solvent tolerance of *Chromobacterium* and some species in the *Bacillus*, *Corynebacterium*, *Brevibacterium*, and *Rhodococcus* genera was low. Other genera showed moderate solvent tolerance as indicated by their limiting log P values, which ranged from 3.4 (cyclohexane) to 5.1 (hexylether).

Based on these results, the solvent tolerance of Gram-negative and Gram-positive bacterium examined was in the order *Pseudomonas* > *Escherichia*, *Serratia*, *Klebsiella* > *Achromobacter*, *Acinetobacter*, *Proteus* > *Alteromonas*, *Aeromonas*, *Flavobacterium* > *Alcaligenes*, *Bacillus* > *Agrobacterium*, *Micrococcus*, *Staphylococcus* > *Streptococcus* > *Leuconostoc*, *Lactobacillus* > *Coryerinebacterium*, *Chromobacterium*, *Brevibacterium*, *Rhodococcus*.

As shown in *Table 8.2.4*, the limiting log P value for the growth of the yeast group ranged from 3.9 to 7.0. Among all the yeasts examined, the *Hansenura*, *Candida*, and *Torulopsis* group showed the greatest solvent tolerance and were able to grow in the presence of *n*-hexane (log P = 3.9). On the other hand, the tolerance of *Sacchromyces uvarum* and *Candida sake* was the lowest in all the yeasts examined, although they grew in the presence of *n*-dodecane (log P = 7.0). Based on these results, the solvent tolerance of yeasts was in the order *Hansenura*, *Candida*, *Torulopsis* > *Saccharomycopsis* > *Shizosaccharomyces*, *Sporidiobolus*, *Rhodotorula*, *Pichia* > *Kloeckera* > *Endomycopsis*, *Sacchromycodes*, *Saccharomyces* > *Leucosporidium* > *Bullera*.

Figure 8.2.1 shows a comparison of the solvent tolerance of each micro population. The Gram-negative bacteria were able to grow in the presence of solvents with growth-limiting log P values ranging from 3.1 to 7.0. The limiting log P values for Gram-positive bacteria and yeasts ranged from 3.4 to 7.0. The solvent tolerance of Gram-negative bacteria was therefore greater than that of Gram-positive bacteria and yeasts, while that of the latter two groups was similar.

These cumulative results suggest that the polarity of solvents affects the cell surface characteristics and thus the growth of bacterial and yeast strains. It may therefore be possible that certain unique cell-surface properties control the growth of some strains in water-saturated organic solvents.

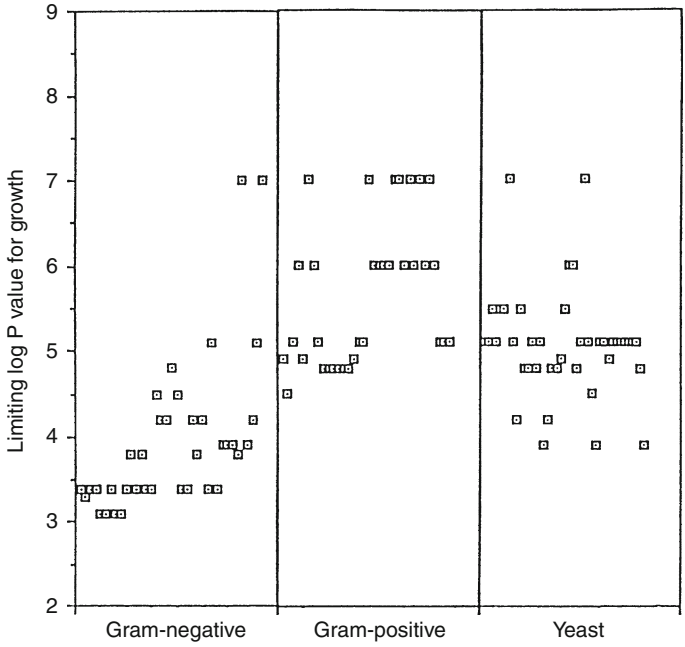

Fig. 8.2.1

Distribution map of limiting log P values of Gram-negative and Gram-positive bacteria, and yeasts

Diversity of Solvent-Tolerant Microorganisms in Deep-Sea Environments

The deep-sea bed is a unique environment that experiences extremely high pressures and low temperatures. Microorganisms living there have developed particular characteristics that allow them to thrive at such extremes. We have isolated and characterized a number of microorganisms from samples of deep-sea mud obtained by the manned submersible Shinkai 6500. This vehicle, which is operated by the Japanese Marine Science and Technology Center, (JAMSIEC), has the ability to submerge to a depth of 6,500 m. It was believed that unknown microbes we have not been able to isolate yet must be thriving in the deep-sea floor, and we attempted to isolate these microbes, especially those that are organic solvent tolerant, from the deep-sea mud samples. Such microorganisms could prove useful for new biotechnology application, such as two-phase (water/organic solvent) bioreactor systems.

Surprisingly, we observed that many organic solvent-tolerant microbes exist even in the deep-sea. Therefore, we are focusing on organic solvent-tolerant microbes and their industrial application.

The Isolation Frequency of Organic Solvent-Tolerant Microbes from Deep-Sea

To obtain information about the presence of solvent-tolerant microorganisms, we had tried to compare the isolation frequency of organic solvent-tolerant microorganisms from land soil and abyssal mud, respectively. As shown in *Table 8.2.5*, the isolation frequency in land soil was 1×10^{-8} to 3.6×10^{-8}. In contract, the isolation frequency in abyssal mud samples was 350×10^{-8} to 700×10^{-8}. From these experiments we found that 100 times as many organic solvent-tolerant microorganisms could be isolated from deep-sea mud samples as from soil samples taken from land.

As a result, we have discovered many useful organic solvent-tolerant strains from the deep-sea environment. Some of these isolates from deep-sea are shown in *Table 8.2.6*.

Benzene-Tolerant Hydrocarbon-Degrading Bacteria

Because various kinds of organic solvents (OSs), such as benzene, toluene, and *p*-xylene, are known to be components in petroleum, and the deep-sea is the ultimate receptor of polluted petroleum-hydrocarbons, we focused on deep-sea bacteria, which showed the characteristics of

Table 8.2.5

Comparison of the frequency of organic solvent-tolerant microbes from different sources

Source	Total microbes ($\times 10^5$ cells/g)	Solvent-tolerant microbes (cells/g)	Isolation frequency ($\times 10^{-8}$)
Land soil	2,800–11,500	7–16	1–3.6
Abyssal mud	54–109	19–52	350–700

Table 8.2.6

Organic solvent-tolerant microorganisms isolated from deep-sea

Strain	Property	Source	Reference
Organic solvent-tolerant bacteria			
DS-711	Degrades crude oil	Suruga Bay, 1,945 m	Moriya and Horikoshi 1993a
DS-944	Utilizes sulfur	Sagami Bay, 1,168 m	Moriya and Horikoshi 1993b
ST-1	Degrades cholesterol	Okushiri Ridge, 1,963 m	Moriya et al. 1995
DS-1906	Degrades polyaromatic hydrocarbons	Sagami Bay, 1,168 m	Abe et al. 1995
Organic solvent-tolerant yeast			
Y-40	Hydrocarbon-degrading yeast	Sagami Ridge, 1,200 m	

halotolerant growth in addition to OS tolerance (Moriya and Horikoshi 1993a, b). We also studied whether isolation of deep-sea bacteria having such characteristics could improve the degradation of polluting petroleum-hydrocarbons in marine environments.

We developed a novel, simple, efficient method for the isolation of a benzene-tolerant bacterium from deep-sea samples. To isolate benzene-tolerant bacteria, which also exhibited the characteristic of halo-tolerant growth, 1 ml of sediment samples was transferred to test tubes containing 3-ml artificial seawater. Benzene was then added at 1 ml (20% v/v), and then test tubes were plugged with silicone-rubber stoppers. The cultures were incubated at 4°C for a week in a test tube shaker. Then, 5 ml (50% v/v) of kerosene was added and the cultures were incubated for a further 5 days at 20°C. After incubation, the cultures were aseptically transferred to separating funnels and allowed to stand for 30 min. Then, the upper layers were carefully separated from the seawater layers, and a portion of each upper layer was directly spread onto M-I agar medium comprising 1 g of proteose peptone no. 3 (Difco), 0.5 g of phytone peptone (BBL), 1 g of $CaCl_2 \cdot 2H_2O$, 0.1g of $MgCl_2 \cdot 6H_2O$, 0.05 g of Na_2SO_3 and per liter of distilled water, at pH 7 and incubated for 2 day at 30°C. For microbial growth, M-II medium which contained 5 g of proteose peptone no. 3, 2.5 g of phytone peptone, 1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$ and 0.05 g of Na_2SO_3 per liter distilled water, and a modified LB-medium (Inoue and Horikoshi 1991a) (tentatively named LB-1 medium in this study) were used. To prepare salt medium, the above media were supplemented with NaCl at 11.8% (w/v) unless otherwise stated. For preparation of agar medium, agar was added at 1.5%. The pH was adjusted at 6.5–7 by 1 N HCI or 1 N NaOH. Artificial seawater consisting of 35 g of NaCl, 10 g of $MgCl_2 \cdot 6H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, and 0.7 g of KCI per liter distilled water was used for the preparation of concentrated cell suspensions. As the *n*-alkane substrate for the microbial degradation experiment, kerosene which contained *n*-alkanes having 7–16 carbon atoms was used at the indicated concentration.

After treatment of the deep-sea sediment samples collected from a depth of 1,945 m in Suruga Bay with 20% v/v benzene followed by 50% (v/v) kerosene, 56 candidate colonies grown on selective medium that combined the characteristics of halotolerant growth and benzene tolerance were isolated as potential hydrocarbon degraders. These candidates belonged to various genera, including *Arthrobacter* sp., *Bacillus* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Flavobacterium* sp., and *Vibrio* sp. Finally, the bacterial strain that showed the

highest growth in the presence of 5% v/v benzene among above 56 isolates was selected as a hydrocarbon degrader that also exhibited the characteristics of halotolerant growth and benzene tolerance. This strain was identified based on its morphological and biochemical characteristics, as outlined in volume I of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). Analysis of the G+C content of DNA was done by the method (Tamaoka and Komagata 1984). The growth temperature of selected isolates was determined using a temperature gradient bio-photo recorder (TN-112D5 Advaptec). Cells of the isolate had peritrichous flagella and showed motility. The isolate formed orange colonies on M-I and M-II salt media. It was Gram-negative, non-spore-forming, and strictly aerobic, oxidase-positive, and catalase-positive. The isolate showed resistance to many antimicrobials including streptomycin, penicillin, lincomycin, nalidixic acid, josamycin, and kanamycin and a susceptibility to novobiocin. Based on its morphological and biochemical characteristics, in addition to its characteristic of benzene tolerance the selected strain seemed to be a variant strain of *Flavobacterium* sp. and was tentatively named strain DS-711.

The OS tolerance of the *Flavobacterium* sp. strain DS-711 was compared with control strains, *E. coli* strain IFO 3806, *B. subtilis* strain IFO 3009, and the toluene-tolerant *P. putida* strain IH-2000. Tested strains were cultured on M-II salt medium and LB-I medium in the presence of OSs such as benzene, toluene, and *p*-xylene. Both, *E. coli* IFO 3806 and *B. subtilis* IFO 3009 were unable to grow not only in the presence of 1% benzene but also 10% toluene and 10% *p*-xylene, as shown in *Table 8.2.7*.

The toluene-tolerant strain IH-2000 showed considerable growth in the presence of 10% toluene, and 10% *p*-xylene, but it could not grow in the presence of more than 1% benzene. In contrast to IH-2000, DS-711 could grow in the presence of not only 10% toluene and 10% *p*-xylene but also 5% benzene. The tolerance of strain DS-711 to various toxic OSs in addition to benzene, toluene, and *p*-xylene, including saturated and unsaturated aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohol, and ethers, was further examined. DS-711 tolerated various toxic OSs, though others such as methoxytoluene, propanol, butanol, heptanol, octylalcohol, benzylether, cyclohexanone, and ethylacetate were not tolerated. Subsequently, we investigated the correlation between the log P value of the OSs and growth on the OSs using the OS-tolerant strains DS-711 and IH-2000.

Table 8.2.7

Comparison of organic solvent-tolerant between strain DS-711 and soil strains

Solvent (log P)	Growth (OD_{660})			
	Strain DS-711	*P.putida* IH-2000	*E.coli* IFO 3806	*Bacillus.subtilis* IFO 3009
No addition	1.36	2.98	3.05	2.95
Benzene 1% (2.1)	1.33	NG	NG	NG
Benzene 5%	1.08	NG	NG	NG
Toluene 10% (2.7)	1.18	2.88	NG	NG
p-Xylene 10% (3.1)	1.06	2.64	NG	NG

Growth was determined by measuring the optical density at 660 nm.
ND, no degradation; NG, no growth

Degradation of *n*-alkanes in kerosene and aromatic hydrocarbons by strain DS-711 was compared with that of the control strains, *Alteromouas* sp. DS-201 and *P. putida* IH-2000. To measure their ability to degrade *n*-alkanes in kerosene and aromatic hydrocarbons, one loop of strains was inoculated in 5,000-ml flasks containing 1,000 ml M-II salt medium and LB-I medium. Incubation was carried out for 2 days at 30°C. After incubation the cultures were centrifuged at 12,000 rpm and washed two times with artificial seawater and sterile distilled water to prepare a 15 ml concentrated cell suspension. The cell suspensions were inoculated into 100 ml fresh M-II salt medium and LB-1 medium at an initial concentration of 1×10^7 or 1×10^9 cells/ml and 10 ml (10% v/v) of kerosene and 1 ml (1% v/v) of aromatic hydrocarbon were overlaid individually. When the stepwise addition of the concentrated cell suspension was complete, the suspensions were added after 2 days and 3 days. An additional no inoculated control was run for measurement through evaporation. Inoculated samples and the additional control were incubated with shaking at 100 rpm for a week at 30°C. At the end of the incubation, cultures were transferred to separating funnels for extraction and analysis of hydrocarbons. The percentage degradation of hydrocarbons is given as the difference between the additional control and the inoculated samples. The total *n*-alkane content was made up of the total content of *n*-alkanes having 7–16 carbon atoms. Cultures on kerosene and aromatic hydrocarbons were centrifuged. Supernatants were transferred to separating funnels for hydrocarbon extraction. Residual kerosene and aromatic hydrocarbon oil droplets were separated from the lower aqueous layer. Then 50 ml of benzene containing 0.1 g of *n*-eicosane as an internal standard was added to the kerosene oil droplets. Kerosene was extracted with benzene as described in detail elsewhere (Walker and Colwell 1974; Walker et al. 1975). Residual aromatic hydrocarbon was extracted with 4-ml of *n*-decane. Any remaining water was removed by drying over Na_2SO_4. Anhydrous extracts were transferred to a vial and stored at −20°C until gas chromatography (GC) analysis was performed.

As shown in *Table 8.2.8*, all the strains used in this experiment could degrade *n*-alkanes in kerosene but did not degrade aromatic hydrocarbons. However, *n*-alkane degradation by strain DS-711 was elevated remarkably compared to that of the control strains, DS-201 and IH-2000. In particular, percentage degradation of *n*-decane and *n*-undecane by both of control strains was approximately 10%, whereas by the strain DS-711 it was 71.4% and 68%, respectively. DS-711 showed the highest *n*-alkane degradation among the strains used, with the degradation of *n*-alkanes by the bacterium reaching approximately 85% after 7 days.

The deep-sea bacterial strain *Flavobacterium* sp. DS-711, which was isolated by our novel, simple, efficient method, exhibited excellent benzene tolerance.

Because microorganisms that are able to grow in the presence of 5% benzene have not yet been found, it will be of interest to compare the cell surface components of the benzene-tolerant strain DS-711 with those of strain IH-2000. We tried to isolate a benzene-tolerant plasmid from DS-711, but no plasmid was obtained. As strain DS-711 harbors no plasmid DNA, the OS tolerance of this bacterium is thought to be dominated by information in genomic genes. Generally, Gram-negative bacteria show higher OS tolerance than Gram-positive ones (Inoue and Horikoshi 1989, 1991a). At present, we are investigating the mechanism of OS tolerance from two aspects: cell surface components and genetics. The discovery of a benzene-tolerant bacterium from the deep-sea is also valuable from the view point of microbial ecology.

Subsequently, hydrocarbon degradation by the benzene-tolerant DS-711 was compared with that of the control strains, DS-201 and the toluene-tolerant IH-2000. As shown in *Table 8.2.8*, with a shaking culture the degradation of *n*-alkanes in kerosene by the

Table 8.2.8

Hydrocarbon degradation by solvent-tolerant strains

Hydrocarbon	Degradation (%)		
	P.putida IH-2000	*Alteromonas* sp. DS-201	DS-711
n-Alkanes			
n-C_7	28	30.5	97
n-C_8	30.9	30.3	82.1
n-C_9	35.7	32.4	82.1
n-C_{10}	10.6	9.7	71.4
n-C_{11}	11.8	10.8	68
n-C_{12}	40.2	41.4	86.7
n-C_{13}	46.2	45.3	85.8
n-C_{14}	39.9	44.6	88
n-C_{15}	35.7	39	92.9
n-C_{16}	40.2	38.1	80.5
Aromatic hydrocarbons			
Benzene	NG	NG	0
Toluene	0	NG	0
p-Xylene	0	NG	0

Degradation (%) was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\text{Residual hydrocarbon conc. of an addtional control} - \text{Residual hydrocarbon conc. of inoculated samples}}{\text{Residual hydrocarbon conc. of an additional control}} \times 100$$

The residual *n*-alkane contents (mg) in 10 ml kerosene after treatment of additional control are : *n*-C_7, 34; *n*-C_8, 39; *n*-C_9, 190; *n*-C_{10}, 245; *n*-C_{11}, 270; *n*-C_{12}, 653; *n*-C_{13}, 501; *n*-C_{14}, 332; *n*-C_{15}, 188, *n*-C_{16}, 39.
NG, no growth.

benzene-tolerant DS-711 was greater than that of our selected control strains DS-201, and IH-2000. In particular, degradation of *n*-decane and *n*-undecane, which were hardly degraded by the control strains, were markedly elevated. However, these strains, including DS-711 showed a percentage degradation of *n*-decane and *n*-undecane lower than that of other *n*-alkanes such as *n*-heptane, *n*-octane, *n*-nonane, *n*-dodecane, *n*-tridecane, *n*-teiradecane, *n*-pentadecane, and *n*-hexadecane. This was thought to be due to lower activity of the emulsifying and solubilizing substance in regard to *n*-decane and *n*-undecane in kerosene. A study of the relationship between hydrocarbon degradation and hydrocarbon emulsification and solubilization is now in progress.

We are examining the identification and characterization of the hydrocarbon emulsifying and solubilizing substance in addition to elucidating OS tolerance and hydrocarbon degradation. Interestingly, neither DS-711 nor IH-2000 degraded aromatic hydrocarbons, such as benzene, toluene, and *p*-xylene. Hydrocarbon degradation is associated with assimilation. Accordingly, these results indicated that tolerance to aromatic hydrocarbons, such as benzene, toluene, and *p*-xylene, is distinguishable from tolerance to *n*-alkanes at least. The *n*-alkane

degrading ability of this bacterium will be useful for the bioremediation of oil spills, especially marine oil spills. Because of the OS tolerance of the bacterium is also available for commercial application in industrial biotransformation processes that involve the use of water insoluble organic substrates, such as biocatalysis in a water-OS two-liquid-phase system, discovery of the benzene-tolerant strain DS-71 1 is worthy of note.

Benzene-Tolerant Sulfur-Degrading Bacteria

We attempted to isolate an OS-tolerant bacterium from the deep-sea to degrade sulfur compounds in the presence of OS (Moriya and Horikoshi 1993b). Deep-sea sediment slurry samples were aseptically collected by sterile core samplers from a depth of 1,168 m in Sagami Bay. Screening the OS-tolerant sulfur degrading bacteria was done by a modification of the method described previously (Moriya and Horikoshi 1993a). Benzene (5 ml) was added to 5 ml of deep-sea sediment slurry, which was then incubated at 25°C for 7 days. After this preincubation the benzene layer was separated from the seawater layer, and the former was spread onto M-II agar medium (Moriya and Horikoshi 1993a) with 5.8% NaCl, 1% $Na_2S_2O_3$, and 0.002% phenol red. The plates were incubated at 37°C for 7 days. The sulfur degrading ability could be detected directly on the plates because of the clear zone that forms around colonies that produce sulfuric acid from $Na_2S_2O_3$, caused by a pH decrease. Thus, microorganisms in which a clear zone formed around colonies were selected and purified. One was designated strain DS-994. This bacterium was gram-positive, spore forming, aerobic, catalase-positive, oxidase-negative, gelatin-liquefying, and motile. It is rod-shaped (0.3–5 × 0.6–3 μm) and has flagella. From its morphological and physiological properties and its characteristics of halophilic growth, sulfur utilization, and OS tolerance, DS-994 seems to be a variant strain of *Bacillus* sp. Strain DS-994 displayed optimal growth at an initial pH 7, NaCl concentration of 12%, and 37°C.

To investigate the influence of OSs on the growth of strain DS-994, LB-II medium which contained 5 g of yeast extract (Difco,USA), 10 g of tryptone (Difco), 1 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter of distilled water was used. The initial pH of the media was adjusted to 7, by NaOH. When incubated for 5 days, the strain showed good growth in the presence of 5% benzene, 10% toluene, and 10% *p*-xylene, respectively. In this OS tolerance experiment, *E. coli* strain IFO 3806, *B. subtilis* strain IFO 3009 and a representative OS-tolerant *P. putida* strain IH-2000 (Inoue and Horikoshi 1989, 1991a) were employed as controls and incubated on LB-I medium and LB-II medium supplemented with 0.5% NaCl in the presence of OS.

The *E. coli* and *B. subtilis* strains did not grow in the presence of any OS, where as the *P. putida* strain did grow in the presence of toluene and *p*-xylene but not benzene (*Table 8.2.9*).

Subsequently, the effect of benzene on the halophilic growth of this bacterium was further examined; it showed growth in the range of 0.01%–29.50% NaCl without influence from 5% v/v benzene. The toxicity of benzene (log P 2.1) is greater than that of the other organic solvents used in this study (toluene log P 2.7, *p*-xylene log P 3.1). Generally, Gram-positive bacteria have lower OS tolerance than Gram-negative ones (Inoue and Horikoshi 1989, 1991a). Therefore, the benzene tolerance of the Gram-positive strain DS-994 is particularly significant. Inoue and Horikoshi (1989, 1991a) also showed that OS-tolerant microorganisms, including the toluene-tolerant *P.putida* strain IH-2000, can grow in the presence of OSs whose log P is

Table 8.2.9

Bacterial growth on organic solvents

Organic solvent	Growth (OD_{660})			
	Bacillus sp. DS-994	*P.putida* IH-2000	*E.coli* IFO 3806	*B.subtilis* IFO 3009
No addition	+ (3.1)	+ (3.8)	+ (3.5)	+ (3.6)
Benzene 5%	+ (2.5)	–	–	–
Toluene 10%	+ (2.6)	+ (3.7)	–	–
p-Xylene 10%	+ (3)	+ (3.8)	–	–

Bacterial growth was determined by measuring the optical density at 660 nm, represented by the numbers in parentheses. Soil strains and strain DS-994 were incubated for 5 days at 37°C on LB-I and LB-II medium, respectively. +, growth; –, no growth.

higher than the specific value. However, our previous study (Moriya and Horikoshi 1993a) indicated that the mechanism of OS tolerance of a. benzene-tolerant bacterium was different from that of the toluene-tolerant *P.putida* strain IH-2000. Accordingly, investigations into the tolerance of strain DS-994 to various kinds of OSs, the mechanism of its OS tolerance, and the differences of OS tolerance between Gram-positive and Gram-negative bacteria are now in progress.

To confirm sulfuric acid production from $Na_2S_2O_3$ by strain DS-994, incubation was done on LB-II medium supplemented with 12% NaCl and 10% $Na_2S_2O_3$. When a 1-day fresh culture of DS-994 was inoculated into the medium and cultured aerobically for 10 days under the optimal growth conditions, the strain showed good growth, and the initial pH value of the culture broth gradually decreased below pH 5. Sulfuric acid from sulfur compounds was measured qualitatively by high-performance liquid chromatography (HPLC). The total sulfur content of organic sulfur compounds was measured on a Perkin-Elmer organic elemental analyzer 2400 II CHNS/O Analyzer (Perkin-Elmer, GmbH, Uberlingen, Germany). From the results of HPLC analysis of the culture broth at the end of the cultivation it was clear that strain DS-994 produced sulfuric acid from $Na_2S_2O_3$, and the sulfuric acid production seemed to cause the decrease of pH.

Because strain DS-994 showed OS tolerance and the ability to produce sulfuric acid from $Na_2S_2O_3$, its sulfur-degrading ability was further investigated in the presence of kerosene with or without 5% benzene. Kerosene and diesel oil were used as the model petroleum. When needed, the model petroleum was supplemented with OSs at the concentrations indicated. Strain DS-994 showed good growth in the presence of kerosene. Furthermore the results of HPLC analysis indicated that DS-994 changed the sulfur of $Na_2S_2O_3$ to sulfuric acid in the presence of kerosene with or without 5% benzene. When kerosene was supplemented with 10% of other organic solvents, sulfuric acid production was also observed. Thus, strain DS-994 has great ability to degrade $Na_2S_2O_3$ as an inorganic sulfur compound in the presence of an organic solvent.

To investigate its ability to utilize organic sulfur compounds such as dibenzothiophene, thiophene, and ethylmethyl sulfide, which are present in petroleum, strain DS-994 was incubated in a water-model petroleum two-liquid-phase system. Dibenzothiophene (DBT), thiophene (T), and ethylmethyl sulfide (EMS) were used as exogenous organic sulfur

Table 8.2.10

Utilization of organic sulfur compounds by strain DS-994

Organic sulfur compound	Total sulfur concentration (%)		
	Control 1	Control 2	Sample
Dibenzothiophene	0.2 (0)	0.195 (2.5)	0.175 (12.5)
Thiophene	0.2 (0)	0.195 (2.5)	0.180 (10)
Ethyl methyl sulfide	0.2 (0)	0.190 (5)	0.170 (15)

Numbers in parentheses represent the percent utilization.

compounds; 0.2% of each organic sulfur compound was added to the model petroleum as total sulfur content. Sterilized seawater (4.5 ml) was employed as the water layer. One-day fresh culture of DS-994 (500 ml) was centrifuged and washed two times with sterilized seawater. The concentrated cell suspension was prepared with 10 ml of sterilized seawater. To the water layer, 0.5 ml of concentrated cell suspension was added at approximately 1×10^9 cells/ml. Finally 5 ml diesel oil with or without 5% OS was overlaid above the water layer. Two additional controls were run: (1) without inoculation and (2) with organic sulfur compounds suspended in the water layer without model petroleum and OS. When incubated for 10 days under optimal growth conditions, strain DS-994 showed good growth on LB-II medium with 12% NaCl. As shown in *Table 8.2.10*, the initial total sulfur concentrations of DBT, T and EMS in the diesel oil decreased to 0.175%, 0.18%, and 0.17%, respectively. The percent sulfur degradation of the organic sulfur compounds solubilized in the diesel oil was higher than that of control 2. In this experiment, no difference in the percent utilization of organic sulfur compounds solubilized in the diesel oil was observed with or without OS.

Furthermore, when vigorous shaking was applied, marked emulsification of the model petroleum was observed. Probably these results indicated that strain DS-994 was in contact with diesel oil droplets, which dispersed in the growth medium and oxidized the sulfur component to sulfuric acid without significant influence of the OS in the model petroleum. Thus, sulfur degradation by this OS-tolerant bacterium was observed in a water-model petroleum two-liquid-phase system.

Organic Solvent-Tolerant Marine Cholesterol-Degrading Bacterium

Microbial cholesterol conversion occurs in aqueous media even when the reactants, such as steroids, are barely soluble in water. The reactants are often in a solid state in such media, and so the reaction rate is usually hindered by the limited availability of the substrate. For industrial microbial cholesterol degradation processes, some detergents, such as Tween and Span, have been mainly used to (1) form stable suspensions of the water-insoluble substrate during fermentation and (2) prevent simultaneous formation of solid particles. Although this detergent addition helps to increase the degradation rate, it does not prevent the simultaneous process of solid particle formation. Accordingly, as a reactant reservoir to keep the product and the substrate in a soluble form until the degradation is complete, the use of OSs is thought to be a logical way to prevent simultaneous formation of the solid particles.

We attempted cholesterol degradation in a water-OS two-liquid-phase system by an OS-tolerant marine bacterium isolated from the deep-sea (Moriya et al. 1995).

Screening OS-tolerant bacteria that possessed the ability to degrade cholesterol was done by modifying our previously described method (Moriya and Horikoshi 1993a). Deep-sea sediment slurry sample were aseptically collected by sterile core samplers from 1,963 m depth of the Okushiri Ridge. The samples (100 ml) were treated with the addition of 100 ml of benzene and kept for 7 days at ambient temperature. After treatment, the upper benzene layer was spread onto M-II agar medium (Moriya and Horikoshi 1993a) supplemented with 5% NaCl and 0.1% cholesterol. Incubation was for 5 days at 30°C. Fifty colonies that formed clear zones due to cholesterol degradation on M-II agar medium were picked up and purified as candidates for benzene-tolerant cholesterol degraders. The candidate that showed the best growth and cholesterol degradation in the presence of 5% (v/v) benzene containing cholesterol 1 mg/ml was selected and tentatively designated strain ST-1. This isolate was Gram-positive, non-spore-forming, strictly aerobic, catalase-positive, oxidase-positive, and nonmotile. It had a typical rodoccus growth cycle. Its cell wall peptidoglycan contains lysine. Optimal growth was at pH 6–8.6 and 35°C, respectively. The mole percent of C+G is 62. Based on the above morphological and biochemical characteristics (Jones and Collins 1984), ST-1 seemed to be a strain of *Arthrobacter* sp.

To compare the OS tolerance and cholesterol conversion ability of the selected isolate, *Arthrobacter symprex* strain ATCC 6946, *Flavobacterium dehydrogenans* strain ATCC 13930, *Mycobacterium* sp. ATCC 29472, and *Mycobacterium smegmatis* strain ATCC 12549 were employed as control cholesterol degraders. Strains ATCC 13930 (Bocren and Laane 1987) and ATCC 29472 (Flygare and Larsson 1987) were used as representative androstene producers. These five strains were individually inoculated into each 10 ml of modified M-II medium (Moriya and Horikoshi 1993a) at approximately 1×10^6 cells/ml and then incubated in the presence of 0.5 ml of various OSs with vigorous shaking for 3 days at 30°C. Six solvents including aromatic hydrocarbons benzene, toluene, and *p*-xylene and the aliphatic hydrocarbons *n*-hexane, *n*-decane, and *n*-dodecane, were used to measure the tolerance to and the effect of these OSs in a 1:20 (v/v) OS-water biphasic system. After incubation, cell growth was determined by measuring viable cell concentration by M-II and LB-II agar plate counts.

As shown in *Table 8.2.11*, the control strains could grow in either *n*-decane (log P 6.0) or *n*-dodecane (log P 7.0) but could not grow in the presence of *n*-hexane (log P 3.9) or aromatic hydrocarbons. In contrast to these controls, strain ST-1 could grow in benzene, toluene, *p*-xylene (log P 3.1), or *n*-hexane in addition to *n*-decane and *n*-dodecane. The control strains and strain ST-1 grew in the presence of 50% (v/v) *n*-decane and *n*-dodecane. Strain ST-1 also grew even in the presence of 10% (v/v) toluene, 10% *p*-xylene, and 10% *n*-hexane in addition to 5% benzene. The final viable cell concentration of strain ST-1 reached approximately 3×10^9 cells/ml with and without OSs. Strain ST-1 was halotolerant and showed good growth in the presence of OS without any effects of NaCl in the range 0–12%. The cholesterol degradation of strain ST-1 and control stains in a buffer-OS two-liquid-phase system was examined. Both cholesterol and androsta-1,4-diene-3,17-dione (ADD), which is one of the androstenes derived from cholesterol, were measured by HPLC. Three additional controls were run: (1) without inoculation; (2) with cholesterol suspended in buffer layer without OS; and (3) with cholesterol suspended in buffer containing 0.1% Tween 80.

The results of a batch experiment are shown in *Table 8.2.12*. When strain ST-1 was incubated in the presence of benzene, toluene, *p*-xylene, or *n*-hexane, the degradation of the initial cholesterol (1 mg/ml) was 22%, 22%, 24%, and 56%, respectively. In the experiments with control 2 and control 3, the cholesterol degradation was 68% and 78%, respectively. In these experiments we also detected ADD produced from cholesterol qualitatively. Therefore,

Table 8.2.11

Growth in the presence of various solvents

Strain	Organic solvents					
	Benzene (log P 2.1)	Toluene (log P 2.9)	*p*-Xylene (log P 3.3)	*n*-Hexane (log P 3.9)	*n*-Decane (log P 6.0)	*n*-Dodecane (log P 7.0)
ST-1	+	+	+	+	+	+
Arthrobacter symplex ATCC 6946	–	–	–	–	+	+
Flavobacterium dehydrogenans ATCC 13930	–	–	–	–	+	+
Mycobacterium sp. ATCC 29472	–	–	–	–	+	+
M. smegmatis ATCC 12549	–	–	–	–	+	+

Cell growth was determined by measuring viable concentration with and without organic.
+, growth; –, no growth.

Table 8.2.12

Cholesterol degradation in two-liquid phase system

Strain	Cholesterol degradation (%)				
	ST-1	*Arthrobacter symplex* ATCC 6946	*Flavobacterium dehydrogenans* ATCC 13930	*Mycobacterium* ATCC 29472	*Mycobacterium smegmatis* ATCC 12549
Water (control 2)	68 (22)	44	52 (12)	49 (10)	53
Tween (control 3)	78 (40)	55	60 (18)	59 (19)	62
Two-liquid system					
Benzene	22 (9)	5	7 (ND)	5 (ND)	4
Toluene	22 (9)	7	9	7	4
p-Xylene	24 (10)	8	9	8	6
n-Hexane	56 (54)	20	38 (44)	32 (40)	38
n-Decane	88 (70)	59	66 (60)	66 (62)	69
n-Dodecane	92 (78)	67	74 (65)	73 (66)	77

Values in parentheses indicate the percent production of anbrosta-1,4-dione –3.17 – dione (ADD) derived from cholesterol.
ND, not detected.

this bacterium had the ability to cleave the side chain of cholesterol presumably due to the activity of a cholesterol dehydrogenase. In contrast, the control strains could scarcely degrade cholesterol in the presence of aromatic hydrocarbons but could degrade cholesterol in an aqueous medium and in a medium supplemented with either *n*-hexane, *n*-decane, or

n-dodecane. However, the percentages of both cholesterol degradation and ADD production by the control strains were lower than those obtained by strain ST-1 in aqueous media and a two-liquid-phase system. When the batch experiment was successively repeated five times using *n*-dodecane, cholesterol degradation was 92% and 90% in the first and second batches, respectively. The degradation efficiency of cholesterol decreased gradually in the third to fifth batches. Changes in the viable cell concentration of the five strains used for batch experiments were measured by conventional agar-plate counts at daily intervals. When incubated in the presence of benzene, toluene, or *p*-xylene, 1×10^9 cells/ml of the initial viable cell concentration of strain ST-1 decreased gradually to approximately 3×10^8 cells/ml after a day and then remained constant. However, the viable cell concentrations of control strains decreased abruptly within a day, and no colony formation was observed on the agar plates after 2 days. When incubated in *n*-hexane, the cell viability, of strain ST-1 was more than 50%, but that of controls was less than 20% at the end of the incubation. After incubation in aqueous media and either *n*-decane or *n*-undecane, all strains tested remained at the initial viable cell concentration.

The marked effect of organic solvent on cholesterol degradation and ADD production might stem from the differences in cell viability. In general, a positive correlation is found between the hydrophobicity of organic solvents and the nontoxicity for the biocatalyst. Organic solvents with a log P value above 4 are highly hydrophobic and generally show no toxic effects on biocatalysts. The log P value is a measure for hydrophobicity suitable for characterization of organic solvents. Accordingly, biocatalysis derived from aromatic hydrocarbon-tolerant microorganisms are of interest for further study. Enzymes associated with cholesterol degradation of strain ST-1, which shows tolerance to aromatic hydrocarbons such as benzene, toluene, and *p*-xylene, are also an attractive object to study in detail.

OS-Tolerant Polyaromatic Hydrocarbon-Degrading Bacteria

We have focused on the deep-sea environment to obtain an OS-tolerant bacterium that possesses the useful characteristics for application in industry (Abe et al. 1995). We describe the polyaromatic hydrocarbon-degrading ability of an OS-tolerant bacterium isolated from the deep-sea.

Screening of OS-tolerant polyaromatic hydrocarbon-degrading bacteria was carried out by a slight modification of the method described previously (Moriya and Horikoshi 1993a). Deep-sea sediment slurry samples were aseptically collected from a depth of 1,168 m in Sagami Bay by the manned submersible Shinkai 2000 in 1992. Deep-sea sediment slurry (5 ml) was transferred to flasks containing 10 ml of artificial seawater. Benzene was overlaid on the aqueous layer (1:1), and these flasks were then closed with silicone-rubber stoppers. The cultures were incubated with shaking at 200 rpm for a week at 30°C. After incubation the benzene layer separated from the artificial seawater, and a portion of the benzene layer was spread directly onto plates of M-II and LB-II media (Moriya and Horikoshi 1993a, b) containing 1% naphthalene. The plates were. incubated for a week at 30°C. Microorganisms that formed clear zones around their colonies were selected and purified.

The isolate was selected as a polyaromatic hydrocarbon degrader that also exhibited organic solvent tolerance; it was named strain DS-1906. Strain DS-1906 was Gram-positive, spore forming, aerobic, oxidase and catalase-positive, gelatin-liquefying, and nonmotile. This strain also grew well at a wide range of pH 5–9, and optimum pH for growth was pH 5–6.

It grew well at NaCl concentrations over the range of 0–0.5 M. Based on the biochemical and morphological characteristics, in addition to its polyaromatic hydrocarbon-degrading ability and organic solvent tolerance, this strain appeared to be a strain of *Bacillus* sp.

The OS tolerance of the strain DS-1906 was compared with that of control strains *E. coli* IFO 3806, *B. subtilis* IFO 3009, and the toluene-tolerant. *P. putida* IH-2000. Various hydrocarbons, includin.g benzene, 1-heptanol, toluene, styrene, *p*-xylene, ethylbenzene, *o*-dichlorobenzene, *n*-propylbenzene, *n*-hexane, diphenylether, cyclooctane, isooctane, *n*-octane, *n*-hexylether, *n*-nonane, *n*-decane, and *n*-dodecane, were employed as organic solvents. Naphthalene, fluorene, phehanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene were used as polyaromatic compounds. The results are shown in *Table 8.2.13*. When incubated for 3 days, the *E.coli* and *B.subtilis* strains did not grow in the presence of any of the organic solvents tested. *P. putida* strain IH-2000 grew in the presence of all the organic solvents except for benzene. In contrast to these strains, strain DS-1906 grew even in the presence of 10% benzene and some other solvents, but it could not grow in the presence of many other OSs, such as 1-heptanol, toluene, styrene, *p*-xylene, and cyclooctane.

Table 8.2.13

Comparison of organic solvent tolerance between strain DS-1906 and soil strains

Solvent	Tolerance				
	log P	*E.coli* IFO 3806	*B.subtilis* IFO 3009	*P.putida* IH-2000	DS-1906
n-Dodecane	7.0	–	–	+ (1.90)	+ (2.42)
n-Decane	6.0	–	–	+ (1.90)	+ (2.40)
n-Nonane	5.0	–	–	+ (1.88)	+ (2.35)
n-Hexylether	5.1	–	–	+ (1.90)	+ (2.30)
n-Octane	4.9	–	–	+ (1.83)	+ (2.30)
Isooctane	4.8	–	–	+ (1.80)	+ (2.27)
Cyclooctane	4.5	–	–	+ (1.80)	–
Diphenylether	4.2	–	–	+ (1.80)	+ (2.25)
n-Hexane	3.9	–	–	+ (1.78)	+ (2.25)
n-Propylbenzene	3.8	–	–	+ (1.75)	–
o-Dichlorobenzene	3.6	–	–	+ (1.75)	+ (2.10)
Cyclohexane	3.4	–	–	+ (1.70)	–
Ethylbenzene	3.3	–	–	+ (1.70)	–
p-Xylene	3.1	–	–	+ (1.65)	–
Styrene	2.9	–	–	+ (165)	–
Toluene	2.8	–	–	+ (1.58)	–
1-Heptanol	2.4	–	–	+ (1.53)	–
Benzene	2.0	–	–	–	+ (1.80)

The initial cell concentration was 1×10^5 cell/ml. Incubation was for 3 days at 30°C. Growth was determined by measuring the optical density at 660 nm (OD_{660}).
+, growth (>5×10^8 cells/ml); –, no growth.

Degradation of naphthalene by DS-1906 was compared with that by the control strains in a medium-OS two-liquid-phase system. Bacteria were incubated in 9 ml of LB-II medium with 1 ml of OSs containing 1% naphthalene. Incubation was for 1 week at 30°C with shaking at 200 rpm. At the end of the incubation, the remaining polyaromatic hydrocarbons were extracted and analyzed by gas chromatograph. The percent degradation of polyaromatic hydrocarbons was given as a difference between the control and the inoculated samples.

We observed a 1-day latency period before growth in the presence of *n*-hexane, and then DS-1906 grew rapidly. After the 1-day of latency period, residual naphthalene decreased gradually. After 7 days the value of residual naphthalene reached about 50%. As shown in *Table 8.2.14*, the control strains did not degrade polyaromatic hydrocarbons, whereas that by strain DS-1906 was 48.3% in the presence of *n*-hexane.

Furthermore, strain DS-1906 could degrade various polyaromatic compounds, such as fluorene, phenanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene in a medium-OS (benzene) two-liquid-phase system (*Table 8.2.15*).

When polyaromatic hydrocarbons were solubilized in an organic solvent, the percent degradation was kept at a constant value, in contrast to the highly variable degradation in the absence of an organic solvent. The percent degradation in the presence of an organic solvent was higher than that obtained from cultures in which polyaromatic hydrocarbons were suspended in the medium without an organic solvent.

Table 8.2.14

Comparison of naphthalene degradation in the different OS by solvent-tolerant strains DS-1906 and soil strains

Solvent	Degradation (%)			
	E.coli IFO 3806	*B.subtilis* IFO 3009	*P.putida* IH-2000	DS-1906
n-Dodecane	NG	NG	NG	ND
n-Decane	NG	NG	NG	ND
n-Octane	NG	NG	NG	33
Isooctane	NG	NG	NG	28
Cyclooctane	NG	NG	NG	48
Diphenylether	NG	NG	NG	26
Toluene	NG	NG	NG	NG
Benzene	NG	NG	NG	17.2
No OS	NG	NG	NG	11.7–35.5

Degradation of naphthalene was examined in the medium-organic solvent (OS) two-liquid phase system. Bacteria were incubated in 9 ml of LB-II medium containing 1 ml of organic solvent (10%) containing 1% naphthalene for 7 days at 30°C.

Degradation was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\text{Residual polyaromatic hydrocarbons conc. of an additional control} - \text{Residual polyaromatic hydrocarbons conc. of inoculated samples}}{\text{Residual polyaromatic hydrocarbons conc. of an additional control}} \times 100$$

NG, no growth; ND, no degradation.

Table 8.2.15

Polyaromatic hydrocarbon degradation by solvent-tolerant strains DS-1906

Substrates	Degradation (%)	
	Without OS	With OS
Naphthalene	11.7–35.5	17.2
Fluorene	10.5–32	17.4
Phenanthrene	10.2–28.7	16.8
Anthracene	2.6–28.5	15.2
Pyrene	2.3–26.7	13.2
Chrysene	3–25.8	14.5
1,2, -Benzopyrene	2.7–24.8	12.6

Degradation was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\text{Residual polyaromatic hydrocarbons conc. of an additional control} - \text{Residual polyaromatic hydrocarbons conc. of inoculated samples}}{\text{Residual polyaromatic hydrocarbons conc. of an additional control}} \times 100$$

NG, no growth; ND, no degradation; OS, organic solvent (benzene).

When cultures were shaken, marked emulsification of the organic solvent was observed. This result possibly indicated that thy OS-tolerant bacterium was in contact with organic solvent dispersed in the medium, which could allow the bacterium to degrade the polyaromatic hydrocarbon substrates efficiently.

Although we tried to isolate OS-tolerant plasmids from this OS-tolerant bacterium, no plasmids were obtained. Accordingly, the OS tolerance is thought to be encoded by information in genomic genes.

Organic Solvent Hydrocarbon-Degrading Marine Yeast

We have reported the isolation, characteristics, and applications such as the degradation of polluting petroleum by microorganisms that tolerate organic solvents (Aono et al. 1991, 1992; Nakajima et al. 1992; Moriya and Horikoshi 1993a, b; Fukumaki et al. 1994). Some such microorganisms can degrade hydrocarbons and organic sulfur compounds in the presence of organic solvents (Moriya and Horikoshi 1993a, b).

We attempted to isolate and investigate marine yeast that tolerates organic solvents and explore its hydrocarbon-degrading ability. Deep-sea sediment samples were collected aseptically with sterile core samplers from a 1,200 m depth in Sagami Bay by a manned submersible vessel, the Shinkai 2000. For yeast growth, YPG medium, which contained 10 g of yeast extract (Difco), 20 g of Bacto-peptone (Difco), and 10 g of glucose per liter of distilled water; YPTC medium, which contained 0.5 g of yeast extract, 4 g of bactorpeptone, 1 g of trypticase peptone (BBL), 0.5 g of glucose, 3 g of $(NH_4)SO_4$, 0.1 g of K_2HPO_4, 0.5 g of $Mg_2SO_4 \cdot 7H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, and 30 g of NaCl per liter of distilled water; and yeast nitrogen base (YNB) medium (Difco) were used. For YPTC agar medium, agar was added at a concentration of 2% (w/v) to

YPTG medium. When needed, both penicillin C potassium (Wako, Osaka, Japan) and streptomycin sulfate (Nacalai Tesque, Kyoto, Japan) were added to these media at 0.010% each, and the pH was adjusted to 2.5.

For isolation of yeasts that could tolerate organic solvents, the deep-sea sediment samples were treated with 50% (v/v) *n*-hexane for 2 days by a slight modification of the described elsewhere method (Moriya and Horikoshi 1993a). Some organic solvent was spread directly on YPTG agar medium with penicillin and streptomycin. Incubation was at 20°C for 1 week. After incubation, yeast-like colonies growing on the medium were picked up and purified.

Altogether 43 yeast strains were isolated and purified from deep-sea sediment sample treated with *n*-hexane. Tolerance of organic solvents was examined on YPG medium. *n*-dodecane, *n*-decane, *n*-nonane, hexyl ether, *n*-octane, isooctane, cyclooctane, diphenylether, *n*-hexane, and kerosene were the organic solvents used.

One of these deep-sea isolates, strain Y-40 tolerated the aliphatic hydrocarbons *n*-dodecane, *n*-decane, *n*-nonane, *n*-octane, isooctane, and cyclooctane and the ethers, hexyl ether and diphenyl ether. This strain could grow even in the presence of 50% (v/v) organic solvents (*Table 8.2.16*). Some other deep-sea isolates and some type strains grew in the presence of diphenyl ether, which had the lowest log P value of the solvents used; but they could not grow in cyclooctane. Accordingly, we selected strain Y-40 as a possible hydrocarbon degrader. These yeast cells were short ovals and formed budding cells during growth in YPG medium. The strain fermented only D-glucose of the sugars tested. It grew in YNB medium, which contained trehalose, L-rhamnose, ribitol, D-mannitol, D-glucitol, or glycerol as the sole carbon source. Based on its morphological and biochemical characteristics, it seemed to be a variant strain of *Candida*. Strain Y-40 could not grow in YNB medium containing any of the organic solvents tested as the sole carbon source.

Table 8.2.16

Tolerance of organic solvents by marine yeast strain Y-40

Solvents	Concentration (%)	Cell growth (OD_{660})
None	–	21.5
n-Octane	10	16.3
	25	9.4
	50	20.1
Isooctane	10	22.2
	25	19.1
	50	17.4
Cyclooctane	10	12.7
	25	7.9
	50	7
Kerosene	10	20.5
	25	14.1
	50	17.8

After incubation at 30°C for 24 h, yeast growth was evaluated by measurement of the OD_{660}.

The ability of yeasts to assimilate *n*-alkanes and organic solvents was examined with 5 ml of YNB medium to which *n*-undecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, or one of the organic solvents listed above was added at 0.5% as the sole carbon source. Incubation was at 30°C. Strains that grew within a week were regarded as being hydrocarbon assimilators.

Degradation of *n*-alkanes in kerosene was examined in YPG medium under a mixture of organic solvents. A 10 µl aliquot from a 1-day culture was used to inoculate 4 ml of YPG medium in a test tube; 1 ml of an organic solvent that contained 10% kerosene was then overlaid on the medium. *n*-octane, isooctane, and cyclooctane were the organic solvents used. The control with no organic solvent and the other control without inoculation were used. Incubation was for 2 days at 30°C with shaking at 200 rpm. At the end of the incubation, the *n*-alkanes remaining in the kerosene were extracted and analyzed. The amount of degraded *n*-alkanes was recorded as the difference between one of the controls and the inoculated sample or control.

For a comparison of the hydrocarbon-degrading ability of strain Y-40 with those of two strains that assimilate *n*-alkane (*C. tropicalis* IFO 1400 and *Yarrowia lipolytica* IFO 1548), kerosene was used because it contains a mixture of *n*-alkanes. The results of degradation of *n*-alkanes are shown in *Table 8.2.17*. The three strains tested degraded *n*-alkane in the absence of an organic solvent. Of the three strains, *C.tropicalis* IFO 1400 degraded *n*-alkanes in kerosene without an organic solvent most. Neither *C.tropicalis* IFO 1400 nor *Y. lipolytipa* IFO 1548 degraded *n*-alkanes in the presence of any of the three organic solvents tested. Strain Y-40 degraded *n*-alkanes in the presence of *n*-octane. The amount of *n*-alkanes in kerosene with *n*-octane degraded by strain Y-40 was greater than that without *n*-octane. In the presence of isooctane or cyclooctane, strain Y-40 grew but did not degrade *n*-alkanes.

Table 8.2.17

Degradation of n-alkanes in organic solvents by yeasts

	n-Alkane degradation (mg)					
	Marin yeast Y – 40		*Candida tropicalis* IFO 1400		*Yarrowia lipolytica* IFO 1548	
n-Alkane	None	*n*-Octane	None	*n*-Octane	None	*n*-Octane
n-Nonane	0.59	1.04	1.20	ND	0.68	NG
n-Decane	1.15	1.92	2.40	ND	1.28	NG
n-Undecane	1.24	2.38	2.80	ND	1.34	NG
n-Dodecane	1.29	2.04	2.38	ND	0.85	NG
n-Tridecane	1.11	1.73	1.94	ND	0.94	NG
n-Tetradecane	0.65	1.02	1.12	ND	0.78	NG
n-Pentadecane	0.40	0.48	0.53	ND	0.30	NG
n-Hexadecane	0.12	0.16	0.24	ND	0.17	NG

Yeast was incubated in 4 ml of YPG with 1 ml (20%) octane containing 2% kerosene at 30°C for 3 days. The controls were incubated without *n*-octane.

ND, no degradation; NG, no growth.

Although *C.tropicalis* IFO 1400 and *Y. lipolytipa* IFO 1548 degraded n-alkanes in kerosene without other organic solvents, neither strain degraded *n*-alkanes in the presence of 20% (v/v) *n*-octane, isooctane, or cyclooctane, which have highly toxic effects on yeast growth. The degradation of *n*-alkanes in kerosene by strain Y-40 increased from 20% to 75% when *n*-octane was added at a concentration of 20% (v/v). The results showed that the metabolic system for *n*-alkanes in strain Y-40 was different from that in *C. tropicalis* IFO 1400 and *Y. lipolytipa* IFO 1548. The increase in *n*-alkane degradation by Y-40 might be due to induction of enzymes that degrade *n*-alkanes after the addition of *n*-octane. Strain Y-40 could grow in the presence of 20% (v/v) isooctane of cyclooctane but did not degrade *n*-alkanes in the presence of either organic solvent. This characteristic would be useful for the microbial conversion of water-insoluble compounds by fermentation in a two-phase system of organic solvent and aqueous medium.

Cross-References

- 8.3 Molecular Responses to Solvent Stress: Strategies for Living in Unpalatable Substrates
- 8.4 Genetics, Evolution and Applications

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8.3 Molecular Responses to Solvent Stress: Strategies for Living in Unpalatable Substrates

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_8.3,

Introduction

This review article covers some general conclusions reached in research studies with respect to a number of solvent-tolerant *Pseudomonas* strains. The seminal observation was performed by Inoue and Korikoshi 1989 when they described in Nature a bacterium, belonging to the genus *Pseudomonas*, which was able to thrive in the presence of high concentrations of toluene. This property of extreme tolerance to solvents makes this *Pseudomonas* strain the first extremophile identified as able to survive in the presence of highly toxic solvents. Following this seminal observation, other solvent-tolerant strains, such as *Pseudomonas putida* DOT-T1E, S12, GM1, and MTB6, were described as able to grow in the presence of highly toxic solvents such as *p*-xylene (log P_{ow} 3.15), styrene (log P_{ow} 3.0), octanol (log P_{ow} 2.92), and toluene (log P_{ow} 2.69) (Aono et al. 1992; Cruden et al. 1992; Huertas et al. 2000; Isken and de Bont 1996; Kim et al. 1998; Ramos et al. 1995; Weber et al. 1994). These microbes can reach high cell densities in culture medium in the presence of 0.3% (v/v) of the mentioned solvents, a concentration that kills most of the microbes we work with in our laboratories. This solvent-tolerant trait allows us to consider this set of *Pseudomonas* strains as extremophiles. Normally solvent toxicity is due to these chemicals dissolving in the cell membranes, disorganizing them and altering the electron flow. They also prevent ATP synthesis and irrevocably provoke cell death. Currently, there is an inherent interest in deciphering the basis for survival and growth of extremophile microbes in such harsh conditions, particularly for the exploitation of these microbes in the bioremediation of heavily-polluted sites and in the biotransformation of water-insoluble compounds into added-value products. The growing interest in biodegradation derives from the fact that many pollutants are toxic above a certain threshold, killing many living organisms. The use of solvent-tolerant microorganisms, provided with an arsenal of enzymes to deal with pollutants, represents a powerful tool for in situ pollutant removal. In the case of biotransformation, many substrates of interest are poorly soluble in water and the use of a double-phase system, made of water and an organic solvent for the production of high added-value chemicals is of great interest in *green chemistry*. Among some examples we can cite the use of solvent-tolerant microorganisms to produce catechols or to carry out biotransformation of aromatic hydrocarbons including nitro substituted ones (Ju and Parales 2006; Neumann et al. 2005, 2006; Ramos-González et al. 2003; Ruhl et al. 2009; Rojas et al. 2004; Verhoef et al. 2009; Wierckx et al. 2005).

Solvent tolerance is an innate character of these microorganisms; however, at the population level the number of microbes that survive a solvent exposure is influenced by the growth conditions; in other words, solvent tolerance is an adaptive process (Ramos et al. 1997, 1998). The specific experimental approach taken to confirm this hypothesis was to grow cells until they reached the exponential phase with citrate as a carbon source. Then the cultures were split in two halves; one was kept as a control and toluene was supplied through the gas phase to the other one. One hour later 0.3% (v/v) toluene was added to the cells and the number of cells that survived the solvent shock was determined. Cells pre-induced with low toluene concentrations survived well, whereas only a minor fraction of the cells that had not been previously exposed to the solvent managed to survive the sudden shock (*Fig. 8.3.1*).

To gain further insight into the most relevant features regarding solvent tolerance in *Pseudomonas*, several complementary approaches have been taken in a number of laboratories around the world (Ramos et al. 2002). With the recent availability of high throughput sequencing facilities, the genomes of at least two of the previously mentioned strains, S12 and DOT-T1E, have been sequenced. The genome of DOT-T1E is made up of a circular

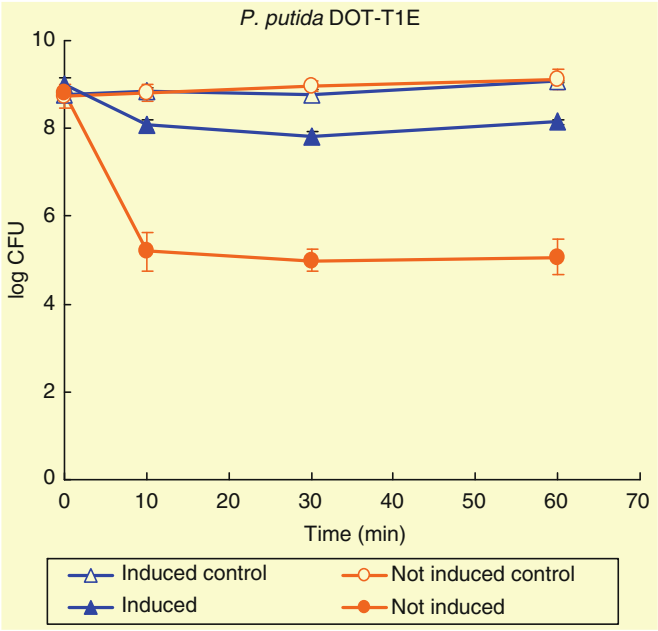

Fig. 8.3.1

Killing kinetics of *Pseudomonas putida* exposed to sudden solvent shock. *Pseudomonas putida* DOT-T1E cells were grown on LB medium without toluene or with low concentrations of toluene supplied via the gas phase. At time 0, cells were split in two halves and toluene was added to reach a concentration of 0.3% (v/v). At the indicated times surviving cells were counted

chromosome of approximately 6 Mb and a medium-sized plasmid of about 130 kb (Segura et al., unpublished). One of the relevant issues of this genome is that it contains a large island full of catabolic pathways for the degradation of aromatic compounds and that it exhibits over 20 ECF-sigma-factors, indicating a high versatility to live in different niches. The DOT-T1E genome also encodes several different families of multidrug extrusion pumps, which can be relevant in tolerance against noxious compounds (García et al. 2009; Rojas et al. 2001). The plasmid has a 70 kb backbone that belongs to the IncI incompatibility group, this backbone encodes the RepA protein for replication and has all of the necessary genes for transfer and mobilization. A 50-kb transposon is integrated on the backbone and this transposon has a set of genes related to resistance to stress conditions (Segura et al., unpublished results). The mere analysis of the genome itself does not reveal why *Pseudomonas putida* DOT-T1E is solvent tolerant. Nonetheless, the analysis of the genome of DOT-T1E allowed us to identify the entire set of genes previously proposed to be involved in solvent tolerance; however, no new key functions were identified since many of the genes remained annotated in very general terms or were recorded as hypothetical proteins or proteins of unknown function. Based on mutant analyses and genome sequences, it seems that solvent tolerance is a multifactoral process in which chromosomally encoded and plasmid-encoded functions are involved. The conclusion drawn from these studies is that the successful combination of these functions leads to the unusual property of solvent tolerance.

Transcriptomic and proteomic assays have also provided relevant information about the immediate response of these extremophile Pseudomonads to solvents. The analysis of cytoplasmic proteins from DOT-T1E cells grown in the presence and absence of toluene by 2-dimensional gel electrophores allows the identification of 17 spots in cells treated with toluene whose expression level increased threefold or more (Segura et al. 2005). This methodology has led to the identification of a number of these proteins as involved in toluene degradation; some were chaperones while others were defined as central metabolism enzymes. Microarray assays of DOT-T1E revealed that around 50 genes increased their expression in response to toluene. Some of the identified genes ranged from efflux pumps that expel solvents and other drugs from the cell membrane to enzymes involved in phospholipid and lipopolysaccharide turnover or to chaperones and other proteins involved in stress responses (our unpublished results).

These approaches have confirmed the mutifactoral response to solvents. What several labs have really been after is the generation of in silico and experimental data to analyze the mechanisms of solvent tolerance and to set up the basis for the biotechnological exploitation of these microbes. Furthermore, classical growth yield analysis, growth kinetics, and behavior of a collection of mutants have shown that solvent tolerance is not a linear process with one step leading to the next, but rather a series of steps that probably function simultaneously and which can be documented as follows: first a rapid alteration of membrane phospholipids takes place to impermeabilize the cells. This involves a *cis* to *trans* isomerisation of unsaturated fatty acids and a slower alteration of phospholipid head composition (Härtig et al. 2005; Heipieper et al. 1992, 1996; Holtwick et al. 1999; Junker and Ramos 1999; Pinkart et al. 1996). This, however, does not suffice and solvents enter the cells, and due to their denaturing character, refolding of denatured proteins becomes very important. To counterbalance this effect several chaperones are induced (Segura et al. 2005; Volkers et al. 2006; Wierckx et al. 2008). Alteration of the cell membranes also leads to the generation of reactive oxygen species, which, in turn, activates an oxidative damage stress programe (Domínguez-Cuevas et al. 2006). The excess solvent that is present in cell membranes, in the periplasmic space, or even in the cytoplasm is removed via efflux pumps. Although all the above elements are of importance, the removal of the toxic through efflux pumps, which literally pump the solvents to the outer medium, represents the most important mechanism of all. In support of this conclusion is the fact that the TtgGHI efflux pump that is encoded in the pGRT1 plasmid (Rodríguez-Herva et al. 2007) confers solvent tolerance when transferred to other *Pseudomonas* strains. This finding is very important because it shows that the solvent tolerance trait can be horizontally transferred to other microbes. In the case of DOT-T1E, but not of other strains, the toxic compound can be metabolized to CO_2 and water, generating carbon skeletons and serving as a source of energy. However, the capacity of *P. putida* DOT-T1E to degrade toluene into Krebs cycle intermediates via the TOD pathway has been shown to have no significant impact on solvent resistance, as shown by the fact that a mutant lacking the initial enzyme of this pathway exhibits a similar solvent resistance phenotype as the wild-type bacterium (Mosqueda et al. 1999). Another relevant feature is that *Pseudomonas* sp. S12, which does not metabolize toluene, tolerates high concentrations of this compound. Therefore, within this model system, toluene resistance and toluene degradation appear to be two independent functions.

Below we analyze relevant features of solvent extrusion and its importance in solvent tolerance, the modifications of membrane phospholipids and the first insight into chaperones and oxidative stress responses, focusing on what we have learned from our studies in *Pseudomonas putida* DOT-T1E.

Multidrug Efflux Pumps in *Pseudomonas putida* Strains Tolerant to Highly Toxic Chemicals

Multidrug resistance (MDR) efflux systems mediate the active extrusion of many structurally and functionally unrelated compounds from the bacterial cytoplasm (or inner membrane) to the external medium. Isken and de Bont (1996) and Ramos et al. (1997) measured the accumulation of [^{14}C]-labeled aromatic hydrocarbons by *P. putida* S-12 and DOT-T1E grown in the absence (non-adapted bacteria) or in the presence (adapted bacteria) of toluene. They found that the amount of [^{14}C]-labelled aromatic hydrocarbon that accumulated in the adapted cells was much lower than in non-adapted bacteria. The addition of the respiratory chain inhibitor potassium cyanide or the protonophore uncoupler CCCP to adapted cells resulted in significant aromatic hydrocarbon accumulation in the cell membranes (Isken and de Bont 1996; Ramos et al. 1997). This finding was interpreted as evidence that these strains had energy-dependent exclusion systems able to decrease the level of the solvent in the membranes.

The isolation and characterization of transposon mutants of *P. putida* S12, DOT-T1E, and GM73 made it possible to identify several efflux pumps involved in solvent tolerance (Kieboom et al. 1998a, b; Kim et al. 1998; Ramos et al. 1998; García et al. 2009). The molecular characterisation of the insertion sites in the mutants led to the early identification the *srpABC* (solvent-resistant pump) genes of *P. putida* S12 (Kieboom et al. 1998a, b) and the *ttgABC* (toluene tolerance genes) of *P. putida* DOT-T1E and GM73. Additionally, Fukumori et al. (1998) isolated a toluene-resistant mutant of the solvent-sensitive *P. putida* KT2440 in which an efflux pump almost identical to the TtgABC pump of *P. putida* DOT-T1E was expressed at high levels; indicating the ubiquitous nature of these pumps in *P. putida*. The *srpA* and *ttgA* genes encode the inner membrane-anchored lipoprotein that spans the periplasm and interacts with the inner membrane transporter (encoded in these systems by *srpB* and *ttgB*); the lipoprotein also appears to interact with the Outer Membrane Protein (OMP) encoded by *srpC* and *ttgC* (Duque et al. 2001; Kieboom et al. 1998a, b). These efflux pumps which expel organic solvents belong to the RND (root-*n*odulation cell *d*ivision) family of pumps (*Table 8.3.1*). In general, these pumps often consist of three basic components: a cytoplasmic membrane export system that acts as an energy-dependent extrusion pump, a membrane fusion protein (MFP), and an outer membrane protein (OMP) (Koronakis et al. 2000; Zgurskaya and Nikaido 1999). The deduced amino-acid sequences of the proteins encoded by these clusters are similar (from 58% to 77% identity depending on the proteins analyzed) to those of the AcrAB-TolC efflux pump of *E. coli* and to the MexAB-OprM multidrug efflux system of *P. aeruginosa*; previously identified to extrude drugs and antibiotics.

A series of assays revealed that pre-exposure of wild-type *P. putida* DOT-T1E to low concentrations of toluene led to survival of almost 100% of the cells after a sudden toluene shock; in contrast, only a fraction (10^{-4}) of the cells that had not been pre-exposed survived (Ramos et al. 1998). When these assays were done with a knock-out mutant in *ttgB*, the strain did not withstand the sudden toluene shock, and only a small fraction (about 1 out of 10^4–10^5 cells) survived the shock if pre-exposed to low toluene concentrations (*Fig. 8.3.1*). This led to the suggestion that the TtgABC pump contributes to the innate tolerance of *P. putida* DOT-T1E to solvents (Ramos et al. 1998), and it was subsequently postulated the existence of other efflux pumps involved in toluene extrusion. A second inducible efflux pump, called TtgDEF, which expels toluene in the solvent-tolerant *P. putida* DOT-T1E was then identified as being linked to the *tod* genes for toluene degradation (Mosqueda and Ramos 2000). Linkage of the

Table 8.3.1

RND efflux pumps involved in solvent extrusion

Inner membrane transporter	Membrane fusion protein	Outer membrane protein	Bacterial species	References
AcrB (1,049)	AcrA (397)	TolC (495)	*E. coli*	Aono et al. (1998), Fralick (1996), Zgurskaya and Nikaido (1999)
AcrF (1,034)	AcrE (385)	TolC (495)	*E. coli*	Kobayashi et al. (2001), Ma et al. (1993)
MexB (1,046)	Mex (383)	OprM (385)	*P. aeruginosa*	Li et al. (1998)
TtgB (1,050)	TtgA (384)	TtgC (484)	*P. putida*	Ramos et al. (1998)
TtgE (1,048)	TtgD (382)	TtgF (480)	*P. putida*	Mosqueda and Ramos (2000)
TtgH (1,049)	TtgG (391)	TtgI (470)	*P. putida*	Rojas et al. (2001)
SrpB (1,049)	SrpA (382)	SrpC (470)	*P. putida*	Kieboom et al. (1998a), Kieboom et al. (1998b), Kim et al. (1998)
ArpB (1,050)	ArpA (371)	ArpC (484)	*P. putida*	Fukumori et al. (1998)

Numbers in parentheses indicate the size in amino acids of the corresponding protein

genes that encode a catabolic pathway with those involved in the efflux of the compound degraded by the strain was conserved in a wide range of *Pseudomonas* strains from different species that degraded toluene through the TOD pathway (Huertas et al. 2000; Phoenix et al. 2003). Subsequently, it was suggested that degradation of a compound with intrinsic toxicity may have coevolved with its exclusion in order to avoid accumulation of the toxic compound above a certain threshold.

Upon inactivation of the TtgABC and TtgDEF pumps in *P. putida* DOT-T1E, a fraction of the cells still survived if they were pre-exposed to low concentrations of toluene, although none ($<10^{-8}$) survived without induction (Mosqueda and Ramos 2000). Mosqueda and Ramos (2000) suggested the presence of at least one other undiscovered pump was necessary to confer tolerance. This pump was later identified by Rojas et al. (2001) and called *ttgGHI*, being almost identical to the *srpABC* pump. Rojas et al. (2001) constructed a *P. putida* strain with a knock out in each of the three efflux pumps. This strain was found to be more sensitive than the *ttgABC*/*ttgDEF* mutant, and each of the three pumps were then tested for the profile of substrates extruded by the pumps. The TtgABC and the TtgGHI pumps were found to be involved in the efflux of toluene, styrene, xylenes, ethylbenzene, and propylbenzene, whereas the TtgDEF efflux pump was shown to be involved in the efflux of only styrene and toluene. It should be noted that the work of Lee et al. (2000) suggested that pumps that operate through a common mechanism have an additive effect in the extrusion of drugs, which seems to be the case for the Ttg efflux systems in *P. putida* DOT-T1E. Efflux pumps can also remove low-water-solubile chemicals and Bugg et al. (2000) showed that *Pseudomonas fluorescens* LP6a, which degrades a number of polycyclic aromatic hydrocarbons, encodes energy-driven efflux pumps that selectively removed phenanthrene, anthracene, and fluoranthene from the cell membrane (Hearn et al. 2003, 2006; Kallimanis et al. 2007).

The physiological role of RND pumps is not yet clearly established, as it has been proposed that RND pumps could be involved in both the extrusion of intracellularly-generated

compounds, as well as the defense against toxic compounds present in the environment (Dong et al. 2007; Mashburn-Warren et al. 2009; Pearson et al. 1999; Skindersoe et al. 2008; Tian et al. 2010; Zhu et al. 2008). The extrusion of a wide range of toxic compounds could be a lateral or a specifically-evolved function related to the ecological fitness of the bacteria. Whatever may be, RND pumps have evolved to become multidrug recognition proteins, which implies that a range of structurally different but related molecules serve as substrates for a given pump (Poole 2004; Yu et al. 2005). Thus, multidrug recognition is a trade-off between affinity of recognition and specificity. This means that a particular pump can only recognize a given number of substrates with physiological affinity. To circumvent the intrinsic chemical limitations of multidrug recognition, bacteria frequently possess several copies of RND pumps with different and often overlapping substrate specificities.

This view is fully supported by data available on the three efflux pumps that contribute to the solvent resistance of *P. putida* DOT-T1E. The TtgABC pump, known for its affinity for aromatic hydrocarbons, was later shown to also act on different antibiotics and plant-derived secondary metabolites (Rojas et al. 2001; Terán et al. 2006). The TtgGHI pump transports aromatic and aliphatic hydrocarbons primarily, while its role in the expulsion of different antibiotics does not appear to be of physiological relevance (Rojas et al. 2001). Although TtgDEF appeared to efficiently extrude aromatic hydrocarbons such as toluene and styrene (Rojas et al. 2001), no physiologically-relevant evidence was obtained for this pump as a transporter for antibiotics (Mosqueda and Ramos 2000). Although these three pumps have overlapping substrate profiles, it is only through their concerted action that the strain achieves the remarkable level of solvent tolerance observed. Another example of efficient self-protection against a wide range of toxic compounds is that of the concerted action of the MexAB, MexCD, and MexXY pumps of *P. aeruginosa*, which were also shown to have overlapping and complementary substrate profiles (Masuda et al. 2000).

Although RND pumps typically have a rather broad substrate spectra, it is possible to cluster the pumps into several groups according to their substrate preference (Hernández-Mendoza et al. 2007). Our recent study using Provalidator defines a profile based on the whole sequence of the inner membrane component that allows the grouping of the efflux pumps according to their main substrates. Some pumps are specific for recognizing metals, others for organic molecules, and a group of pumps extrude both simultaneously (Godoy et al. 2010) (*Fig. 8.3.2*). Other studies have performed clustering taking into account only the region proposed by Yu et al. (2003) to be involved in substrate recognition, clustering did, in fact, occur according to substrate preference (Hernández-Mendoza et al. 2007). This indicates that RND pumps share the same overall structure, but differ within the sequence of amino acids involved in substrate recognition. This also implies that the structural and functional features of well-characterized family members, such as AcrB, can be extrapolated to uncharacterized solvent efflux pumps.

The Ttg and Srp efflux pumps are similar to the Mex antibiotic efflux pumps of *P. aeruginosa* in respect to structure. In fact, TtgABC and TtgGHI also remove certain antibiotics in addition to aromatic hydrocarbons (Li et al. 1998; Rojas et al. 2001). Li et al. (1998) showed that *P. aeruginosa* strains that express wild-type levels of MexAB-OprM, as well as mutants that hyperexpress this efflux pump – as is the case for MexR mutants, in which the *mexR* gene product controls expression of the *mexAB-oprM* operon (Evans et al. 2001; Köhler et al. 1999; Srikumar et al. 2000; Ziha-Zarifi et al. 1999) – tolerated *n*-hexane and *p*-xylene, whereas strains where these genes were partially or totally deleted showed no resistance. These data suggest that the MexAB-OprM multidrug efflux systems of *P. aeruginosa*, which is a well-characterized

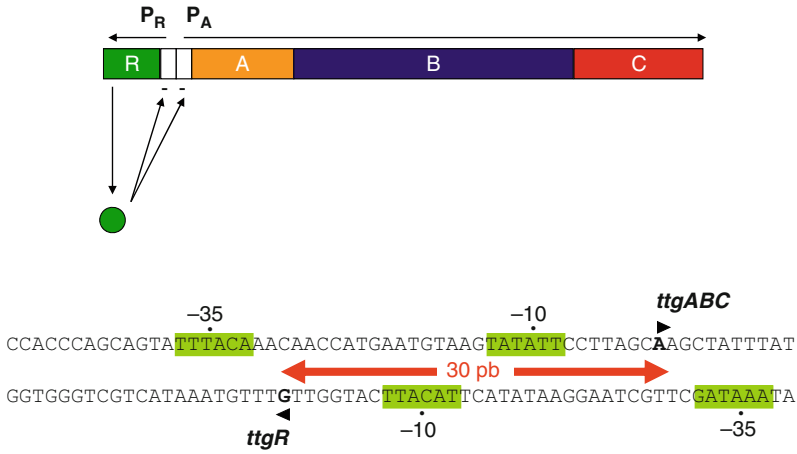

Fig. 8.3.2

Proposed regulatory model for TtgR and the promoter region of the *ttgA* and *ttgR* genes. The −10/−35 regions of both promoters overlap and within these the overlapping RNA polymerase binding sites is the region recognized by the TtgR regulator

antibiotic extrusion system (Poole 2004; Nikaido 1996), can accommodate organic solvents as well as antibiotics.

The Assembly of the Tripartite RND Pump Remains Uncertain

RND pumps contain multiple components, which are assembled as a functional pump, spanning the periplasm. The three-dimensional structures of the three individual components of an RND pump have been solved for the AcrAB-TolC system of *E. coli* (Koronakis et al. 2000; Murakami et al. 2006). However, no structural information is available on the functional protein complex or a complex of any two types of individual components. An interesting feature is that the two integral membrane proteins, AcrB (inner membrane) and TolC (outer membrane), protrude deeply into the periplasmic space where both proteins are thought to contact. This observation is further supported by in vivo cross-linking experiments with both proteins (Touzé et al. 2004). Since the adaptor protein, AcrA, is able to bind to AcrB and TolC, structural models for subunit assembly have been developed (Ge et al. 2009; Symmons et al. 2009; Dawson and Locher 2006; Eda et al. 2003). Given that the inner and outer membrane proteins crystallize as trimers, all models concur that an RND pump contains a trimer of each protein. In contrast, the adaptor protein was found to be monomeric in solution at neutral pH, dimeric in crystals grown at acidic pH and associated as 13-mers in crystals grown at neutral pH. This diversity of oligomeric states leaves uncertainties about the physiological oligomeric state of this protein. Hence, the models proposed for the arrangement of the RND pumps differ in the number of adaptor proteins, which were proposed to be either 3, 6, or 9 per functional RND pump (Fernandez-Recio et al. 2004; Akama et al. 2004; Eswaran et al. 2004).

Recently, X-ray crystallographic studies of AcrB provided new evidence for the existence of a fourth component in an RND pump. One copy of the helical protein, YajC, was found to be bound to each monomer of AcrB. This protein is embedded in the membrane where it appears

to make contacts with the transmembrane regions of AcrB. Bacterial mutants lacking YajC showed a modest increase in susceptibility toward different antibiotics. YajC, as well as its binding site on AcrB, are highly conserved (Törnroth-Horsefield et al. 2007); however, the contribution of YajC to the AcrAB-TolC pump function remains to be established. More recently, Symmons and colleagues (2009) developed a new model of the AcrAB-TolC efflux pump by solving the structure of the apparently disordered N and C termini of the adaptor protein (AcrA). This new data was combined with the previously available high-resolution structures of all three components and in vivo cross-linking experiments to map the intermolecular contacts between the adaptor AcrA and the transporter AcrB; previous resolution data of the AcrA hairpin–TolC interaction aided in development of a model of the assembled tripartite complex. Their model AcrA(3)-AcrB(3)-TolC(3) represents a 610-kDa, efflux pump capable of crossing the entire bacterial cell envelope (Symmons et al. 2009).

Regulation of Efflux Pump Expression

Although multidrug efflux pumps are often constitutively expressed at a low basal level they are also controlled in their expression by cognate DNA binding repressors. Most of the regulatory genes that encode proteins involved in the transcriptional control of RND efflux pumps are located adjacent to the structural genes of the pump. The expression of efflux systems is generally controlled by the presence of at least one of their substrates in the culture medium, and it has been shown that this regulation occurs at the transcriptional level (Duque et al. 2001; Terán et al. 2003; Guazzaroni et al. 2004, 2005, 2007; Su et al. 2007). The repressors generally belong to one of two main families; the TetR family of repressors and the IclR protein family (Ramos et al. 2005; Molina-Henares et al. 2006).

The inherent solvent resistance of *P. putida* DOT-T1E cells not previously adapted to toxic compounds is mediated by both the TtgABC and TtgGHI efflux pumps, which are both expressed at a relatively high basal level. The expression level of TtgABC increases in the presence of hydrophobic antibiotics such as tetracycline and chloramphenicol (Terán et al. 2003), but is not altered in response to solvents (Duque et al. 2001). TtgR, a member of the TetR family of transcriptional regulators (Ramos et al. 2005), modulates transcription of the *ttgABC* operon in response to antibiotics, aromatic solvents, and plant antimicrobials (Terán et al. 2003, 2006). Expression levels of both TtgDEF and TtgGHI increase in response to organic solvents such as toluene, xylenes, and others (Rojas et al. 2001; Mosqueda and Ramos 2000). Divergently transcribed with respect to *ttgGHI* and *ttgDEF* are the *ttgV* and the *ttgT* genes, respectively, which belong to the IclR family of transcriptional regulators (Molina-Henares et al. 2006; Krell et al. 2006).

Further in vitro studies revealed that TtgV and TtgT each bind to the same operator sites in both the *ttgDEF* and the *ttgGHI* promoters. Interestingly, TtgV dominates in vivo for the regulation of these two efflux pump operons because the affinity of TtgV for the *ttgDEF* operator is higher than that of TtgT, and the *ttgV* promoter is almost twofold stronger than the *ttgT* promoter (Duque et al. 2001; Guazzaroni et al. 2005, 2007; Terán et al. 2007; Fillet et al. 2009). Expression of the *ttgGHI* and *ttgDEF* efflux pumps is under the influenced of the alternative sigma factor RpoT, however, whether its role is direct or indirect remains unknown (Duque et al. 2007).

TtgV binds a wide range of mono- and bicyclic aromatic compounds. Binding of these effectors to the DNA–TtgV complex leads to the release of TtgV leading to efflux pump gene

transcription (Rojas et al. 2003; Guazzaroni et al. 2004, 2005). It has been shown that the *ttgGHI* operon and the adjacent *ttgV* regulatory gene are both borne by the pGRT1 megaplasmid (Rodríguez-Herva et al. 2007). In contrast to their parental strain, mutants lacking pGRT1 were unable to grow in LB medium supplemented with 0.3% (v/v) toluene and, reciprocally, the transfer of the megaplasmid to a toluene-sensitive strain conferred solvent tolerance to the latter (Rodríguez-Herva et al. 2007). TtgT also belongs to the IclR family of repressors, and shares high sequence identity (63%) with TtgV. This is an interesting observation because although the gene encoding TtgV is located on a plasmid and is likely more newly acquired, it is dominant over the chromosomally encoded TtgT in the regulation of efflux pump expression.

No three-dimensional structures have yet been reported for either TtgV or TtgT; however, the TtgR protein has recently been crystallized both alone and with several different effectors (Alguel et al. 2007, *Fig. 8.3.3*). It has a highly-conserved structure and like TetR (Hinrichs et al. 1994) and QacR (Schumacher et al. 2002), TtgR is made up of 9 α-helices with α_2 and α_3 constituting the HTH DNA binding domain. Co-crystallization of TtgR with effectors

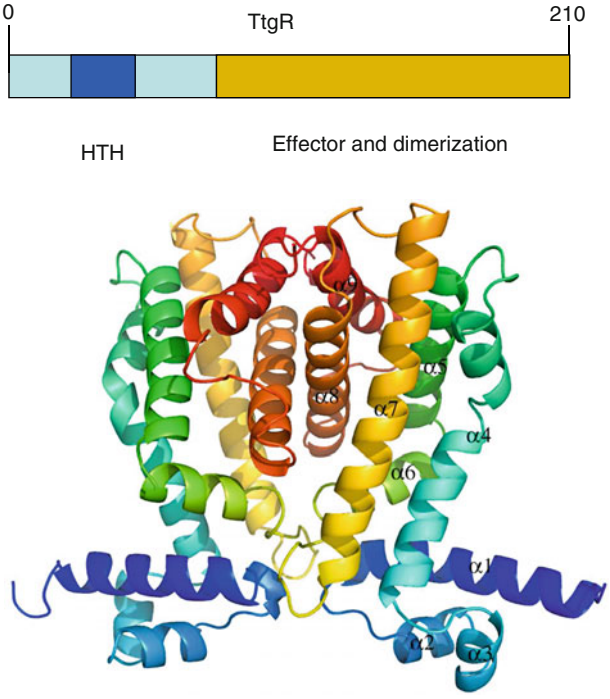

Fig. 8.3.3

Ribbon representation of the TtgR regulator and its domains. The HTH DNA binding domain is located at the N-terminus of the protein and it is composed of the alpha helices 2 and 3 in the ribbon representation. The central region of TtgR represents the dimerization motif and makes a cavity where effectors are bound. For further details see Alguel et al. (2007)

revealed that the binding pocket of this regulator exhibits a wide cavity that allows it to interact with a multitude of effector molecules by establishing different van der Waals and ionic bridges between the effectors and numerous amino-acid residues in the effector binding pocket (Alguel et al. 2007). Interestingly, it has been recently found that changing some of the amino acid in the effector binding region of TtgR results in a marked increase in affinity for the operator DNA and a subsequent decrease in affinity for certain effector molecules (Daniels et al. 2010), suggesting that the targeted residues form an important part of the effector binding pocket and that there is functional cross-talk between the effector binding and the DNA binding domain.

Alterations in the Phospholipid Composition of the Membranes Lead to Cells More Sensitive to Solvent Stress

Bacterial cells are surrounded by a cellular envelope composed of two elements: the cytoplasmic membrane and the cell wall. This envelope constitutes the first contact point between microbes and the environment. The cytoplasmic membrane is a phospholipid bilayer in which many different types of proteins are intercalated. In this structure, the fatty acid chains (hydrophobic) of the phospholipids are facing towards the inner part of the bilayer while the polar headgroups are facing toward the outer surfaces of the bilayer. In *Pseudomonas putida*, as in other Gram-negative bacteria, the main membrane phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). PE represents about 75% of total phospholipids, while the relative amounts of PG and CL depend on the growth phase of the cultures, with PG being more abundant in exponential phase and CL being more abundant during stationary phase (Bernal et al. 2007a). Fatty acids attached to phospholipids can be saturated or unsaturated. Among the main saturated fatty acids in *P. putida* membranes are myristic (tetradecanoic, C14:0), palmitic (hexadecanoic, C16:0), and stearic acid (octadecanoic, C18:0). The more abundant unsaturated fatty acids (UFA) are palmitoleic (*cis*-9-hexadecenoic, *cis*-$\Delta^{9,10}$-16:1) and *cis*-vaccenic acid (*cis*-11-octadecenoic, *cis*-$\Delta^{11,12}$-18:1). Other fatty acids that are present in bacterial membranes are cyclopropane fatty acids (CFAs). In *E. coli* and *P. putida,* the most abundant fatty acids are C17:cyclopropane (9,10-methyl-hexadecanoic acid) and the C19:cyclopropane (11,12-methyl-octadecanoic acid). Cyclopropane fatty acid abundance is growth-phase dependent representing almost 30% of total fatty acids when bacterial cultures reach the stationary phase (Grogan and Cronan 1997; Muñoz-Rojas et al. 2006; Pini et al. 2009).

Changes in temperature or a rapid increase in the concentration of toxic compounds produce alterations in membrane properties and, if the resulting disturbance in membrane integrity is severe enough, growth inhibition or cell death may result (Sikkema et al. 1995). For this reason, bacteria have developed several mechanisms to overcome the membrane effects produced by a changing environment. The major adaptive response of bacterial cells to compensate for the changes in membrane fluidity produced by environmental challenges is called "homeoviscous adaptation" and is largely achieved by changes in fatty acid composition (Sinensky 1974).

We discuss the major changes that *Pseudomonas* sp. use to modify membrane composition in response to solvent exposure and the key membrane components involved in solvent tolerance.

Changes in Fatty Acid Composition in Response to Organic Solvents

Variations in the length of fatty acids (Ingram 1986), in the degree of saturation (Suutari, and Laakso 1994; Hamamoto et al. 1994), and in the ratio of *cis* to *trans* of the unsaturated fatty acids (Heipieper et al. 2003) are the main factors that affect membrane fluidity. Of the three mechanisms, probably the best studied is the change in the configuration from *cis* to *trans* of the double bond in the unsaturated fatty acids. This reaction is carried out by the periplasmic enzyme *cis-trans* isomerase (Cti; Okuyama et al. 1991; Heipieper et al. 1992; Okuyama et al. 1998; Pinkart et al. 1996; Härtig et al. 2005; Pedrotta and Witholt 1999) without de novo synthesis of fatty acids (Diefenbach and Keweloh 1994); it is therefore a post-synthetic modification of the unsaturated fatty acids – one that does not include the transient saturation of the double bond (von Wallbrunn et al. 2003). It has been suggested that Fe^{3+} plays a crucial role in the catalytic reaction (Okuyama et al. 1998) and in fact, a putative heme-binding site has been found in all the predicted Cti sequences reported so far (Holtwick et al. 1999).

It has been well established that the change of the double bond from *cis* configuration to *trans* provokes a conformational change in the fatty acid molecule. Fatty acids with the *trans* configuration have a long extended structure (similar to that of the saturated fatty acids) that allows tight packing of the membrane molecules; however, the conversion to the *cis* configuration provokes a kink of 30° in the acyl chain that disturbs the ordered conformation of the fatty acids in the membrane. Thus, membranes with *cis* unsaturated fatty acids (*cis*-UFA) are more fluid than those with a higher content of *trans* unsaturated fatty acids (*trans*-UFA) .Only a few bacterial species (mainly belonging to the genus *Pseudomonas* and *Vibrio*) contain *trans*-UFAs in their membranes, which are present at very low quantities under normal laboratory conditions. However, the proportion of *trans*-UFA increases in the presence of certain environmental stresses such as organic solvents, heat shock, osmotic stress, or heavy metals (Diefenbach et al. 1992; Weber et al. 1993; Heipieper et al. 1996; Ramos et al. 1997; Junker and Ramos 1999). Most of these insults cause an increase in membrane fluidity and therefore bacteria respond by synthesizing *trans*-UFA to increase the rigidity of the membrane to counteract the effect of the stress. Changes in membrane fluidity that occur when toluene is added to the medium have been measured with fluorescence anisotropy using probes such as DPH (1,6-diphenyl-1,3,5-hexatriene). Addition of toluene to *P. putida* DOT-T1E culture leads to a decrease in fluorescence polarization, indicating increased fluidity of the membranes; however, when the cells are grown in the presence of sublethal concentrations of toluene, the polarization values are higher than those of cells without toluene pre-incubation, indicating a more rigid state of the membrane as a consequence of adaptive mechanisms in response to toluene (Bernal et. al. 2007a).

However, these changes are subject to synthesis de novo and are not as fast as the changes in the configuration of the double bond. In fact, *cis/trans* isomerization can be detected within 5 minutes after solvent exposure (Ramos et al 1997), and is versatile in that it allows bacteria to regulate membrane fluidity when growth or fatty acid biosynthesis is inhibited by toxic compounds (Segura et al. 2004a; Härtig et al. 2005).

Surprisingly, the expression of the *P. putida* DOT-T1E *cti* gene is not induced by the presence of organic solvents or by temperature shifts (Junker and Ramos 1999; Bernal et al. 2007b). Activation of the constitutively expressed Cti is still an open question. One plausible explanation for Cti activation is that in the presence of the stress, membranes became more fluid allowing the enzyme to gain access to the substrate (the double bond) that under normal conditions is deeply immersed in the phospholipid bilayer (Heipieper et al. 2001).

The benefits of increased membrane rigidity to counteract the increased fluidity that occurs due to environmental stress appear to be marginal, as shown in experiments using *P. putida* DOT-T1E. In these studies, *cti* knock-out mutants were only slightly more sensitive to toluene shocks or temperatures above 37°C than the wild-type (Junker and Ramos 1999).

Other Lipid Membrane Components Involved in Solvent Tolerance

The increase in cardiolipin (CL) content in the presence of organic solvents has been reported in *P. putida* Idaho and *P. putida* DOT-T1E (Weber et al. 1994; Ramos et al. 1997). Bernal et al. (2007a) constructed a mutant in the cardiolipin synthase (*cls*) gene of *P. putida* DOT-T1E, the enzyme responsible for the transfer of a phosphatidyl group from one PG molecule to another to synthesize CL (*Fig. 8.3.4*). Although the *cls* mutant showed traces of cardiolipin in its membranes (as shown in other strains; von Wallbrunn et al. 2002), it was more sensitive to toluene shock than the wild-type strain, demonstrating the importance of CL in solvent tolerance in this strain. CL has a higher transition temperature than PE and, therefore, increases in CL have a stabilizing effect. In fact, the *P. putida* DOT-T1E *cls* strain has less fluid membranes than the wild-type. It has been shown that the *cis-trans* isomerisation is not affected in cardiolipin synthase mutants (von Wallbrunn et al. 2002). Interestingly, in the *cls*-deficient mutant, the function of the organic solvent extrusion efflux pumps was affected suggesting a structural role of CL domains in the stability of the efflux pumps.

Recently, work carried out by Pini et al. (2009) has implicated cyclopropane fatty acids in solvent tolerance. A *cfaB* (cyclopropane synthase) gene knock out was more sensitive to the sudden addition of 0.3% (v/v) toluene than the wild-type when cells were in the stationary growth phase. This sensibility does not seem to correlate with changes in membrane fluidity

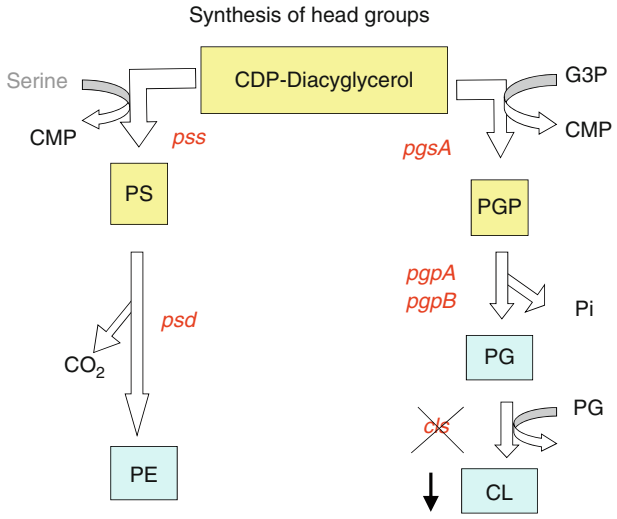

Fig. 8.3.4

Proposed pathways for phospholipid head group synthesis based on the annotation of the genome of *Pseudomonas putida* DOT-T1E (Segura et al., unpublished)

as the wild-type and mutant cells did not show significant differences in fluorescence polarization when analyzed using DPH. *cfaB* expression is not induced by organic solvents and the content of CFA in the membrane does not increase when cells are grown in the presence of organic solvents.

Other Functions Involved in Solvent Tolerance

The advance of new "-omics" technologies have allowed a more global approach toward understanding solvent tolerance in bacteria. Proteomic studies have been performed in two highly solvent-tolerant strains, namely *P. putida* DOT-T1E and *P. putida* S12 (Segura et al. 2005; Volkers et al. 2006). Apart from the obvious induction of toluene degrading proteins in *P. putida* DOT-T1E (*P. putida* S12 does not metabolize toluene), in both strains it was demonstrated that proteins required for energy production are overexpressed in the presence of solvent – a logical consequence of the higher energetic demands required to cope with the toxicity of the organic solvents. It is known that organic solvents cause protein denaturation, and that most probably the presence of misfolded proteins in the cytoplasm provides the signal that triggers the induction of chaperones, and a number of chaperones are indeed induced, including a set of classical chaperones like DnaK, J, GroESL, GrpE, and ClpB. The proposed role of these chaperones is to refold denatured proteins or to help proper folding of newly synthesized proteins in response to toluene and other solvents (Segura et al. 2005; Domínguez-Cuevas et al. 2006). Several stress-related proteins (XenA, alkyl hydroperoxidases, and other oxygen stress-related proteins) were also induced, indicating that as a consequence of the alteration of the phospholipid bilayer by toluene, cells suffer oxidative stress at concentrations below the killing threshold. Construction of mutants in two stress-related genes (*cspA2* and *xenA*) in *P. putida* DOT-T1E confirmed their role in solvent tolerance, as both mutants were less resistant to toluene shock (0.3% v/v) than the wild-type strain, and both grew more slowly than the wild-type when this solvent was present in the media. Induction of stress-related genes, mainly heat-shock genes, was also observed by Domínguez-Cuevas et al. (2006) when the transcriptomic response of the solvent-sensitive strain *P. putida* KT2440 towards non-toxic toluene concentrations was analyzed. Collectively these data again emphasize the importance of a multi-factorial response to cope with the sudden presence of a membrane altering stimuli.

Parasitism of the Flagellar Transport System for Solvent Tolerance

Random mutagenesis using transposon derivatives lead to the unexpected finding that mutants in flagellar genes were involved in solvent tolerance (Segura et al. 2001; Kieboom et al. 2001). So far there is no evidence of a correlation between motility and solvent tolerance, and the more plausible hypothesis is that some proteins involved in solvent tolerance can highjack the flagellar export system in order to reach their final location. This parasitisation of the flagellar system was reported in *Yersinia enterocolitica* and *Xenorhadbus nematophilus* where virulence proteins are exported to the outer medium via the flagellar system. One of the *P. putida* DOT-T1E genes identified by transposon inactivation was *flhB* – a gene that encodes one of the proteins of the flagellar export apparatus. Interestingly this gene is subject to phase variation in *P. putida* DOT-T1E (Segura et al. 2004b). Translational variation caused by

frameshift mutations has been shown to be a widespread mechanism for adaptation to new environments. Cells extracted from a liquid culture carried an *flhB* gene that contained a track of 7, 9, or 10 Gs; translation of any of these gene variants resulted in a truncated FlhB protein. However, cells extracted from the swimming halo of a semisolid LB medium plate contained 8 or 11 Gs and their translation gave rise to full-length FlhB (or +1 amino acid). The presence/absence of the full-length FlhB protein correlated with solvent tolerance. As such, in liquid cultures only 1 out of 10^4 cells survived the sudden toluene shock (0.3% v/v); however, in cells obtained from semisolid medium plates, the survival rate was two orders of magnitude higher. Clearly, further studies will be required to elucidate the precise mechanism of flagellar protein mediated resistance.

Concluding Remarks and Some Research Perspectives

Solvent tolerance in *Pseudomonas* strains is a multifactoral response that involves impermeabilization of the cell membrane, removal of the solvent from cell compartments via efflux pumps, and establishment of a global stress response in which chaperones and oxidative stress proteins play a relevant role. Some of these microbes are also able to degrade the pollutant, but in itself this property is not relevant for solvent tolerance because mutants deficient in catabolic pathways exhibit a level of tolerance equivalent to that of the wild-type strain.

Specific signals triggering some of these solvent tolerance responses remain to a certain extent unknown. For instance, what is the chemical signal that leads to activation of the *cis-trans* isomerase that through isomerisation of unsaturated fatty acids increases membrane endurance? How does the turnover of phospholipid head groups take place? And are patchy regions on the cell membrane involved in response to the solvents?

Multidrug efflux pumps have been characterized in antibiotic resistance strains. A number of studies are needed at the structural and functional level to discern the molecular basis of recognition of substrates and the intimate mechanism of drug/solvent extrusion. The genes encoding these pumps form part of an intricate finely tuned regulatory network. Further insight into the specific regulators that influence efflux pump expression is also needed. However, we anticipate that the structures of some of these regulators, i.e., TtgV, will soon be resolved and will help in understanding the details of effector recognition and the consequent pump regulation.

While further investigation of the accessory functions, such as the parasitism of the flagellar apparatus, and the activation of oxidative stress systems are anticipated to reveal new insights that will aid in providing a full picture of the processes involved in solvent tolerance.

Beyond all these questions related to basic science, the exploitation of solvent-tolerant microorganisms in bioremediation and biotransformation are where we envisage significant future advances.

Acknowledgments

Work in our laboratory was supported by grants BIO2008-04419-E, Consolider-Ingenio (CSD2007-00005) of the Spanish Ministry, CVI-3010 from Junta de Andalucia and BACSIN (FP7-KBBE-2007-1) from the European Commission.

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8.4 Genetics, Evolution, and Applications

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_8.4,

There is increasing interest in culturing microorganisms in two-liquid-phase systems consisting of an aqueous medium and a hydrophobic organic solvent. This culture is potentially advantageous for the bioconversion of hydrophobic compounds with low solubility in water. Hydrophobic organic solvents can be toxic to microorganisms. Therefore, organic solvent-tolerant microorganisms can be used as effective biocatalysts in an aqueous-organic solvent two-phase system.

The mechanisms of adaptation and tolerance toward organic solvents, particularly in *Pseudomonas putida* and *Escherichia coli* strains, have been extensively studied. These include morphological adaptation, changes of the energetic status, modification of the membrane's fluidity, changes in the cell wall and outer membrane, modification of surface properties, transformation or degradation of the solvent, active transport of solvents from the membrane into the environment by energy-consuming efflux systems, and modification of membrane proteins (Aono 1998; Isken and de Bont 1998; Tsukagoshi and Aono 2000; Ramos et al. 2002; Heipieper et al. 2007). The mechanisms of organic solvent-tolerance in *P. putida* are discussed in the previous section. In this section, genes involved in the organic solvent-tolerance in *Escherichia coli* are discussed. In addition, some of the selected applications of organic solvent-tolerant bacteria are described.

Genes Involved in Determination of Organic Solvent-Tolerant Levels in *Escherichia coli*

The biochemical and genetic properties of the cell structures of *E. coli* are well understood. This knowledge is useful in understanding the organic solvent-tolerance mechanisms. Although *E. coli* is less tolerant to organic solvents than several *Pseudomonas* spp., its tolerance level is relatively high among various microorganisms (Aono 1998).

E. coli strains in general can form colonies on an agar medium overlaid with *n*-hexane (log P_{ow} 3.9) but not on the medium overlaid with organic solvents with log $P_{ow} < 3.9$. Aono et al. isolated several organic solvent-tolerant mutants from *E. coli* JA300, a strain K-12 derivative (Aono 1998). The organic solvent-tolerant mutants exhibited improved tolerance to organic solvents such as cyclohexane (log P_{ow} 3.4), *n*-pentane (log P_{ow} 3.3), and *p*-xylene (log P_{ow} 3.1). Characterization of these organic solvent-tolerant mutants gave evidence that *E. coli* has genes involved in determining organic solvent-tolerance levels. Various genes implicated in the solvent-tolerance of *E. coli* have been reported so far. These genes are listed in *Table 8.4.1* and are summarized below.

acrAB and *tolC*

Bacteria have evolved to cope with various environments containing toxic compounds such as antibiotics and endogenous metabolic products. Bacteria bearing multidrug efflux systems are able to survive in adverse ecological niches. Energy-dependent efflux belonging to the resistance/nodulation/cell division (RND) family (Paulsen et al. 1996) has been shown to serve to maintain solvent-tolerance in gram-negative bacteria (White et al. 1997; Kieboom et al. 1998; Li et al. 1998; Ramos et al. 1998; Tsukagoshi and Aono 2000). These efflux pumps commonly consist of three components, a transporter protein located in the cytoplasmic membrane acting as an energy-dependent extrusion pump, a membrane fusion protein anchored to the

Table 8.4.1

Genes involved in determination of organic solvent-tolerant levels in *Escherichia coli*

Gene	Function	Effects of the gene expressions on the organic solvent-tolerance of *E. coli*	Reference
acrAB	*acrA* and *acrB* code for AcrA, a membrane fusion protein and AcrB, a transporter protein. These proteins are components of the AcrAB-TolC multidrug efflux pump, a member of the RND family. The AcrAB-TolC system was positively regulated by high levels of expression of *marA, robA,* and *soxS*.	Strain JA300 was tolerant to *n*-hexane (log P_{ow} 3.9). JA300Δ*acrAB* was sensitive to organic solvents with log P_{ow} values lower than 5.5, but was tolerant to decane (log P_{ow} 6.0). Introduction of *acrAB* genes into JA300Δ*acrAB* resulted in the complementation of *n*-hexane-tolerance.	Tsukagoshi and Aono (2000)
tolC	*tolC* codes for an outer membrane protein. TolC is a component of the AcrAB-TolC efflux pump.	JA300Δ *tolC* was sensitive to organic solvents with log P_{ow} values lower than 5.5, but was tolerant to decane (log P_{ow} 6.0). Introduction of *tolC* into JA300Δ *tolC* resulted in the complementation of *n*-hexane-tolerance.	Aono et al. (1998)
marA	*marA* codes for a DNA-binding transcriptional activator belonging to the AraC subfamily. MarA is implicated in multiple antibiotic resistances.	JA300 transformed with a high copy plasmid containing the *marA* gene is tolerant to organic solvents with log P_{ow} values higher than 3.4, including cyclohexane (log P_{ow} 3.4).	Asako et al. (1997)
robA	*robA* codes for a DNA-binding transcriptional activator belonging to the AraC subfamily.	JA300 transformed with a high copy plasmid containing the *robA* gene is tolerant to organic solvents with log P_{ow} values higher than 3.4.	Nakajima et al. (1995a)
soxS	*soxS* codes for a DNA-binding transcriptional activator belonging to the AraC subfamily. SoxS is implicated in the regulation of superoxide response regulon.	JA300 transformed with a high copy plasmid containing the *soxS* gene is tolerant to organic solvents with log P_{ow} values higher than 3.4.	Nakajima et al. (1995b)
acrEF	*acrE* and *acrF* code for AcrE, a membrane fusion protein and AcrF, a transporter protein. AcrE and AcrF are highly homologous to AcrA and AcrB, respectively.	The *acrB* deficient mutant, OST5500, was sensitive to organic solvents with log P_{ow} values lower than 4.9. OST5500 expressing *acrEF* was tolerant to organic solvents with log P_{ow} values higher than 3.4.	Kobayashi et al. (2003)

Table 8.4.1 (Continued)

Gene	Function	Effects of the gene expressions on the organic solvent-tolerance of *E. coli*	Reference
emrAB	*emrAB* code for EmrA, a membrane fusion protein, and EmrB, a major facilitator superfamily (MFS) transporter. The EmrAB-TolC system is responsible for the efflux of various toxins.	JA300Δ*acrAB* was sensitive to organic solvents with log P_{ow} values lower than 5.5, such as nonane (log P_{ow} 5.5) and octane (log P_{ow} 4.9). JA300Δ*acrAB* transformed with a plasmid containing *emrAB* was tolerant to nonane and octane, but not to heptane (log P_{ow} 4.4).	Tsukagoshi and Aono (2000)
yhiUV	*yhiU* and *yhiV* code for YhiU, a putative membrane protein and YhiV, a putative transporter. YhiU and YhiV are highly homologous to AcrA and AcrB, respectively.	JA300Δ*acrAB* transformed with a plasmid containing *yhiUV* was tolerant to nonane, but not to octane.	Tsukagoshi and Aono (2000)
ostA	*ostA*/*imp* codes for a protein involved in the transport of lipopolysaccharides to the cell surface.	An *n*-hexane sensitive strain OST4251 was converted to the *n*-hexane tolerant phenotype by transformation of a plasmid containing the *ostA* gene.	Aono et al. (1994b), Ohtsu et al. (2004)
pspA	*pspA* codes for a phage-shock protein which is induced under extreme stress conditions.	The survival frequency of JA300 cells exposed suddenly to *n*-hexane was improved by introduction of a plasmid containing the *psp* operon.	Kobayashi et al. (1998)
glpC	*glpC* codes for a subunit of GlpABC, an anaerobic glycerol-3-phosphate dehydrogenase.	The colony-forming frequency of JA300 grown on an agar medium overlaid with *n*-hexane was improved about 100-fold by introduction of a plasmid containing *glpC*.	Shimizu et al. (2005b)
fruA	*fruA* codes for a fructose-specific transport protein.	The colony-forming frequency of JA300 grown on an agar medium overlaid with *n*-hexane was improved about 100-fold by introduction of a plasmid containing *fruA*.	Shimizu et al. (2005b)
purR	*purR* codes for a DNA-binding transcriptional repressor. Most genes of the *purR* regulon function as the enzymes of nucleotide metabolism.	The colony-forming frequency of JA300 grown on an agar medium overlaid with *n*-hexane was improved about 10-fold by introduction of a plasmid containing *purA*.	Shimizu et al. (2005a)

Table 8.4.1 (Continued)

Gene	Function	Effects of the gene expressions on the organic solvent-tolerance of *E. coli*	Reference
manXYZ	*manXYZ* operon codes a sugar transporter of the phosphotransferase system.	The colony-forming frequency of JA300 grown on an agar medium overlaid with *n*-hexane was improved about 100-fold by introduction of a plasmid containing *manXYZ*.	Okochi et al. (2007)
crp	*crp* codes for a cyclic AMP receptor that acts as a DNA-binding transcriptional regulator.	The colony-forming frequency of BW25113 grown on an agar medium overlaid with the organic solvent mixture of *n*-hexane and cyclohexane (1:1) was improved about 1,000-fold by deletion of the *crp* gene.	Okochi et al. (2008)
cyaA	*cyaA* codes for an adenylate cyclase involved in cAMP biosynthetic processes.	The colony-forming frequency of BW25113 grown on an agar medium overlaid with organic solvent mixture of *n*-hexane and cyclohexane (1:1) was improved about 1,000-fold by deletion of *cyaA*.	Okochi et al. (2008)
gadB	*gadB* codes for a glutamate decarboxylase B subunit.	Deletion of *gadB* decreases the solvent-tolerance of *E. coli* BW25113.	Okochi et al. (2008)
nuoG	*nuoG* codes for a peripheral subunit of NADH:ubiquinone oxidoreductase.	Deletion of *nuoG* decreases the solvent-tolerance of *E. coli* BW25113.	Okochi et al. (2008)
ahpCF	*ahpCF* codes for an alkyl hydroperoxide reductase.	A tetralin-tolerant *E. coli* mutant was tolerant to cyclohexane, propylbenzene, and 1,2-dihydronaphthalene. By contrast, a wild type strain was sensitive to these solvents. The gene involved in the solvent-tolerance was identified as *ahpCF*.	Ferrante et al. (1995)

cytoplasmic membrane, and an outer membrane protein that is supposedly a channel which circumvents the outer membrane, allowing the discharge of the solvent into the external environment. The AcrAB-TolC efflux pump, a member of the RND family, is a major pump exporting various hydrophobic compounds in *E. coli* (Ma et al. 1995; Fralick 1996). AcrA, AcrB, and TolC are a membrane fusion protein, a transporter protein, and an outer membrane protein, respectively. Aono et al. isolated spontaneous cyclohexane-tolerant mutants from JA300 (Aono 1998). In these mutants, the enhanced expression of AcrA and TolC are observed. In contrast, the deletion of *acrAB* and *tolC* decreases the solvent-tolerance of *E. coli* (Aono et al. 1998; Tsukagoshi and Aono 2000). The amount of solvent entering *E. coli* cells was measured after the cells were incubated in an organic solvent-aqueous two phase system (Tsukagoshi and Aono 2000). In wild type strain JA300, the intracellular levels of solvents with a log P_{OW} higher than 4.4 were maintained at low levels. By contrast, the Δ*tolC* or Δ*acrAB* mutants accumulated the solvents more abundantly than the parent stain.

marA, *robA*, and *soxS*

Three genes, *marA*, *robA*, and *soxS*, elevating the organic solvent-tolerance of JA300, were cloned by the shotgun method from the JA300 chromosome itself (Nakajima et al. 1995a, b; Asako et al. 1997). These genes code for DNA-binding proteins that are transcriptional activators belonging to the AraC subfamily with the helix-turn-helix motif. The products of these genes are known to be cytoplasmic proteins positively regulating the expression of several genes belonging to the *mar-sox* regulon. *marA* and *soxS* confer tolerance on *E. coli* to multiple antibiotics and superoxide anion. It was shown that the expression of the AcrAB-TolC system was positively regulated by high levels of the expression of *marA*, *robA*, and *soxS* (Aono et al. 1995).

Cyclohexane-tolerant mutants were defective in *marR*, a repressor protein for *mar* operon including *marA* (Asako et al. 1997). Mutations in the *marR* locus generally depress the *mar* operon. Derepression of the *mar* operon causes an increase in MarA and elevates the expression of the *mar* regulon genes including *acrA*, *acrB*, and *tolC*.

acrEF

E. coli strain OST5500 is hypersensitive to solvents because *acrB* was defective by the insertion of IS30 (Kobayashi et al. 2003). Suppressor mutants showing high solvent-tolerance were isolated from OST5500. These mutants produced high levels of AcrE and AcrF proteins, which were not produced in OST5500, and in each mutant an insertion sequence (IS1 or IS2) was found integrated upstream of the *acrEF* operon. AcrE and AcrF are highly homologous to AcrA and AcrB, respectively. The suppressor mutants lost solvent-tolerance by inactivation of the *acrEF* operon. The solvent hypersensitivity of OST5500 was suppressed by introduction of the *acrEF* operon with IS1 or IS2 integrated upstream of the operon but not by introduction of the operon lacking the integrated IS. These results indicated that IS integration activated *acrEF*, resulting in functional complementation of the *acrB* mutation.

emrAB

The Emr transporter system is known to extrude various drugs. This system is comprised of EmrB (a major facilitator superfamily transporter), EmrA (a membrane fusion protein) and TolC (an outer membrane channel). *E. coli* JA300Δ*acrAB* was sensitive to nonane and octane (Tsukagoshi and Aono 2000). When JA300Δ*acrAB* was transformed with a plasmid containing *emrAB*, the transformed cells improved the nonane or octane-tolerance levels. The Emr transporter seemed to confer tolerance on *E. coli* to weakly toxic solvents such as nonane and octane. However, *emrAB* disruptants derived from JA300 and JA300Δ*acrAB* were as tolerant to solvents as JA300 and JA300Δ*acrAB*, respectively.

yhiUV

yhiUV encodes for a putative multidrug transporter which is homologous to *acrAB*. JA300Δ*acrAB* cells improved colony-forming efficiency in the presence of nonane by transformation of a plasmid containing *yhiUV* (Tsukagoshi and Aono 2000). However, the

transformed cells were sensitive to octane and *n*-hexane. JA300Δ*acrAB* cells harboring a plasmid containing *acrAB* or *acrEF* acquired tolerance against octane and *n*-hexane. Therefore, the expression of *yhiUV* seemed not to be as effective in improving the organic solvent-tolerance levels as the expression of *acrAB* or *acrEF*.

ostA

A *n*-hexane sensitivity determinant at 1.2 min was mapped on the *E. coli* genetic map by genetic analysis of the *n*-hexane-tolerant strain JA300 and its *n*-hexane-sensitive derivative OST4251 (Aono et al. 1994b). This determinant was named *ostA* (organic solvent-tolerance) and was shown to be identical to the *imp* (increased membrane permeability) gene (Aono et al. 1994c; Ohtsu et al. 2004). The *ostA* gene, when cloned on a plasmid, suppressed the *n*-hexane-sensitive phenotype of OST4251. The nucleotide sequence of the *ostA* structural gene of OST4251 was identical to that of JA300. Instead, IS2 was integrated in upstream of *ostA* gene in OST4251. The integration of IS2 at this position seemed to disrupt the putative promoter sequence. Western blotting analysis showed that the amount of OstA protein was significantly decreased in OST4251. These results showed that the *n*-hexane-sensitivity of OST4251 was caused by the lower production of the OstA production.

OstA is a minor protein associated with the outer membrane known to be essential for growth in *E. coli*, except for LolB lipoproteins. OstA mediates the transport of lipopolysaccharides to the cell surface (Bos et al. 2004). These results indicated that cell envelope biogenesis might be involved in the organic solvent-tolerance in *E. coli*.

pspA

PspA, a phage-shock protein, was strongly induced in strain JA300 cells grown in the presence of *n*-hexane or cyclooctane (Kobayashi et al. 1998). PspA is known to be induced in *E. coli* cells under extreme stress conditions and to play a role in maintenance of the proton motive force under stress conditions (Kleerebezem et al. 1996). Introduction of a multi-copy plasmid carrying the *psp* operon into *E. coli* improved the survival frequency of cells exposed suddenly to *n*-hexane, but not the growth rate of cells growing in the presence of *n*-hexane.

glpC and *fruA*

DNA microarrays were used to investigate the biological mechanism of organic solvent-tolerance in *E. coli* (Shimizu et al. 2005b). Gene expression profiles were collected from several spontaneous organic solvent-tolerant strains before and after exposure to organic solvents. Among several genes showing higher gene expression, the overexpression of the *glpC* gene improved the colony-forming efficiency of strain JA300 in the presence of *n*-hexane. In addition, the overexpression of *glpC* in the *E. coli* strain decreased the hydrophobicity of the cell surface. The gene *glpC* is one of the genes of the *glpABC* operon encoding the anaerobic glycerol-3-phosphate dehydrogenase (Cole et al. 1988). The GlpC subunit functions not as the catalytic subunit, but as the membrane anchor for the catalytic GlpAB dimmer.

The gene *fruA* was also upregulated in the solvent-tolerant strains after exposure to organic solvents. Overexpression of *fruA* slightly increased the colony formation efficiency of the wild type strain in the presence of *n*-hexane. Since FruA, as well as GlpC, is a membrane-associated protein (Geerse et al. 1986), it is likely that the expression of *fruA* increases the organic solvent-tolerance through a change in the cell surface properties.

purR

Time-course analysis of gene expression using DNA microarrays showed that *purR* regulon genes were strongly repressed after exposure to organic solvent. *purR* codes for a purine nucleotide synthesis repressor (Shimizu et al. 2005a). Most genes of the *purR* regulon function as the enzymes of nucleotide metabolism. Overexpression of *purR* in strain JA300 increased the colony-forming frequency in the presence of *n*-hexane. By contrast, deletion of *purR* decreases the solvent-tolerance of *E. coli*.

manXYZ

Transcriptional analysis using DNA microarrays revealed that the expression levels of the *manX*, *manY*, and *manZ* genes in *E. coli* were strongly upregulated after exposure to organic solvents (Okochi et al. 2007). JA300 overexpressing *manXYZ* remarkably improved colony-forming efficiency in the presence of *n*-hexane. The *manXYZ* operon encodes a sugar transporter of the phosphotransferase system (Erni and Zanolari 1985). The amount of *n*-hexane accumulated in *E. coli* was measured after the cells were incubated in an *n*-hexane-aqueous two phase system. The intracellular hexane level stayed lower in *E. coli* cells overexpressing *manXYZ* after incubation with *n*-hexane. Overexpression of *manXYZ* seemed to decrease the membrane permeability or increase the efflux of organic solvents. Adherence of the cells expressing *manXYZ* to hydrocarbons was also investigated in a two-phase mixture consisting of toluene and aqueous buffer. It was shown that *E. coli* cells expressing *manXYZ* can bind more abundantly to the organic solvent compared with the control cells. This result indicated that the expression of *manXYZ* changed the cell surface property to obtain a higher affinity to hydrocarbons.

crp, *cyaA*, *gadB*, and *nuoG*

The *glpC*, *purR*, and *manXYZ* genes described above were related to the metabolic pathway of glucose catabolism. Therefore, it was speculated that changes in metabolic flux affect the organic solvent-tolerance of *E. coli*. In *E. coli*, there are seven global transcriptional regulators (i.e., *arcA*, *arcB*, *cra*, *crp*, *cyaA*, *fnr*, and *mlc*) involved in carbon catabolism (Martinez-Antonio and Collado-Vides 2003). The organic solvent-tolerances of the knockout mutants of these seven global regulator genes were investigated to identify the metabolic pathway related to organic solvent-tolerance (Okochi et al. 2008). Among these genes, the deletion of *crp* and *cyaA* increased the solvent-tolerance of *E. coli*. The *crp* and *cyaA* genes code for a cyclic AMP receptor protein and an adenylate cyclase, respectively. Since Crp and cAMP, synthesized from Cya, are involved in catabolite repression in *E. coli*, it was indicated that the formation of the cAMP-Crp complex is related to the solvent-tolerance. In the knockout mutants of *crp* and *cyaA*, the

expression levels of *gadB* and *nuoG* were higher than those of the wild type strain. *gadB* and *nuoG* code for a glutamate decarboxylase B subunit (Smith et al. 1992) and a peripheral subunit of NADH: ubiquinone oxidoreductase (Falk-Krzesinski and Wolfe 1998), respectively. Δ*gadB* and Δ*nuoG* mutants decreased the organic solvent-tolerance levels, suggesting that the expressions of these proteins are involved in the organic solvent-tolerance in the Δ*crp* and Δ*cyaA* mutants. Since GadB and NuoG function as proton transporters, proton transport might affect the organic solvent-tolerance in *E. coli*.

ahpCF

Organic solvent-tolerant *E. coli* mutant was isolated on an agar medium overlaid with a solvent, tetralin (1,2,3,4-tetrahydronaphthalene) (Ferrante et al. 1995). The mutant was tolerant not only to tetralin, but also to cyclohexane, propylbenzene, and 1,2-dihydronaphthalene. The wild type strain was not tolerant to these solvents. The gene involved in the solvent-tolerance was cloned from a recombinant library from mutant DNA and identified as alkylhydroperoxide reductase operon *aphCF*. A mutation was localized to a substitution of valine for glycine at position 142 in the coding region of *ahpC*. The *ahpC* mutant was found to have three times higher activity compared with that of the wild type strain in reducing tetralin hydroperoxide to 1,2,3,4-tetrahydro-1-naphthol. These results showed that the toxicity of solvents such as tetralin seemed to be caused by the formation of toxic hydroperoxides in the cell. The *ahpC* mutation increased the enzyme activity and acquired the tolerance to hydrophobic hydroperoxides.

Other Genes

Aono reported that the expression of two genes, *ostB* and *ostC*, improved the organic solvent-tolerance level of the *ostA*-defective *E. coli* mutant (Aono 1998). However, these genes did not improve other strains. *ostB* seemed to encode a 26-kDa cytoplasmic protein having a helix-turn-helix motif like a GntR family protein. OstB probably regulates the expression of *ostC*. OstC is thought to encode a 52-kDa inner membrane protein having 14 transmembrane domains. The OstC protein is homologous to AraE family proteins, indicating that this protein might be a H^+/drug antiporter. This gene seems to correspond to the *bglT* gene.

Asako et al. observed that the levels of OmpF protein were markedly decreased in the *E. coli* mutants because of mutations in *marR* (Asako et al. 1999). It was reported that the OprF porin protein was absent in a toluene-tolerant mutant of *Pseudomonas aeruginosa* (Li et al. 1995). Hydrophobic β-lactam antibiotics passed through OmpF channels faster than through OmpC channels (Nikaido et al. 1983). It thus seemed likely that organic solvent molecules could also pass through the OmpF porin. Therefore, it was considered likely that the decreased levels of OmpF or loss of OmpF might contribute to improving the organic solvent-tolerance of *E. coli*. The organic solvent-tolerance of *E. coli* was measured under conditions in which the OmpF levels were controlled by various means as follows: alteration of NaCl concentration in the medium, transformation with a stress-responsive gene (*marA*, *robA*, or *soxS*), or disruption of the *ompF* gene (Asako et al. 1999). These results indicated that the solvent-tolerance of *E. coli* did not depend upon the OmpF levels in the membrane.

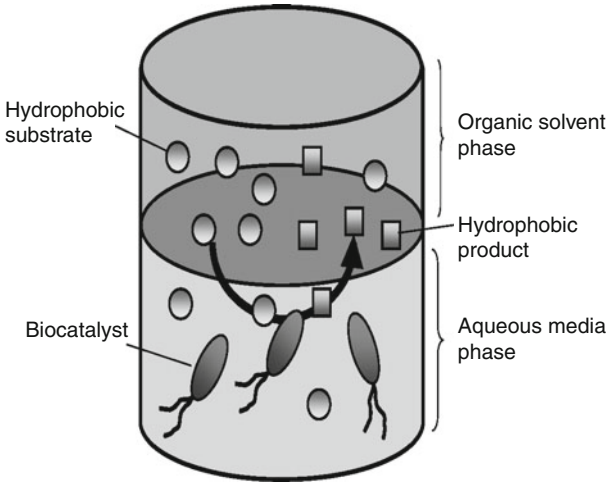

Fig. 8.4.1

Scheme of a two-phase biotransformation system. The hydrophobic substrate partitions from the organic solvent phase into the water phase, bioconversion by whole cells takes place, and then the hydrophobic product partitions into the solvent phase

Applications of Organic Solvent-Tolerant Microorganisms

The use of whole-cell biocatalysts has been employed for biotransformations of hydrophobic compounds in aqueous-organic solvent two-phase systems (*Fig. 8.4.1*). The advantages of the two-phase systems include not only the production of hydrophobic compounds, but also the maintenance of a low concentration of toxic or inhibitory compounds in the aqueous phase which reduces substrate and/or end-product inhibition and an easier recovery of both product and biocatalyst. Whole-cell biocatalysts are beneficial in the biotransformations involving in their internal cofactor regeneration and bioconversions requiring multi-step metabolic pathways. The formation of epoxides from alkenes and alkanes by *Pseudomonas oleovorans* or *E. coli* recombinant carrying alkane mono-oxygenase genes from *P. oleovorans* is one of the well studied examples for bioconversion in the two-phase system (Schwartz and McCoy 1977; Witholt et al. 1990; Wubbolts et al. 1996).

When organic solvent-sensitive microorganisms are used in the bioconversion in the two-phase system, the range of available organic solvents is limited and the productivity levels are often lowered. The solvent-tolerant organisms are considered to expand the usability for bioconversion in the presence of a wide range of the solvents. A number of two-phase biotransformation systems using organic solvent-tolerant microorganisms have been studied. Several selected examples of these bioconversions are listed in *Table 8.4.2* and some are described below.

Bioconversion of Cholesterol

There are several water-insoluble compounds of commercial importance. Cholesterol, being typical of insoluble materials, is usually suspended in bioconversion systems containing

Table 8.4.2

Selected applications of organic solvent-tolerant microorganisms

Biocatalyst	Biocatalytic properties	Additional information	Reference
Pseudomonas putida DOT-T1	Degradation of toluene	Strain DOT-T1 grew at the expense of toluene as a sole carbon source provided at 0.1 to 90% (vol/vol) in the culture medium. The strain uses the toluene-4-monooxygenase pathway to metabolize toluene. The catabolic potential of this strain was expanded by transfer of the TOL plasmid pWW0-Km.	Ramos et al. (1995)
Pseudomonas putida S12	Degradation of stylene, octanol, and heptanol	Strain S12 grew in a mineral salts medium containing 1% (vol/vol) stylene, octanol, and heptanol as a sole carbon source.	Weber et al. (1993)
Burkholderia cepacia ST-200	Oxidative modification of cholesterol	Strain ST-200 effectively converted cholesterol to 6β-hydroxycholest-4-en-3-one and cholest-4-ene-3,6-dione in a medium overlaid with a 10% volume of various organic solvents such as cyclooctane and a mixture of diphenylmethane and *p*-xylene.	Aono et al. (1994a), Aono and Doukyu (1996), Doukyu et al. (1996)
Pseudomonas putida ST-491	Production of steroid hormone precursors from lithocholic acid	Strain ST-491 produced steroid hormone precursors, androsta-4-dien-3,17-dione and androsta-1,4-diene-3,17-dione from lithocholic acid in a medium overlaid with a 20% volume of diphenyl ether.	Suzuki et al. (1998)
Burkholderia cepacia ST-200	Production of indigo from indole	Strain ST-200 produced indigo from indole in a medium overlaid with a 20% volume of cyclooctane, diphenylmethane and propylbenzene.	Doukyu and Aono (1997), Doukyu et al. (1998)
Acinetobacter sp. ST-550	Production of indigo from indole	Strain ST-550 effectively produced indigo from indole in a medium overlaid with a 3–30% volume of diphenylmethane.	Doukyu et al. (2002)
Escherichia coli mutant (recombinant)	Production of indigo from indole	A cyclohexane-tolerant *E. coli* mutant carrying the phenol hydroxylase gene from *Acinetobacter* sp. ST-550 effectively produced indigo from indole in a medium overlaid with a 10% volume of diphenylmethane.	Doukyu et al. (2003)
Rhodococcus opacus B-4	Production of indigo from indole	Strain B-4 cells that were suspended in bis(2-ethylhexyl)phalate effectively produced indigo from indole.	Yamashita et al. (2007)

Table 8.4.2 (Continued)

Biocatalyst	Biocatalytic properties	Additional information	Reference
Pseudomonas putida MC2 (recombinant)	Production of 3-methylcatechol from toluene	Strain MC2 carrying *todC1C2BAD* genes produced 3-methylcatechol from toluene in a medium overlaid with a 50% volume of octanol.	Hüsken et al. (2001a)
Pseudomonas putida S12 (recombinant)	Production of 3-methylcatechol from toluene	Strain S12 carrying *todC1C2BAD* genes produced 3-methylcatechol from toluene in a medium overlaid with a 40% volume of octanol.	Wery et al. (2000a)
Pseudomonas putida S12 (recombinant)	Production of phenol from glucose	Strain S12 carrying the tyrosine phenol lyase gene from *Pantoea agglomerans* effectively produced phenol from glucose in a medium overlaid with a 16% volume of octanol.	Wierckx et al. (2005)
Flavobacterium sp. DS-711	Degradation of crude oils and various *n*-alkanes	Strain DS-711 effectively degraded crude oils and various *n*-alkanes (C_7–C_{16}).	Moriya and Horikoshi (1993b)
Bacillus sp. DS-1906	Degradation of polyaromatic compounds	Strain DS-1906 effectively degraded polyaromatic compounds such as naphthalene, fluorine, phenanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene in a medium overlaid with a 10% volume of *n*-hexane.	Abe et al. (1995)
Bacillus sp. DS-994	Degradation of organic sulfur compound	Strain DS-1906 degraded organic sulfur compounds such as dibenzothiophene, thiophene, and ethylmethylsulfide in a medium overlaid with a 50% volume of model petroleum.	Moriya and Horikoshi (1993a)
Arthrobacter sp. ST-1	Production of steroid hormone precursors from cholesterol	Strain ST-1 produced androsta-1,4-diene-3,17-dione from cholesterol in a medium overlaid with a 50% volume of organic solvents such as dodecane, *n*-hexane, toluene, and benzene.	Moriya et al. (1995)
Pseudomonas putida A4 (recombinant)	Degradation of dibenzothiophene	Strain A4 carrying the biodesulfurizing gene cluster *dszABCD* from *Rhodococcus erythropolis* XP effectively degraded dibenzothiophene in a medium overlaid with a 10% volume of *p*-xylene.	Tao et al. (2006)
Moraxella sp. MB1	Production of decarboxycitrinin, a mycotoxin, from citrinin	Strain MB1 converted citrinin to decarboxycitrinin in a medium overlaid with a 50% volume of ethyl acetate.	Devi et al. (2006)

Table 8.4.2 (Continued)

Biocatalyst	Biocatalytic properties	Additional information	Reference
Bacillus sp. BC1	Production of cholest-4-en-3,6-dione from cholesterol	Strain BC1 transformed cholesterol to cholest-4-en-3,6-dione in a medium overlaid with a 50% volume of chloroform.	Sardessai and Bhosle (2003)

surfactants to improve the conversion reaction rate. It has also been established that cholesterol dissolved in organic solvents at a high concentration is converted by immobilized enzymes or resting cells in the water phase of a water-organic solvent two-phase system. Thus, cholesterol was used to examine the potential advantage of organic solvent-tolerant microorganisms in the two-phase system.

An organic solvent-tolerant and cholesterol-converting microorganism, *Pseudomonas* sp. (reclassified as *Burkholderia cepacia*) strain ST-200 was isolated from humus soil (Aono et al. 1994a). Strain ST-200 was tolerant to various organic solvents such as decane, nonane, cyclooctane, *n*-octane, diphenylmethane, and cyclohexane, but not *p*-xylene. Strain ST-200 grew in a medium overlaid with a 10% volume of organic solvents containing cholesterol and effectively converted cholesterol to 6β-hydroperoxycholest-4-en-3-one and cholest-4-ene-3,6-dione (*Fig. 8.4.2*). The conversion was not effective in a uniphasic system in which cholesterol was suspended (*Fig. 8.4.3*). This result indicated that a two phase fermentation system using organic solvent-tolerant microorganisms is effective for bioconversion of a hydrophobic compound.

Yield of the conversion products can be controlled by changing the organic solvent used to dissolve cholesterol (Aono and Doukyu 1996; Doukyu et al. 1996. When a 10% volume of cyclooctane containing 2% (wt/vol) cholesterol was added to the medium, the predominant product was cholest-4-ene-3,6-dione and the recovery was 60% of the initial cholesterol. When a 10% volume of a mixed solvent of diphenylmethae-*p*-xylene (7:3, vol/vol) containing 2% (wt/vol) cholesterol was used, 6β-hydroperoxycholest-4-en-3-one was predominant and recovery was 70% of the initial cholesterol.

Bioconversion of Lithocholic Acid to Steroid Hormone Precursors

Microbial cleavage of the side chains of steroids has received a great deal of attention, since it allows the use of inexpensive steroids as raw materials for the production of steroid hormones. Suzuki et al. planned to use a two-phase fermentation system for cleavage of the side chains of organic solvent-insoluble steroids (Suzuki et al. 1998). Lithocholic acid and deoxycholic acid have C-24 carboxyl groups on their side chains. These bile acids are insoluble in hydrophobic organic solvents due to the polar carboxyl groups. When the polar carboxyl groups of lithocholic acid were cleaved and the 3-hydroxyl group of the compound was oxidized by microorganisms in the two phase system, more hydrophobic steroid hormone precursors such as androsta-4-dien-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) were formed and extracted into the organic solvent phase (*Fig. 8.4.4*). Thus, the two phase system can be used for an extractive fermentation of hydrophobic steroid hormone precursors from polar bile acids. Organic solvent-tolerant microorganisms are expected to be effective for the

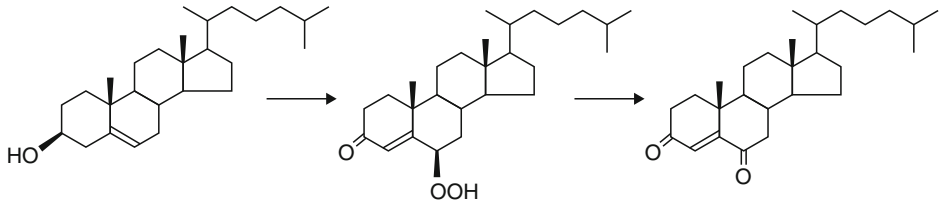

Fig. 8.4.2

Scheme of cholesterol oxidation by *Burkholderia cepacia* strain ST-200

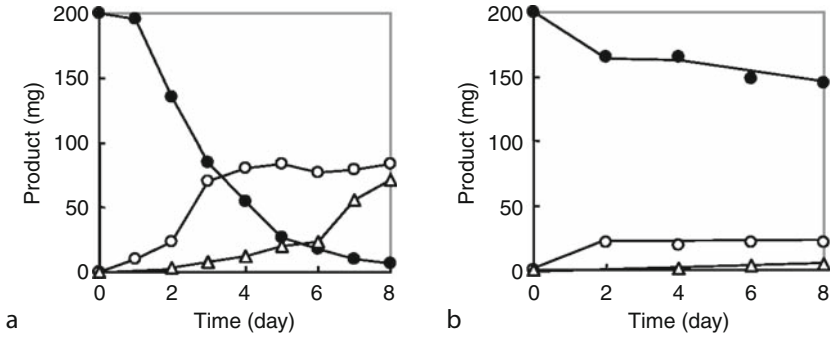

Fig. 8.4.3

Conversion of cholesterol by strain ST-200. Strain ST-200 was inoculated in 100 ml of conversion medium containing 200 mg of cholesterol. (a) Cholesterol was added to the medium as 10 ml of a 2% (wt/vol) cholesterol solution in the mixed solvent of *p*-xylene and diphenylmethane (3:7, vol/vol). (b) Soild cholesterol was suspended in the medium to give a 0.2% (wt/vol) concentration. The organism was grown at 30°C with shaking. Symbols: *closed circle*, cholesterol; *open circle*, 6β-hydroperoxycholest-4-en-3-one; *open triangle*, cholest-4-ene-3,6-dione

fermentation in the presence of organic solvents. A microorganism capable of converting bile acids to steroid hormone precursors in the presence of cyclooctane was isolated from humus soil and identified as *Pseudomonas putida* strain ST-491. Strain ST-491 grew on an agar medium overlaid with various organic solvents, such as cyclooctane, diphenyl ether, *n*-hexane, or *p*-xylene.

The effects of organic solvents on the cleavage of lithocholic acid were examined for strain ST-491 (*Fig. 8.4.5*). In the absence of solvent, strain ST-491 grown on 0.5% (wt/vol) lithocholic acid catabolized approximately 30% of the substrate as a carbon source and transiently accumulated ADD in an amount corresponding to 5% of the substrate added. When 20% (vol/vol) diphenyl ether was added to the medium containing 0.5% (wt/vol) lithocholic acid, 60% of the substrate was converted to 17-ketosteroids (AD and ADD) or a 22-aldehyde steroid (pregne-1,4-dien-3-on-20-al) (PDOA). The amounts of the products were responsible for 45% (ADD), 10% (PDOA), and 5% (AD) of the substrate, respectively. These results showed that diphenyl ether facilitates catabolism from lithocholic acid to ADD rather than suppressing the assimilation of ADD. In addition, these results indicated that a two

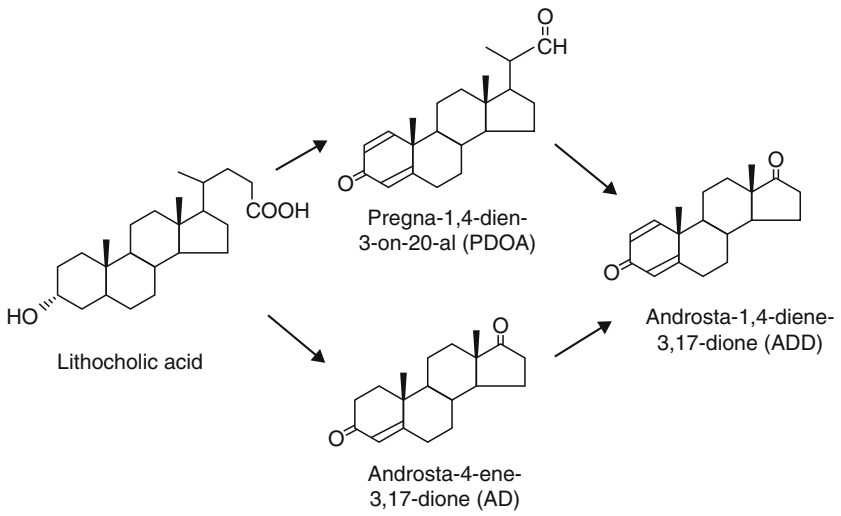

Fig. 8.4.4

Proposed scheme of conversion of lithocholic acid by *Pseudomonas putida* strain ST-491

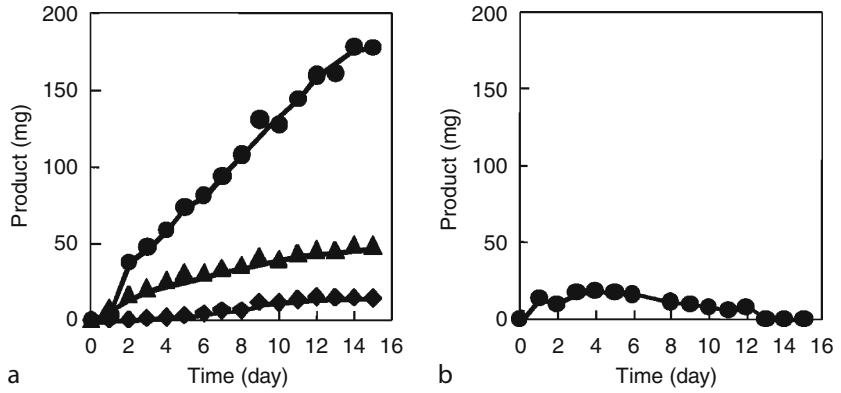

Fig. 8.4.5

Conversion of lithocholic acid by strain ST-491. Strain ST-491 was grown at 30°C with shaking in 100 ml of a medium containing 500 mg of lithocholic acid. (a) To the medium, 20 ml of diphenyl ether was added. (b) No solvent was added. Symbols: *circle*, ADD; *triangle*, PDOA; *diamond*, AD

phase fermentation system can be used not only for bioconversion of a hydrophobic compound but also for that of a compound with low solubility in organic solvents.

Bioconversion of Indole to Indigo

Several fine chemicals and their raw materials such as aromatic compounds and low molecular weight aliphatic compounds are toxic to microorganisms. Therefore, in many cases, the

production of fine chemicals by viable cells is impeded by their toxicity to the cells. Many hydrophobic compounds, including several fine chemicals, are more soluble in organic solvents than in water.

Indigo is one of the world's largest-selling textile dyes used on cotton and wool fabrics. It was traditionally produced from plants of the genus *Indigofera*. Plant-derived indigo has been replaced by synthetic indigo in the textile industry. Several attempts to produce indigo from indole by microorganisms expressing monooxygenase or dioxygenase have been reported (Murdock et al. 1993; Doukyu et al. 1997). One obstacle to the production of indigo is the toxicity of the substrate indole to the producers. The concentration of indole in the medium must be kept low to avoid the toxic effect (Doukyu and Aono 1997). However, the substrates are readily consumed by the microorganisms when little indole is supplied. Thus, indigo formation is not very effective.

We isolated *Acinetobacter* sp. strain ST-550 that effectively produced indigo from indole in the two phase system (Doukyu et al. 2002). ST-550 produced a slight amount of indigo (less than 0.1 mg/ml) when grown in the presence of indole at concentrations of 0.05–0.3 mg/ml. However, ST-550 effectively produced indigo when ST-550 was grown in the presence of a large volume of diphenylmethane and a high level of indole. One of the optimized conditions for indigo production was that ST-550 was grown in 3 ml of a medium containing 0.3 ml of diphenylmethane and 2.7 mg of indole. Under the condition, ST-550 produced 0.88 mg of indigo (292 μg/ml-medium).

Particular proteins increased remarkably in strain ST-550 grown in the two-phase culture system for indigo production (Doukyu et al. 2003). Among these proteins, the N-terminal amino acid sequence of the 60 kDa-protein was identical to that of the largest subunit of phenol hydroxylase (MopN) from *Acinetobacter calcoaceticus* NCBI8250. The indigo-producing activity was strongly induced when ST-550 was grown with phenol as a sole carbon source. Genes coding for the multicomponent phenol hydroxylase were cloned based on the homology with *mopKLMOP* from *A. calcoaceticus* NCBI8250. *E. coli* JA300 and its cyclohexane-tolerant mutant OST3410 carrying the hydroxylase genes and the NADH regeneration system were grown in the two-phase culture system. OST3410 recombinant produced 52 μg indigo/ml of the medium in the presence of diphenylmethane. This productivity was 4.3-fold higher than that of JA300 recombinant.

3-Methylcatechol Production from Toluene

Catechol and its derivatives are important chemicals that are used in the manufacture of synthetic flavors such as vanillin and are used as precursors for pharmaceutical production. They are difficult to synthesize chemically (Hüsken et al. 2002).

Wery et al. improved 3-methylcatechol production from toluene using the solvent-tolerant *Pseudomonas putida* S12 in a two-liquid-phase system consisting of aqueous media and a water-immiscible octanol phase (Wery et al. 2000b). The solvent-tolerant *P. putida* S12 is not able to metabolize toluene. In order to obtain a strain S12 that produces 3-methylcatechol from toluene, the genes from *P. putida* F1 coding for toluene dioxygenase, *todC1C2BA*, and *cis*-toluene dihydrodiol dehydrogenase, *todD*, were introduced into strain S12. Production of 3-methylcatechol was monitored in different incubation systems using a single medium and a two-liquid medium-octanol system. The maximum concentration of 3-methylcatechol increased twofold by the use of the two-liquid medium-octanol system.

Husken et al. also investigated the bioproduction of 3-methylcatechol from toluene by *P. putida* MC2 in the presence of an additional 1-octanol phase (Hüsken et al. 2001b). The strain originally contained a natural toluene degradation pathway. This native pathway was mutated such that enzymatic degradation of the product was no longer possible. Additional sets of *todC1C2BAD* genes for 3-methylcatechol production were introduced into this strain. In the presence of 50% (vol/vol) of octanol, the 3-methylcatechol concentration per overall reactor volume (octanol and water) reached 25 mM. This product concentration is much higher than in aqueous media without organic solvent.

Bioproduction of Phenol from Glucose

Processes for the production of chemicals from natural renewable resources (green processes) have attracted a great deal of attention. These processes are expected to reduce fossil resources dependency and maintain closed carbon cycles. Wierckx et al. constructed a solvent-tolerant *Pseudomonas putida* S12 effectively producing phenol from glucose by introducing the *tpl* gene from *Pantoea agglomerans*, encoding tyrosine phenol lyase, into the solvent-tolerant strain *P. putida* S12 (Wierckx et al. 2005). Tyrosine availability was a bottleneck for efficient production. The production host was optimized by overexpressing the *aroF-1* gene, which codes for the first enzyme in the tyrosine biosynthetic pathway, and by random mutagenesis procedures involving selection with the toxic antimetabolites. In a fed-batch process, the productivity was limited by accumulation of 5 mM phenol in the medium. This toxicity was overcome by use of octanol as an extractant for phenol in a biphasic medium-octanol system. This approach resulted in the accumulation of 58 mM phenol in the octanol phase, and there was a twofold increase in the overall production compared to a single-phase fed batch.

Bioremediation

Much attention has been focused on bioremediation technology as a means of recovering from environmental pollution. This technique utilizes microorganisms to decompose or remove pollutants such as crude oil, aromatic hydrocarbons, and sulfur compounds.

Kato et al. found that 100 times more organic solvent-tolerant microorganisms can be isolated from deep-sea mud samples than from soil samples taken from land (Kato et al. 1996). Many useful organic solvent-tolerant microorganisms have been isolated from the deep sea. Among these microorganisms, *Flavobacterium* sp. strain DS-711 showed halotolerant growth and tolerance to various organic solvents such as benzene, toluene, and *p*-xylene (Moriya and Horikoshi 1993b). Strain DS-711 effectively degraded crude oils and various *n*-alkanes. *Bacillus* sp. strain DS-1906 was also isolated from the deep sea (Abe et al. 1995). This strain was tolerant to benzene, cyclohexane, and *n*-hexane. It degraded polyaromatic compounds such as naphthalene, fluorine, phenanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene in a liquid *n*-hexane-medium two-phase system.

Sulfur oxides generated by the combustion of sulfur-containing fossil fuel cause severe environmental pollution. Biodesulfurization is thought to be an interesting alternative for the development of a new petroleum-refining process. The derivatives of dibenzothiophene (DBT) and benzothiophene, as well as other polycyclic aromatic sulfur heterocyclic compounds, are the most abundant heterocyclic compounds in petroleum. *Bacillus* sp. strain DS-994 isolated

from the deep sea was tolerant to various organic solvents such as benzene, toluene, and *p*-xylene (Moriya and Horikoshi 1993a). This strain grew in a liquid medium overlaid with model petroleum oil containing DBT and degraded DBT dissolved in the oil.

A solvent-tolerant desulfurizing bacterium, *Pseudomonas putida* A4, was constructed by introducing the biodesulfurizing gene cluster *dszABCD*, which was from *Rhodococcus erythropolis* XP, into the solvent-tolerant strain *P. putida* Idaho (Tao et al. 2006). Desulfurization of various sulfur-containing heterocyclic compounds by *P. putida* strain A4 was investigated in the presence of various organic solvents. Strain A4 degraded DBT at a specific rate of 1.29 mM g (dry weight) of cells^{-1} h^{-1} for the first 2 h in the presence of 10% (vol/vol) *p*-xylene.

Closing Remarks

Various genes implicated in the organic solvent-tolerance of *E. coli* have been reported so far. Among these genes, genes encoding for efflux pumps which belong to the RND family are crucial for organic solvent-tolerance in *E. coli*. *E. coli* strains become hypersensitive to organic solvents by disruption of the genes encoding for the efflux pumps including *acrAB* or *tolC*. This efflux pump system seems to discharge organic solvent molecules incorporated into the cells and to play an important role in maintenance of the organic solvent-tolerance of *E. coli* (Tsukagoshi and Aono 2000). As well as *E. coli*, this pump system confers tolerance on *Pseudomonas* species to organic solvents (Ramos et al. 2002). The efflux pump systems are considered to contribute to determining the intrinsic levels of organic solvent-tolerance in a wide range of gram-negative bacteria. There are many other genes contributing to improving organic solvent-tolerance levels. These findings indicate that *E. coli* strains have various strategies for organic solvent-tolerance. However, the mechanisms of solvent-tolerance by the expression of these genes are not clear. Further studies on mechanisms to improve organic solvent-tolerance levels will be conducted in the future.

There are several approaches to obtaining organic solvent-tolerant microorganisms which possess biocatalytic properties available for the two-phase bioconversion system. The approaches described in this section can be roughly divided into two groups as follows: (1) screening of organic solvent-tolerant microorganisms which naturally possess useful biocatalytic properties and (2) introduction of genes encoding for useful enzymes into organic solvent-tolerant microorganisms. Both of these approaches require broadening of the knowledge of the mechanism of organic solvent-tolerance to construct highly efficient bioconversion systems in the presence of organic solvents.

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Part 9

New Frontiers: Deep Biosphere

9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_9.1,

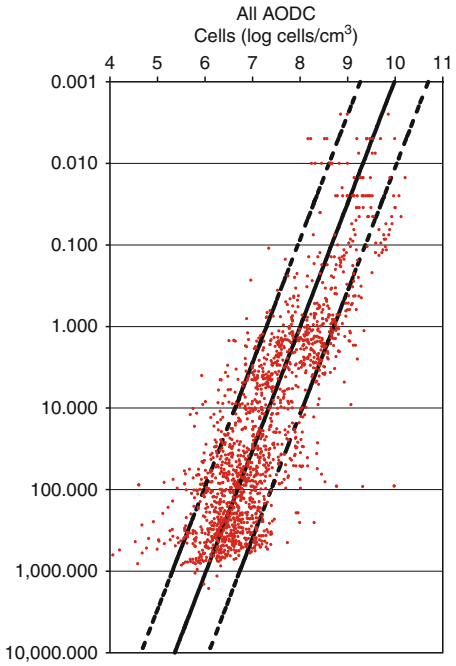
The Marine Biosphere in Deep Sediments

Oceans cover about 70% of the Earth's surface and beneath these there are normally sediments accumulated from the 5–10 billion tons of particulate matter that are constantly sinking within them. Hence, these sediments are very extensive and can be up to 10 km thick, although average depths are about 500 m. They are a major global reservoir for compounds such as organic carbon, nitrogen, iron, and sulfur which impacts element cycles, climate, and the redox state of the Earth on geological timescales. The average ocean depth above these sediments is about 3,800 m, which exerts a pressure of 38 MPa. Hence, ocean sediments are characteristically high-pressure environments. Surface sediments are cold, around 2°C, although temperatures do gradually increase with depth (~30°C/km). But so does pressure, which squeezes the sediment, increasing compaction and reducing porosity. These high-pressure conditions and initially low temperatures, together with limited input of photosynthetically produced organic matter (<1% of sea surface production), have meant that ocean sediments have been traditionally considered an extreme environment for microorganisms. Initial investigations seemed to confirm this view, with Morita and Zobell in the 1950s claiming that they had reached the limit of the biosphere at 7.47 m depth, as they could not culture microorganisms from deeper layers (Morita and ZoBell 1955). Sporadic reports of microorganisms much deeper were usually dismissed as contaminants from surface sediments introduced during coring.

Newer data from deep sediments obtained by the Ocean Drilling Program (ODP), such as subsurface biogenic methane gas occurring to hundreds of meters depth and continued alteration of organic compounds, started to suggest that microorganisms might, after all, be present in subseafloor sediments to considerable depths. This combined with the knowledge that cultivation severely underestimates the number of microorganisms detected in environmental samples and that both low (<2°C) and high temperature (>100°C) adapted prokaryotes exist, as well as high-pressure adapted organisms, made researchers to look again for evidence of deep, subseafloor microbial activity (Whelan et al. 1986). In 1986 microbiologists conducted the first comprehensive investigation of the presence and activity of prokaryotes in subseafloor sediments (Parkes et al. 1990) using a range of complimentary approaches (direct microscopy, cultivation, radiotracer activity measurements) alongside indirect measurement of activity reflected in changes in pore water chemistry (sulfate) and gas concentrations (methane). These and subsequent studies demonstrated surprisingly large prokaryotic populations to hundreds of meters depth, as reported in a seminal *Nature* paper in 1994 by Parkes et al., which suggested that an additional 10% of all known biomass may be present as prokaryotic cells in subseafloor sediments, globally.

Prokaryotic Distributions and Concentrations in Marine Sediments

Prokaryotic cells determined by direct microscopy have been shown to be ubiquitous in a broad range of different subsurface sediment types (e.g., deep-sea clays, carbonates, chalk, nannofossil and siliceous oozes, terrigenous and hydrothermal sediments, turbidites, sapropels, gas hydrate containing sediments) and oceanographic settings (continental, deep [~6,000 m] and cold [~2°C] water sediments, Pacific and Atlantic Oceans, hydrothermal systems (up to 150°C), subduction zones, Mediterranean and Japan Seas, Amazon Fan, carbonate mounds, ridges and basins). In general, cell numbers are highest in near-surface sediments (~10^9/cm^3) and then decrease exponentially with increasing depth (*Fig. 9.1.1*;



Prokaryotic cell distributions in deep sediments from ODP legs worldwide.

Deep bacteria ubiquitous.

Ranges

- Water depth to 5,867 m.
- Sediment depth 1,626 mbsf.
- Low to high OM (~0.4 – 6.8%).
- Low to high temperature (2-~150C-hydrothermal).
- Ancient deposits (~111 Mya)
- Many environments bacteria continuously decrease with depth.
- Biomass to 500 m, globally equals ~10% of surface biosphere carbon, with both higher (~30%, extrapolated to 4 km) and lower estimates (minimum based on detectable living cells).
- Regression log cells = 8.0014 –0.6599 × Log depth(m), N = 1,889

Fig. 9.1.1

Distribution of prokaryotic cells in subseafloor sediments worldwide. The *solid line* is the regression line and the *dotted lines* either side are the 95% prediction limits. ODP Ocean Drilling Program, OM organic matter (Modified from Parkes et al. 1994)

Parkes et al. 2000). This probably is due to the preferential degradation of the most labile sedimentary organic matter leading to the remaining organic matter becoming increasingly recalcitrant during burial and supporting a lower prokaryotic population. At the average ocean sediment depth of 500 m, there is a ~97% decrease in cell numbers, but cell numbers are still substantial (~3×10^6/cm^3). Globally prokaryotic cell numbers down to 500 m could represent ~10% of all biomass carbon (Parkes et al. 1994) and an extrapolation to 4 km (Whitman et al. 1998) suggests that up to 70% of all prokaryotes might live in subseafloor sediments.

Currently, the deepest sediments with quantified prokaryotic populations is 1,626 mbsf (meters below seafloor) in the Newfoundland Margin (Roussel et al. 2008, hemipelagic mudrocks with gravity flow deposits), at ~1.5×10^6/cm^3. As cell depth distributions followed the above general global trend (Parkes et al. 2000) and the decrease in cell numbers did not steepen with depth, it is likely that prokaryotes will be present in even deeper sediments. Temperatures in the deepest layers of the Newfoundland Margin were estimated to be between 60 and 100°C, and there was molecular genetic evidence for the presence of appropriate thermophiles and hyperthermophiles (novel *Pyrococcus* and *Thermococcus*). These sediments were 111 Myr old, and hence, only ancient and recalcitrant organic matter should be present, but there was also evidence that some of the prokaryotes present (Anaerobic Methane Oxidizing, ANME, sequences) were utilizing hydrocarbons, potentially of deep thermogenic origin.

The large estimates of cell numbers in subseafloor sediments have been questioned due to perceived energy limitation, suggesting that "Most microorganisms in subseafloor sediments are either inactive or adapted for extraordinary low metabolic activity" (D'Hondt et al. 2002). Direct measurement of active cells (CARD-FISH which targets ribosomes, Schippers et al. 2005), however, showed that a large fraction of subseafloor prokaryotes were alive even in deep and ancient sediments. This result is supported by measurement of depth distributions of another biomolecule for living prokaryotes, intact polar membrane lipids (Zink et al. 2003; Lipp et al. 2008), and prokaryotic community and activity changes at deep interfaces (7–11 Myr old, Parkes et al. 2005). Active prokaryotes have also been detected in the top ~9 m (~70 Myr old) of South Pacific Gyre sediments (D'Hondt et al. 2009), which have the lowest organic matter concentrations (~0.2%) and sediment accumulation rates of all ocean sediments. At this site hydrogen supplied from radiolysis of water by the radioactive decay of minerals may be as important as organic matter degradation for sustaining prokaryotic populations. However, activity and prokaryotic biomass is extremely low, orders of magnitude lower than at other subseafloor sites and, if this is representative of other ocean gyre sediments, it would reduce subseafloor biosphere global estimates.

Diversity of Marine Deep Biosphere Prokaryotes

Molecular Biological Surveys of *Bacteria* and *Archaea*

Molecular surveys on deep subsurface sediments reveal a microbial community that strongly differs from those found in surface sediments. But as the dominant archaeal and some of the bacterial phyla present have not been cultured, we can only speculate about the metabolic diversity and the ecological niches of most subseafloor prokaryotes.

The bacterial community in the deep biosphere is dominated by certain subphyla of the *Chloroflexi*, the *Gammaproteobacteria*, and the candidate division JS1 (*Table 9.1.1*). These have been found in almost all subsurface sediments and represent the majority of bacterial clones and DGGE (denaturing gradient gel electrophoresis) bands detected. For example, members of the *Chloroflexi* have been shown by quantitative PCR to represent 40–80% of all 16S rRNA genes in subsurface sediments of the Eastern Mediterranean (Coolen et al. 2002). *Deltaproteobacteria* are also frequently found but appear to be less numerous. Most deltaproteobacterial sequences are only distantly related to known organisms and it is unclear what physiological groups they represent (Coolen et al. 2002; Reed et al. 2002; Inagaki et al. 2003; Parkes et al. 2005). This is different from the subsurface *Gammaproteobacteria* that are generally closely related to cultured genera (e.g., *Pseudomonas*, *Halomonas*, *Acinetobacter*). Other bacterial phyla like the *Alphaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, the novel candidate division OP8, and the NT-B2 and NT-B6 clusters have less frequently been found, although the NT-B6 cluster may be dominant when it is present (Reed et al. 2002).

Molecular surveys of *Archaea* in marine subsurface sediments revealed an unexpected high diversity of *Crenarchaeota*, *Euryarchaeota*, and the Deep-Sea Archaeal Group (DSAG, also called Marine Benthic Group B) which does not closely affiliate with the *Eury-* nor the *Crenarchaeota* (Coolen et al. 2002; Reed et al. 2002; Inagaki et al. 2003; Kormas et al. 2003; Parkes et al. 2005; Biddle et al. 2006). At most sites the Miscellaneous Crenarchaeotic Group (MCG) is dominant, while several other groups are also widespread but appear to be numerically less abundant as inferred from clone libraries or the number of DGGE bands (*Table 9.1.2*). These include the

Table 9.1.1

Contribution of different bacterial groups detected by molecular biological approaches in different subsurface sediments

Site	Percent of all clones or DGGE bands									Total no. of clones or DGGE bands
			Proteobacteria							
	Chlorofl.	JS1	*Alpha*	*Gamma*	*Delta*	OP8	*Firmic.*	*Actinob.*	others	
Peru Margin, ODP site 1229 (water depth 150 m)[a]										
6.7 mbsf	51	2	–	36	–	2	–	–	9	***58***
30 mbsf	76	2	–	1	–	4	–	–	17	***86***
87 mbsf	34	–	3	47	1	1	–	–	14	***92***
Nankai Trough forearc basin (water depth 945 m)[b]										
Above hydrate zone (160–176 mbsf)	14	–	–	19	3	–	24	24	16	***37***
Within hydrate zone (209–252 mbsf)	4	–	–	–	18	–	28	46	4	***50***
Below hydrate zone (291–309 mbsf)	15	–	–	–	3	–	24	47	11	***34***
Sea of Okhotsk, clay layers (water depth 1,225 m)[c]										
7.5 mbsf	60	17	–	–	17	–	–	–	6	***64***
58 mbsf	13	73	3	–	5	–	–	–	6	***75***
Sea of Okhotsk, ash layer (water depth 1,225 m)[c]										
46 mbsf	3	3	6	87	–	–	–	–	–	***150***
Mediterranean Sea (water depth 2,155 m)[d]										
Sapopels S1–S8 (0.2–3.7 mbsf)	100	–	–	–	–	–	–	–	–	***8***
Surface and hemipelagic layers (0.1–3.4 mbsf)	75	–	–	–	25	–	–	–	–	***4***

Table 9.1.1 (Continued)

Site	Percent of all clones or DGGE bands									Total no. of clones or DGGE bands
	Chlorofl.	JS1	Proteobacteria			OP8	*Firmic.*	*Actinob.*	others	
			Alpha	*Gamma*	*Delta*					
Cascadia Margin (water depth 870–1,216 m)[e]										
Site 1251, slope, hydrate poor (5–331 mbsf)	12	53	0.3	7.9	1	1.4	0.7	0.3	23	***292***
Sites 1244 & 1245, hydrate rich (0.1–195 mbsf)	10	53	0.8	0.8	4.6	3	5	0.8	19	***131***
South China Sea (water depth 1,508 m)[f]										
4.9 mbsf	–	–	15	79	2.4	–	3.6	–	–	***82***
Western Pacific Warm Pool (water depth 2,068 m)[g]										
2.3 mbsf	–	–	51	–	–	–	9	1	39	***95***
Arctic Ocean, Lomonossow Ridge (water depth 1,209 m)[h]										
Silty clay, 55–103 mbsf	–	–	–	31	–	–	2	53	14	***215***
Diatom ooze, 242 mbsf	61	–	–	16	–	–	–	–	23	***76***

[a]Data from Webster et al. 2006a; [b]data from Reed et al. 2002; [c]data from Inagaki et al. 2003; [d]data from Coolen et al. 2002; [e]data from Nunoura et al. 2008; [f]data from Jiang et al. 2007; [g]data from Jing and RunYing 2008; [h]data from Forschner et al. 2009.

Chlorofl.: *Chloroflexi*; *Actinob.*: *Actinobacteria*; *Firmic.*: *Firmicutes*; JS1: candidate division JS1; OP8: candidate division OP8.

Table 9.1.2

Contribution of different archaeal groups to archaeal diversity in different subsurface sediments

Site	Percent of all archaeal clones or DGGE bands							Total no. of clones or DGGE bands
		Euryarchaeota			*Crenarchaeota*			
	DSAG	SAGMEG	MBG-D	TMEG	MCG	MG-I	Others	
Peru Margin, ODP site 1229 (water depth 150 m)[a]								
6.7 mbsf	4	4	–	–	92	–	–	***23***
30 mbsf	–	–	–	–	100	–	–	***24***
87 mbsf	–	7	4	–	89	–	–	***27***
Peru Margin, ODP site 1227 (water depth 427 m)[b]								
6–7 mbsf	2	8	5	2	84	–	–	***127***
37–38 mbsf	89	10	–	–	–	–	1	***176***
40–46 mbsf	–	–	–	–	100	–	–	***109***
Nankai Trough, ODP site 1173 (water depth 4,791 m)[c]								
4.3 mbsf	44	–	18	–	–	15	25	***34***
Nankai Trough forearc basin (water depth 945 m)[d]								
Above hydrate zone (160–176 mbsf)	86	–	–	–	–	14	–	***42***
Within hydrate zone (209–252 mbsf)	–	–	–	–	–	100	–	***35***
Below hydrate zone (291–309 mbsf)	–	71	–	–	21	8	–	***49***
Mediterranean Sea (water depth 2,155 m)[e]								
Sapopels S1–S8 (0.2–3.7 mbsf)	–	3	–	–	97	–	–	***29***
Surface and hemipelagic layers (0.1–3.4 mbsf)	5	16	–	–	52	11	16	***19***

Table 9.1.2 (Continued)

Site	Percent of all archaeal clones or DGGE bands							Total no. of clones or DGGE bands
	DSAG	*Euryarchaeota*			*Crenarchaeota*		Others	
		SAGMEG	MBG-D	TMEG	MCG	MG-I		
Cascadia Margin (water depth 870–1,216 m)[f]								
Site 1251, slope, hydrate poor (5–331 mbsf)	46	2.5	4	0.8	25	10	12	***353***
Sites 1244 & 1245, hydrate rich (0.1–195 mbsf)	67	8	3	–	19	–	3	***150***
South China Sea (water depth 1,508 m)[g]								
4.9 mbsf	27	5	6	3	27	–	32	***100***
Arctic Ocean, Lomonossow Ridge (water depth 1,209 m)[h]								
Diatom ooze, 242 mbsf	–	–	–	–	100	–	–	***214***

[a]Data from Webster et al. 2006a; [b]data from Sørensen and Teske 2006; [c]Newberry et al. 2004; [d]Reed et al. 2002; [e]data from Coolen et al. 2002; [f]data from Nunoura et al. 2008; [g]data from Jiang et al. 2007; [h]data from Forschner et al. 2009.

DSAG: Deep Sea Archaeal Group; SAGMEG: South African Gold Mine Euryarchaeotal Group; MBG-D: Marine Benthic Group D, TMEG: Terrestrial Miscellaneous Euryarchaeotal Group; MCG: Miscellaneous Crenarchaeotal Group; MG-1: Marine Group I.

crenarchaeotal Marine Group I (MG-I) and the euryarchaeotal South African Gold Mine Euryarchaeotal Group (SAGMEG), Marine Benthic Group D (MBG-D), and Terrestrial Miscellaneous Euryarchaeotic Group (TMEG). Other archaeal groups (e.g., *Thermococcales*, *Archaeoglobales*, Deep-Sea Hydrothermal Vent Euryarchaeota) are less frequently found but may represent a significant percentage of all retrieved sequences in some sediments (e.g., Roussel et al. 2009a).

Most of the widespread bacterial and archaeal sequences (e.g., within the *Chloroflexi*, candidate phylum JS1, MCG, DSAG, SAGMEG) were novel and previously unknown, suggesting that these organisms are specific to subsurface environments (Coolen et al. 2002; Reed et al. 2002; Fry et al. 2008). However, during more recent investigations many of these groups have been detected also in relatively shallow subsurface sediments like the younger sapropels of the Eastern Mediterranean (Coolen et al. 2002) or a few meters deep in coastal sediments (Wilms et al. 2006; Webster et al. 2007; Roussel et al. 2009b). However, these sediments are already several hundred to thousand years old and they may represent the upper boundary of the marine subseafloor biosphere.

There is an ongoing debate whether *Archaea* or *Bacteria* are numerically more abundant in subseafloor sediments. Different approaches even on the same sediments (e.g., Peru Margin, Mauclaire et al. 2004; Biddle et al. 2006; Schippers et al. 2005; Sørensen and Teske 2006; Webster et al. 2006a; Lipp et al. 2008) have yielded contradictory results, despite most of them detecting a similar set of bacterial and archaeal 16S rRNA sequences. Most of this disagreement may be due to the use of different experimental procedures (clone libraries, FISH, DGGE) and target molecules (DNA, RNA, lipids) or the use of different nucleic acid extraction protocols. For example, bacteria possessing a Gram-positive type cell wall (*Actinobacteria*, *Firmicutes*) have rarely been detected by molecular methods although cultivation based methods suggested that they represent up to a few percent of the in situ community (Süß et al. 2004). It appears, however, that RNA-targeting methods favor *Archaea* (Biddle et al. 2006; Sørensen and Teske 2006), while DNA-targeted methods more often detect *Bacteria* (Schippers and Neretin 2006; Webster et al. 2006a). Unfortunately, a number of studies investigated either *Bacteria* or *Archaea* making a direct comparison of these two domains impossible.

It may also be that some approaches detect dead or dormant cells (e.g., bacterial cysts or endospores). Under some circumstances DNA and some lipid biomarkers show considerable long-term survival, particularly if adsorbed onto a kerogen matrix (e.g., sapropels or lignite) or clay minerals, and apparently have been protected against microbial degradation. For example, quantifiable amounts of nucleic acids of *Chlorobiaceae* (green sulfur bacteria) have been found in 217,000-year old sapropels of the Mediterranean Sea, representing up to 0.5% of the total community DNA (Coolen and Overmann 2007). As the obligately photolithoautotrophic *Chlorobiaceae* are not able to thrive in the deep-sea sediments, either cells or their DNA must have been persisting since the time of burial.

Analysis of Functional Genes

Sulfate-reducing bacteria (SRB) and methanogenic archaea are the most important terminal oxidizers during organic matter degradation in anoxic near-surface sediments (Wellsbury et al. 1996) representing up to 12% of all bacterial and archaeal cells, respectively (e.g., Wilms et al. 2007). It was therefore expected that significant numbers of these prokaryotes would be present in deep sediments, particularly as geochemical modeling and radiotracer experiments detected

low but widespread sulfate reduction and methanogenesis in subseafloor sediments (D'Hondt et al. 2002; Parkes et al. 2005; Webster et al. 2006a, 2009). Surprisingly, however, both groups are very rarely detected by molecular biological approaches, even those using genes specific for their key metabolic pathways (*dsrA* and *mcrA*, Parkes et al. 2005; Schippers and Neretin 2006; Webster et al. 2006a, 2009). This may indicate that the populations are very small but active, or alternatively, carried out by so far unknown prokaryotes not matching the primers used. For example, novel sulfite reductase (*dsrAB*) genes have been found in the shallow subsurface sediments of Aarhus Bay associated with the anaerobic oxidation of methane (Thomsen et al. 2001). Although methanogens, similarly, are only found in some molecular surveys, they are generally more often detected than sulfate-reducing bacteria, particularly when the *mcrA* functional gene is used, and their sequences are more similar to those of well described genera, as for example *Methanosarcina*, *Methanobrevibacter*, or *Methanococcoides*.

Diversity of Deep Biosphere Isolates

Enrichment and Isolation of Prokaryotes from Marine Subsurface Sediments

In general, only very few marine subsurface sediments have been analyzed using cultivation-based methods (Parkes et al. 1990; Parkes et al. 1994; Inagaki et al. 2003; D'Hondt et al. 2004; Süβ et al. 2004; Toffin et al. 2004b; Biddle et al. 2005; Köpke et al. 2005; Batzke et al. 2007; Kobayashi et al. 2008; Webster et al. 2009; Parkes et al. 2009), and consequently only few bacterial and archaeal isolates have been obtained from this habitat. There are a number of reasons for this underrepresentation of deep subsurface microorganisms in culture. Subsurface sites are highly inaccessible and sampling requires a drilling vessel or at least gravity or piston coring. A number of measures have to be taken to limit and determine any contamination of samples with microorganisms from drilling fluid or seawater (Smith et al. 2000; Masui et al. 2008). Enrichment and isolation of deep biosphere bacteria is also hampered by the low culturability of deep biosphere bacteria. In most studies, far less than 0.1% of all prokaryotic cells were growing in culture media (Parkes et al. 1990; Inagaki et al. 2003). This low culturability has been explained by the fact that bacteria in deep sediments are strongly energy-limited and often unable to grow in and/or severely harmed by a sudden exposure to high substrate concentration media ("substrate-accelerated death"). This hypothesis is supported by a study on Eastern Mediterranean sapropels in which the use of substrates in submillimolar concentrations helped to increase the cultivation success by up to four orders of magnitude, compared to use of standard rich microbiological media (Süβ et al. 2004). However, "culturability" of subsurface sapropel bacteria was still one order of magnitude lower than that of coastal surface sediment bacteria when the same low-substrate media was used (Köpke et al. 2005).

In marine sediments, the percentage of cells that grow in culture media generally decreases with depth (e.g., Morita and ZoBell 1955; Parkes et al. 1994; Köpke et al. 2005). In deeper sediments, however, there are some exceptions. Culturability appears to be higher in the organic-matter-rich layers like the sapropels of the Eastern Mediterranean Sea (Süβ et al. 2004), where deep methane gas is present (Parkes et al. 1994), or in deep layers receiving brines or fluids from the underlying oceanic crust (Mather and Parkes 2000; Engelen et al. 2008). In addition, porous sediments seem to harbor higher numbers of culturable bacteria, while

clayey sediments, despite having similar total cell numbers and microbial activities, seem to yield lower viable counts (Inagaki et al. 2003). This can lead to a reversal in declining cell numbers with depth with very low viable counts along the upper layers and higher numbers in very deep layers at some sites (Kobayashi et al. 2008; Webster et al. 2009).

Another possible reason for the low culturability of microorganisms from deep submarine sediment is potential damage or even inactivation of cells due to depressurisation during sample recovery. Although sampling devices can be recovered slowly to reduce the speed of pressure change, bacteria may still not be able to adapt quickly enough. In a recent study on gas-hydrate containing sediments, pressure-retaining sampling (HYACINTH) and processing (DeepIsoBug) equipment was applied (Parkes et al. 2009). This allowed the parallel setup of enrichment cultures using undecompressed or decompressed sediments. Surprisingly, all anaerobic enrichment cultures, irrespective of pressure incubation conditions (undecompressed, decompressed and re-pressurized or incubated at atmospheric pressure), resulted in a very similar set of bacterial genera (*Acetobacterium*, *Carnobacterium*, *Clostridium*, *Marinilactibacillus*, *Pseudomonas*), despite some enrichments growing up to 80 MPa, while no *Archaea* were enriched under any incubation conditions. This appears surprising as Yanagibayashi et al. (1999) found no overlap between enrichments without (*Shewanella* and *Moritella* spp.) and after decompression (*Pseudomonas* spp.). However, their sediment was retrieved from a water depth of 6,292 m while the sediments used by Parkes et al. (2009) had total depths (water + sediment) of less than 1600 m, and it may be that these samples were not deep enough to obtain obligate piezophiles.

In addition, Parkes et al. (2009) also found the same bacterial genera enriched using different "selective" media targeting physiological groups like fermenters, sulfate-reducing bacteria (SRB), or methanogens. This effect seems to be typical for deep subsurface sediments from which only a limited bacterial diversity is obtained in spite a range of different media being used and a relatively large number of bacterial isolates being obtained (Süβ et al. 2004; Batzke et al. 2007; Kobayashi et al. 2008), particularly when compared to surface sediments (Köpke et al. 2005). However, as indicated by rarefaction analysis, the culturable diversity at some sites has been well represented by the isolates obtained (Süβ et al. 2004; Batzke et al. 2007), indicating that for the isolation of additional microbial diversity novel cultivation methods need to be established. The isolation of fermentative bacteria in MPN series with media targeting sulfate-reducing bacteria (SRB) or methanogens reinforces molecular diversity results suggesting that in situ numbers of sulfate reducers and methanogens may be very small. In fact, only very few SRB (*Desulfofrigus*, *Desulfomicrobium*, *Desulfovibrio* spp.) or methanogens (*Methanoculleus*, *Methanococcus* spp.) have been isolated from deep marine sediments (Bale et al. 1997; Barnes et al. 1998; Mikucki et al. 2003; Süβ et al. 2004; Kendall et al. 2006; Batzke et al. 2007). However, substrates such as lactate and propionate allow only little growth of fermentative bacteria, and consistent with this in many cases colonies obtained in agar shakes or on plates are extremely small, often only detectable using a stereo microscope and easily overlooked (Süβ et al. 2004).

Phylogenetic Affiliation of Deep Biosphere Isolates

Bacterial isolates from marine subsurface sediments so far belong to the *Alpha*, *Beta*, *Gamma*, *Delta*-Subgroups of the *Proteobacteria*, to the *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (*Table 9.1.3*; *Fig. 9.1.2*; Bale et al. 1997; Barnes et al. 1998; Inagaki et al. 2003; Süβ et al. 2004; Lee et al. 2005; Takai et al. 2005; Batzke et al. 2007; Kobayashi et al. 2008; Parkes et al. 2009),

Table 9.1.3

Number of bacterial isolates obtained from different subseafloor sediments

Genus	Pacific Ocean[a]		Sea of Okhotsk[b]	Mediterranean Sea[a]		Hidaka Trough[b]	
	Open Ocean sites	Peru Margin		Hemipelagic sediments	Sapropels	Isolated at 4°C	Isolated at 30°C
Alphaproteobacteria							
Erythrobacter	–	–	3	1	–	–	–
Paracoccous	–	–	–	–	2	–	8
Rhizobium	9	32	–	9	25	–	–
Roseobacter	–	–	–	1	–	–	–
Sulfitobacter	–	–	26	–	–	–	–
Gammaproteobacteria							
Acinetobacter	–	–	–	2	–	2	–
Alteromonas	–	–	–	–	2	–	–
Enterobacter	–	–	–	–	–	2	1
Halomonas	–	–	82	–	2	8	12
Oceanospirillum	–	–	–	1	1	–	–
Photobacterium	–	1	–	13	18	–	–
Pseudalteromonas	–	–	–	–	–	11	1
Pseudomonas	–	–	–	–	–	10	12
Psychrobacter	–	3	58	–	–	–	–
Shewanella	–	–	–	–	–	1	–
Vibrio	1	22	–	3	3	–	–
Firmicutes							
Bacillus	27	43	4	4	11	–	5
Marinilactibacillus	–	–	10	–	–	–	–
Paenibacillus	1	–	2	–	2	–	1
Actinobacteria							
Aeromicrobium	–	–	–	–	–	–	6
Brachybacterium	–	–	–	10	19	–	–
Dietzia	–	–	1	–	–	–	–
Kocuria	–	–	1	–	–	–	–
Micrococcus	3	–	2	10	10	–	–
Rhodocoocus	–	–	1	–	1	4	2
Total no. of isolates	**48**	**118**	**190**	**54**	**96**	**38**	**48**
References	D'Hondt et al. 2004		Inagaki et al. 2003	Süß et al. 2004, Sass unpublished.		Kobayashi et al. 2008	

[a]Isolates obtained under oxic and anoxic conditions, [b]Isolated obtained under oxic conditions.

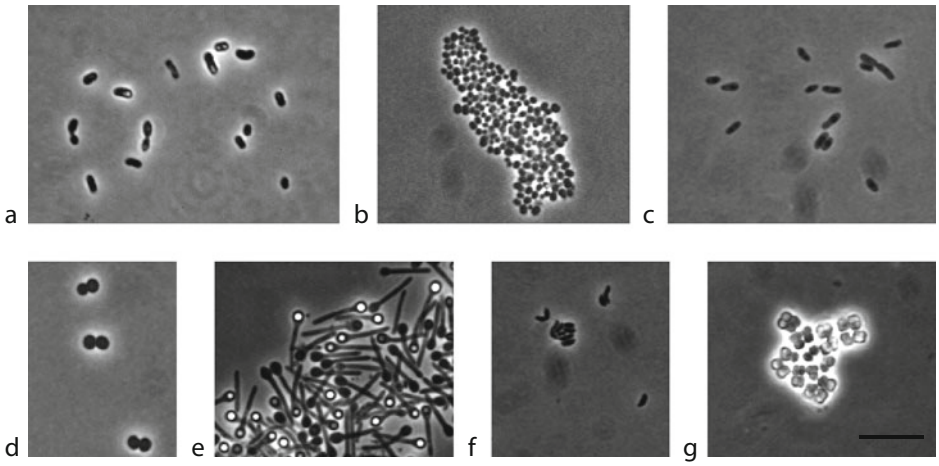


Fig. 9.1.2

Bacterial isolates from Eastern Mediterranean sapropels (a) *Photobacterium* sp. 67FSb; (b) *Alteromonas* sp. S1FS1; (c) *Halomonas* sp. S7A; (d) *Acinetobacter* sp. Z7TS1; (e) *Clostridium* sp. SO1; (f) *Thalassospira* sp. Z0A; (g) *Micrococcus* sp. SM4; Bar = 10 μm

while archaeal isolates are affiliated with the euryarchaeotic genera *Methanoculleus* and *Methanococcus* (Mikucki et al. 2003; Kendall et al. 2006). Most of the genera isolated from marine subsurface sediments are also typical for surface sediments or pelagic environments, and it could be argued that their cells have just remained viable during burial. But if this was the case, numbers should strongly decrease with depth. The presence of some of the genera, however, has been confirmed by cultivation-independent molecular biological techniques, e.g., for *Pseudomonas*, *Halomonas, Marinobacter*, and *Acinetobacter* spp. (Reed et al. 2002; Inagaki et al. 2003; Kormas et al. 2003; Parkes et al. 2005; Inagaki et al. 2006), indicating that they contribute significantly to the in situ microbial communities. Close relatives of *Rhizobium radiobacter* were repeatedly isolated from sediments of the Eastern Mediterranean (Süβ et al. 2004), and quantitative PCR showed that *Rhizobium* spp. are more abundant in subsurface than surface sediment layers and even represent up to 5% of the in situ bacterial population in some of the sapropel layers (Süβ et al. 2006).

Members of *Actinobacteria* and *Firmicutes* have frequently been isolated from subsurface sediments and were often dominating (D'Hondt et al. 2004; Batzke et al. 2007; Kobayashi et al. 2008; Parkes et al. 2009). However, as mentioned earlier, they are rarely detected using molecular methods, suggesting that their cells are present either in very low numbers or in an inactive state. Members of both phyla, *Actinobacteria* (*Micrococcus*) and *Firmicutes* (*Bacillus*), have been isolated from several million years old amber and subsurface halite crystals, indicating that they possess extreme longevity. Endospores, formed by certain members of the *Firmicutes*, are resting stages that are inactive, strongly dehydrated, and highly resistant to environmental stresses. Endospores do contain enough energy for germination and are specifically adapted to quickly respond to substrate availability and to form a vegetative cell able to replicate. Therefore, it can be expected that they easily outgrow other organisms after transfer to culture media, explaining their dominance in subsurface cultures from Peru Margin and Hidaka Trough sediments (D'Hondt et al. 2004; Kobayashi et al. 2008). Although there is

only little information about the number of endospores in marine sediments, there appears to be a permanent supply by sedimentation. Hubert et al. (2009) estimated that about 10^8 endospores of thermophilic bacteria are deposited per square meter per year in Arctic surface sediments. A cultivation-independent analysis based on dipicolinic acid contents, unique to endospores, estimated between 10^6 and 10^7 endospores per cm^3 of coastal sediment (Fichtel et al. 2008) and showed that their numbers do not significantly decrease with depth within the upper six meters. This suggests that their relative contribution to the microbial community increases in deeper layers. One reason why they were not detected using molecular biological techniques may be their ultrastructure. Endospores possess a very rigid cell wall and it is unclear to what extent their DNA is extracted using standard procedures. In a study of Arctic sediments, thermophilic endospore-forming bacteria could only be detected after the endospores were stimulated to germinate and were present as vegetative cells (Hubert et al. 2009).

Uncultured Deep Biosphere Prokaryotes

Some of the bacterial (e.g., candidate phyla JS1 and OP8) and archaeal phyla (e.g., Marine Benthic Group-D, Miscellaneous Crenarchaeotal Group) that dominate marine subsurface sediments in molecular studies have yet to be cultured. It is one of the major challenges for future subsurface microbiology to enrich and isolate some of these organisms, as pure cultures are still required to reveal the whole spectrum of physiological characteristics. These are necessary to understand the ecology of a single bacterial species and their community interactions, their metabolism, pressure, temperature and physiological range and optima, starvation survival strategies and to identify the types of biomarkers they contain, such as specific phospholipids. However, the phylogenetic diversity of these "yet-to-be-cultured" groups suggests a wide range of metabolic types and metagenomic studies (Biddle et al. 2008) may help to unravel the major metabolic pathways facilitating the design of specific culture media. While this metabolic diversity may include highly specialized lithotrophic organisms, like the recently isolated nitrifying pelagic Marine Group I crenarchaeon "*Nitrosopumilus maritimus*," it can be expected that at least some of the "yet-to-be-cultured" microorganisms have heterotrophic metabolism (Coolen et al. 2002; Biddle et al. 2006; Fry et al. 2008). For example, representatives of the phylum *Chloroflexi* have already been successfully enriched in "standard" anoxic culture media containing peptone or sugars but not obtained in pure culture (Lysnes et al. 2004; Köpke et al. 2005). The reason for the failure to obtain *Chloroflexi* in pure culture may be that these supposedly slow-growing bacteria were outgrown by opportunistic fast growing bacteria like some *Proteobacteria* or that they failed to form visible colonies. Stable isotope probing has also demonstrated that members of the candidate division JS1 are able to incorporate acetate and glucose (or glucose metabolites) under anoxic sulfate-reducing conditions and that DSAG and MCG *Archaea* assimilate organic carbon (Webster et al. 2006b, 2010).

Physiology of Marine Deep Biosphere Prokaryotes

As described above, only a few studies have focused on enrichment and isolation of marine subsurface microorganisms and only a small proportion of these isolates has been investigated in detail (Bale et al. 1997; Barnes et al. 1998; Mikucki et al. 2003; Lee et al. 2005; Takai et al. 2005;

Kendall et al. 2006; Batzke et al. 2007; Süß et al. 2008). Most of these isolates are closely related to organisms known from surface sediments or pelagic environments. Some of these isolates (e.g., *Photobacterium* and *Shewanella* spp.) do not seem to differ significantly with respect to their metabolic or physiological capacities from their surface counterparts (Süß et al. 2008). However, although the number of isolates investigated in detail is too small to draw a firm conclusion, a significant number of isolates from deep sediments do appear to be adapted to a broader range of environmental conditions, allowing them to inhabit large parts of the deep biosphere. Most of these isolates have an extraordinary broad pH and temperature range for growth, such as *Desulfovibrio profundus*, *Marinobacter alkaliphilus*, *Methanococcus aeolicus*, and *Methanoculleus submarinus* (Bale et al. 1997; Mikucki et al. 2003; Takai et al. 2005; Kendall et al. 2006). All these isolates are able to grow at temperatures from around 10°C to about 50°C and more (*Table 9.1.4*). Deeper sediment layers (>800 mbsf, Wellsbury et al. 2002) have not yet been successfully analyzed using cultivation-based methods. However, it can be expected they harbor mostly thermophilic *Bacteria* and *Archaea*, as suggested by molecular biological studies.

As pressure increases with water and sediment depth it is obvious that bacteria in subseafloor sediments have to be at least pressure-tolerant (piezotolerant) if not pressure-requiring (piezophilic). As an example, the optimum pressure of a sulfate-reducing bacterium, *Desulfovibrio profundus* from subsurface sediments of the Japan Sea, reflects well the in situ pressure as it has its optimum at pressures of 10–20 MPa and was still active at pressures between 30 and 40 MPa (Bale et al. 1997; Barnes et al. 1998).

Little is known how subsurface microorganisms cope with the limited energy supply and whether they are more starvation resistant than microorganisms from surface environments. In a study on *Arthrobacter* and *Pseudomonas* strains from terrestrial subsurface environments, cells were starved for over 60 weeks and showed little decline in total cell counts, while viable counts decreased by one to two orders of magnitude (Kieft et al. 1997). However, when compared to closely related surface isolates, no significant differences were found. Whether these results can be extrapolated to other genera and habitats remains unclear, but it may explain why *Gammaproteobacteria* and *Actinobacteria* have repeatedly been isolated from deep sediments.

Ecology of Marine Deep Biosphere Prokaryotes

Depth Distribution of Respiratory Processes

The majority of the bacteria isolated from subsurface environments are facultative anaerobes. At first sight, this may seem surprising, as sediments are generally considered anoxic at a few milli- to centimeters below surface. However, in deep-sea sediments, the low input by sedimentation and the recalcitrant nature of organic matter allows only limited metabolic activities. Consequently only slow oxygen consumption and production of reduced compounds like hydrogen sulfide occurs and the redox potential in many subsurface sediments is not highly reduced when compared to coastal and shelf sediments. In a recent study on sediments underlying the ultra-oligotrophic South Pacific Gyre, oxygen was even detected in the deepest sediments investigated (9 mbsf, D'Hondt et al. 2009). Likewise, other electron acceptors like nitrate, manganese, or iron oxides are present much deeper than in coastal sediments and strictly anaerobic processes such as sulfate reduction and methanogenesis occur only at much

Table 9.1.4

Pressure, temperature, and salinity range for growth of some bacterial and archaeal isolates from marine subsurface sediments

Strain	Origin depth (mbsf)		Temperature in situ (°C)	Pressure range (MPa)	Temperature range (°C)	Salinity range (%)	Reference
	Water	Sediment					
Shewanella profunda DSM 15900^T	4,791	4.2	1–2	0.1–50	4–37	0–6	Toffin et al. 2004a
Photobacterium. sp. S14	2,150	4.5	13–16	n.a.	4–35	1–7.5	Süß et al. 2008
Marinilactibacillus piezotolerans DSM 16108^T	4,791	4.2	1–2	0.1–30	4–50	0–12	Toffin et al. 2005
Marinobacter alkaliphilus JCM 12291^T	2,942	1.5	1.5–2	n.a.	10–45	0–21	Takai et al. 2005
Desulfovibrio profundus DSM 11384^T	900	500	16	0.1–40	15–65	0.2–10	Bale et al. 1997
Methanoculleus submarinus DSM 15122^T	950	247	15–16	n.a.	10–50	0.6–9	Mikucki et al. 2003
Methanococcus aeolicus DSM 17508^T	950	247	15–16	n.a.	15–55	0.3–6	Kendall et al. 2006

n.a.: data not available.

greater depths. For example, in the Mediterranean Sea nitrate, oxidized manganese or iron species are present at least as deep as sapropel S1 which is approximately 12,000 years old (van Santvoort et al. 1996). Active reduction of manganese oxides still occurs in 7–11 Myr old diatom layers between 250 and 320 mbsf in Pacific Ocean sediments (Parkes et al. 2005). It appears that potentially competitive anaerobic respiratory processes often occur together in subseafloor sediments, e.g., manganese and iron reduction, sulfate reduction and methanogenesis (Cragg et al. 2003; D'Hondt et al. 2004; Parkes et al. 2005), presumably due to the low energy conditions preventing any particular terminal-oxidizing prokaryotic group being able to outcompete other groups. But electron acceptors like nitrate or sulfate are not only supplied by diffusion from the sediment surface, in addition deep circulation of fluids through the Ocean crust may provide oxidants to subsurface sediments (Mather and Parkes 2000; Wellsbury et al. 2002; D'Hondt et al. 2004).

Lithotrophy in the Marine Subsurface

Potential substrates for deep subseafloor microbial communities are inorganic compounds, most prominently H_2. In addition to formation from aromatization of sedimentary organic matter at elevated temperature (Parkes et al. 2007) it can be formed by the oxidation of ferrous iron in ultramafic rocks (serpentinization, Bach and Edwards 2003). Diffusion of hydrogen formed by serpentinization (typically at ~150–250°) into shallow cooler sediments would allow a microbial community to thrive independently from photosynthesis. Such SLIME (Subsurface, Lithoautotrophic Microbial Ecosystem) communities have been postulated (Stevens and McKinley 1995; Takai et al. 2004), although their widespread existence remains controversial as H_2 formation from common minerals such as basalt appears to be a negligible reaction under in situ conditions (Anderson et al. 1998). Another mechanism for hydrogen formation is by radiolysis of water. Although this is a very slow process, it can be expected to support microbial activities for millions of years (Lin et al. 2006) and be particularly important when organic matter concentrations are very low (D'Hondt et al. 2009).

Another abundant inorganic substrate is ammonium. The anaerobic oxidation of ammonium can be coupled to nitrite reduction (ANAMMOX). This process requires the availability of oxidized nitrogen (nitrate, nitrite) species and may be restricted to layers close to the surface and the sediment/crust. However, ammonium oxidation coupled to manganese oxide is thermodynamically feasible (Luther et al. 1997) and appears to be a possible process in sediments of the Izu-Bonin Trench (Cragg et al. 2003). Recent reports even suggested ammonium oxidation with sulfate as inferred from porewater profiles (Schrum et al. 2009).

Deep Biosphere Hot Spots

Although it appears that vast areas of the marine subsurface are extremely energy limited, with organic matter generally present as highly degradation resistant macromolecular kerogen, there are sediments that are characterized by exceptionally high cell numbers and activities and can be seen as deep biosphere "hot spots." Among these are organic-matter-rich sediments like the sapropels of the Eastern Mediterranean (*Fig. 9.1.3*; Parkes et al. 2000; Coolen et al. 2002), diatom-rich layers of the Pacific Ocean (*Fig. 9.1.4*; Parkes et al. 2005), or marine Cretaceous shales, even when uplifted onto land by tectonic activity (Krumholz et al. 2002).

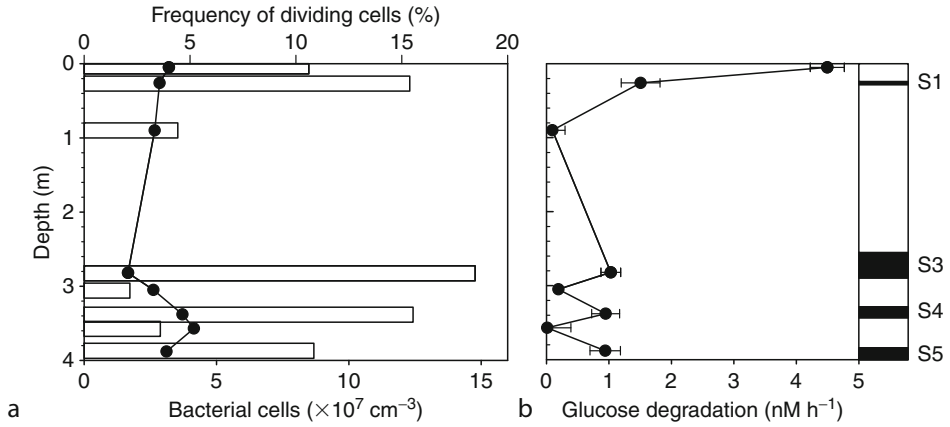


Fig. 9.1.3

(a) Total numbers of prokaryotic cells (*bars*), frequency of dividing cells (*circles*); and (b) glucose degradation rates in sapropels (S1–S5) and intermediate layers of the gravity core #69-2 taken from the Eastern Mediterranean Sea (after Coolen et al. 2002)

Whether this stimulation is caused by the very slow degradation of kerogen itself or by slow desorption of smaller and more easily degradable molecules is unclear. However, it is clear that in these sediments microbial communities are being supported by ancient organic matter that is utilized by prokaryotes on geological time scales. But despite having elevated cell numbers the energy turnover in these layers is still is extremely low and not comparable to surface environments. Consequently, the available energy is used predominantly for cell maintenance rather than growth. For example, microbial communities in the sapropels of the Eastern Mediterranean were estimated to have an average doubling time of 100 kyr (Parkes et al. 2000).

At greater depth the recalcitrant kerogen is exposed to increasing temperatures and it has been shown that this thermal activation can enhance degradability leading to production of low molecular weight compounds, including volatile fatty acids like acetate and H_2, which can directly stimulate thermophilic prokaryotic communities. At high temperatures these and other compounds (e.g., methane and other hydrocarbons) can be thermogenically produced and diffuse upward into cooler sediment layers where they can fuel the base of the hot subseafloor biosphere (Wellsbury et al. 1997; Horsfield et al. 2006; Parkes et al. 2007). These communities may even receive volatile compounds diffusing upwards from hot Ocean crust (Cowen et al. 2003). These elevated substrate concentrations by thermal breakdown or deep fluid flows are directly reflected by increasing cell numbers, as for example in sediments of the Nankai Trough or the Juan de Fuca Ridge (Mather and Parkes 2000; Parkes et al. 2007; Engelen et al. 2008). The presence of thermophilic hydrocarbon-degrading anaerobes in the deep biosphere sediments may also explain the presence of microorganisms in some oil reservoirs that have previously been considered to be introduced during exploration.

As mentioned above, deep fluid flows may also provide electron acceptors for deep microbial communities, as for example sulfate at IODP site 1229 on the Peru Margin. This deep sulfate supply unusually enables two sulfate–methane transition zones (SMTZ) at 30 and 90 mbsf at this site. Both interfaces are characterized by elevated microbial populations and activities (*Fig. 9.1.5*; Parkes et al. 2005), whereby cell numbers at the deeper SMTZ even reach

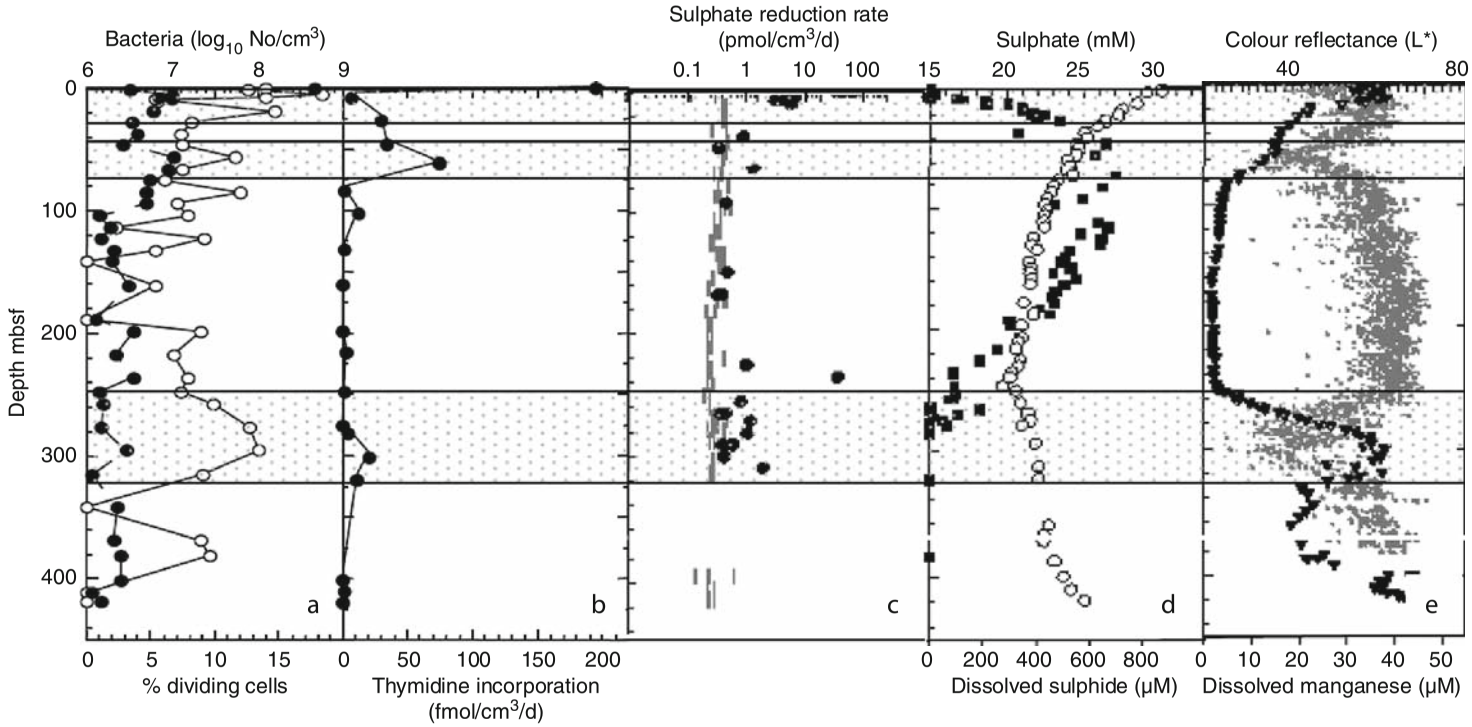


Fig. 9.1.4

Stimulation of biogeochemical activity and prokaryotic populations at a Pacific Open Ocean Site (ODP 1226) within ancient diatom-rich layers (oldest 7–11 Myr). (a) Prokaryotic profiles: ● Total prokaryotic population (Log_{10} Nos./cm^3), O percentage dividing and divided cells; (b) rates of prokaryotic growth ● thymidine incorporation (fmol/cm^3/d); (c) ● sulfate reduction rates, | minimum detection limits (pmol/cm^3/d); (d) geochemistry: O pore water sulfate (mM), and ■ hydrogen sulfide (μM); and (e) color reflectance as a measure of diatom abundance, low reflectance equals high diatom abundance, ♦ pore water manganese (μM). Shaded boxes highlight elevated prokaryotic processes and high diatom layers

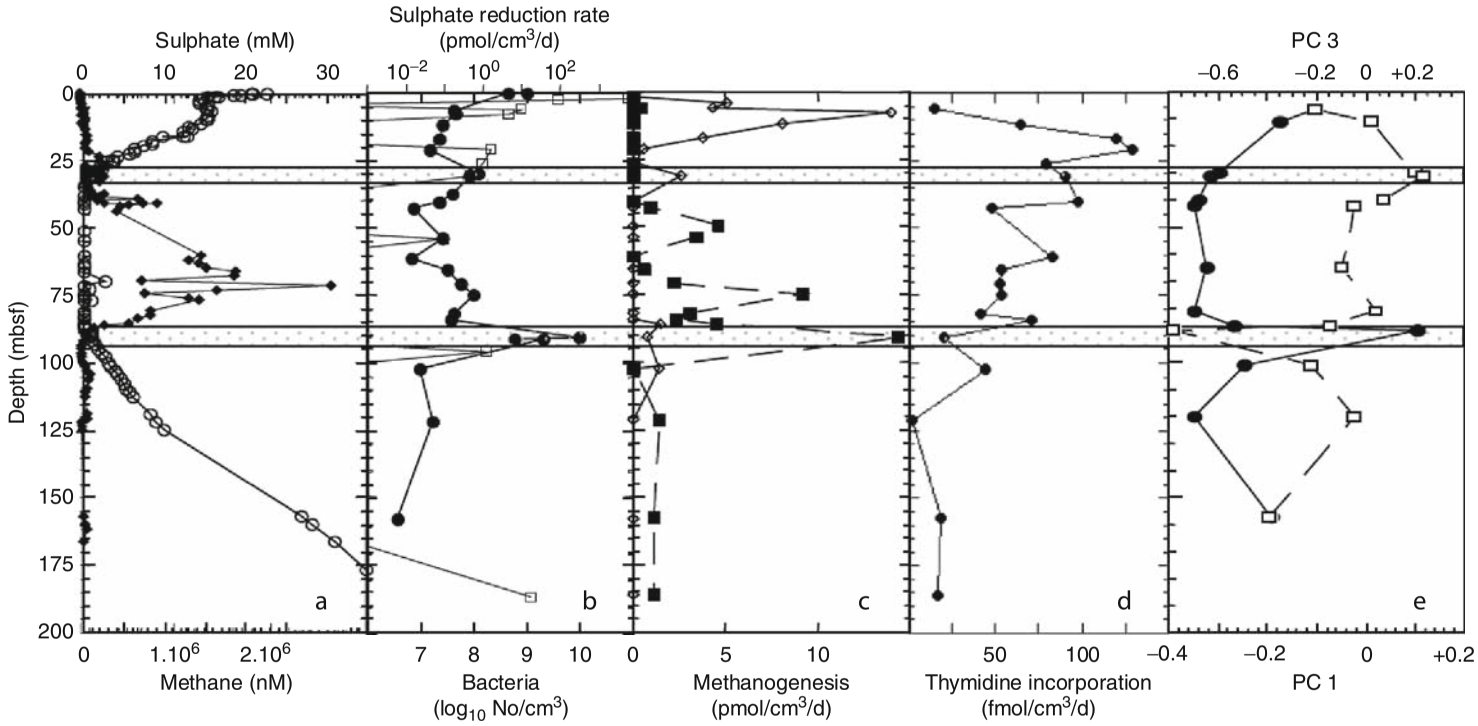


Fig. 9.1.5

Stimulation of biogeochemical activity, prokaryotic biomass, and biodiversity in Peru Margin sediments (ODP 1229) at two sulfate:methane interfaces. (a) geochemistry: O pore water sulfate (mM), ♦ CH_4 (nM); (b) □ sulfate reduction rates, ● total population; (c) methanogenic rates, ◇ H_2/CO_2, acetate ■; (d) growth rates ● thymidine incorporation; (e) principal components profile of diversity of *Bacteria* from DGGE analysis of 16S rRNA gene sequences: ● Component 1 (56% of variation), □ Component 3 (9% of variation); Component 2 (24% of variation) has a similar profile to Component 1. Shaded boxes highlight sulfate:methane interfaces (Parkes et al. 2005)

10^{10} cm^3 which is higher than in most coastal surface sediments. In both SMTZs bacterial populations are distinct from those in the layers above and below (Parkes et al. 2005), indicating a specialized dynamic community presumably fuelled by the anaerobic oxidation of methane (AOM). Many coastal and shelf sediments contain gas hydrates with very high methane concentrations within an ice matrix. These gas hydrates have been estimated to contain twice the amount of carbon than other fossil fuels and are stable only at low temperatures and high pressures. Above and below this gas hydrate stability zone hydrates melt due to too low pressures or too high temperatures, respectively, providing high methane concentrations for AOM communities. In addition, at these high organic matter accumulation sites low temperature activation of buried organic matter occurs at relatively shallow depths, particularly within and below the hydrate stability zone and low molecular weight organic acids and H_2 concentrations increase. This process seems to be similar to the higher temperature activation of kerogen at deeper depths, and it also stimulates prokaryotic activity. This process may be enhanced by deep fluid flow around the base of the gas hydrate stability zone. Why these very high organic acid concentrations (e.g., acetate up to 10 $mmol \cdot l^{-1}$, *Fig. 9.1.6*) are not more completely utilized by the in situ prokaryotes is puzzling, but some other factor than substrate availability may be limiting.

Is the Marine Deep Biosphere an Extreme Habitat?

Deep marine sediments are potentially the largest microbial habitat on Earth and one of the most diverse microbial environments. They contain a multitude of different physicochemical settings from completely oxidized sediments like in the South Pacific Gyre (D'Hondt et al. 2009) to reduced sediments with a range of anaerobic respiratory processes occurring together,

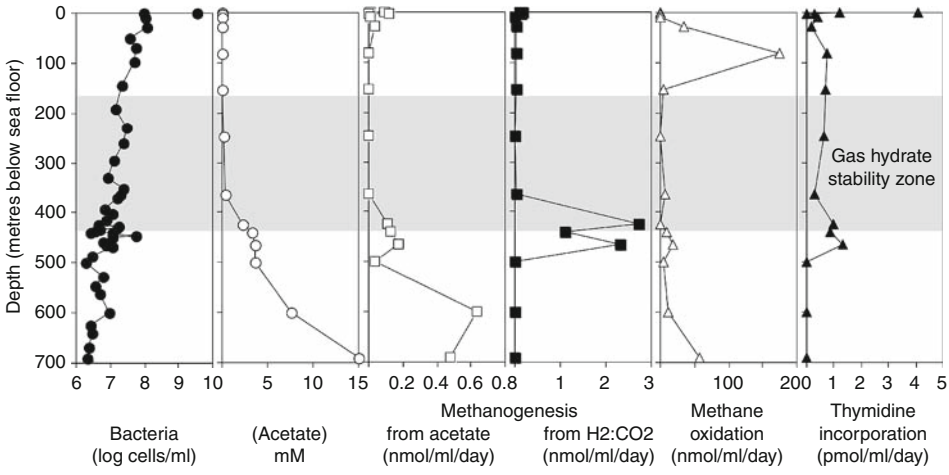


Fig. 9.1.6

Deep stimulated prokaryotic populations and activities in gas hydrate sediments from Blake Ridge, Atlantic Ocean. Thymidine incorporation into bacterial DNA is a measure of growth rates (Modified from Parkes et al. 2000)

including sulfate reduction and methanogenesis (D'Hondt et al. 2004; Parkes et al. 2005). Porewater salinity ranges from lower than marine due to clay dewatering at depth, reflected in some mud volcanoes, to hypersaline due to deep brine incursions (D'Hondt et al. 2004; Parkes et al. 2005), pH values from slightly acidic to alkaline (Takai et al. 2005). Deep sediments are characterized by extreme energy limitation, by high pressures and show a temperature gradient from psychrophilic to hyperthermophilic conditions at depth (Mather and Parkes 2000; Roussel et al. 2008). But as long as microbial life is physically possible even under the most oligotrophic conditions and over geological timescales, substantial numbers of microorganisms are present. In conclusion, deep marine sediments represent the largest reservoir of extremophilic microorganisms on Earth and under these circumstances it could be argued whether the term extremophile should, in fact, be reserved for near-surface prokaryotes.

Cross-References

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- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.4 Anaerobic Alkaliphiles and Alkaliphilic Poly-Extremophiles
- 2.5 General Physiology of Alkaliphiles
- 2.6 Adaptive Mechanisms of Extreme Alkaliphiles
- 2.7 Bioenergetics: Cell Motility and Chemotoxis of Extreme Alkaliphiles
- 2.8 Enzymes Isolated from Alkaliphiles
- 2.9 Genomics and Evolution of Alkaliphilic *Bacillus* Species
- 2.10 Beta-Cyclomaltodextrin Glucanotransferase of a Species of Alkaliphilic *Bacillus* for the Production of Beta-cyclodextrin
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- 3.1 Taxonomy of Halophiles
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- 6.1 Ecology of Psychrophiles: Subglacial and Permafrost Environments
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- 11.1 Ecology and Cultivation of Marine Oligotrophic Bacteria
- 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria
- 12.1 Actinobacteria of the Extremobiosphere

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9.2 Physiology

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_9.2,

Cultivation of Microorganisms from Deep Biosphere

Scientific investigations into deep biosphere were stimulated by demands for petroleum, and subsequent exploration and study of oil fields and oil-bearing environments. Several of the early investigations provided successful cultivations of sulfate-reducing bacteria from groundwater samples collected from oil fields at depths of 150–600 m below land surface (mbls) (Bastin 1926). Although such early studies suggested the presence of microorganisms in deep subsurface environments, it was still very unclear due to the primitive microbiological sampling procedures whether the microbial isolates were truly indigenous to the subsurface environments or not, and from where the microbial cells were derived.

In the late 1970s and early 1980s, emerging groundwater quality issues in the USA and other countries led to further investigations of microorganisms living in shallow and deep groundwater and aquifer environments (Fredrickson and Fletcher 2001). Technological and methodological advances allowed researchers to collect deep water, sediment, and rock samples while minimizing external chemical and microbial contaminations. Most of these studies were conducted by traditional cultivation-dependent methods, and some of the cultivated microorganisms were further characterized with respect to their phylogenetic and physiological properties. In the 1990s, a number of studies, most of which were still based on the cultivation-dependent techniques, were conducted for indigenous microorganisms inhabiting aquatic and oil-bearing deep subsurface environments (Magot et al. 2000). In addition, exploration of deep biosphere began to extend to several deep subsurface mine biota and deep subseafloor sedimentary environments (Kieft et al. 1999; Kotelnikova et al. 1998; Parkes et al. 2000). To date, together with rapidly developed culture-independent molecular techniques, knowledge in biomass, phylogenetic diversity, and biogeography of microbial components has been extensively characterized in various terrestrial and oceanic habitats of deep biosphere. However, the general sketch of phenotypic and physiological diversity of subsurface microorganisms has remained poorly understood as most of the indigenous subsurface microbial components still escape laboratory cultivations.

In this chapter, the physiological properties of the microorganisms that have been cultivated from various subsurface environments are overviewed. As a tentative definition of the potentially indigenous subsurface microorganisms, this chapter deals with the microorganisms directly obtained from the subsurface environmental samples or from the subsurface fluids with a relatively poor degree of hydrogeologic connectivity to the surface environments. Thus, the microorganisms isolated from the surface geothermal, the submarine hydrothermal, and cold seepage environments are for the most part excluded from the list. In addition, this chapter focuses on the physiological aspects of various subsurface microorganisms cultivated so far and the taxonomic features are reviewed in another chapter (Chap. 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)).

Physiological Diversity of Subsurface Microorganisms

The potentially indigenous subsurface microorganisms isolated from various habitats and their representative physiological features characterized are summarized in *Table 9.2.1*. In addition, the subsurface microbial isolates, of which physiological properties have been characterized only in part, are summarized in *Table 9.2.2*. Magot et al. (2000) overviewed the phylogenetic and physiological diversity of microorganisms cultivated and isolated from the petroleum environments. Subsequent to this review the data in *Tables 9.2.1* and *9.2.2*

Table 9.2.1

Potential subsurface microorganisms of which physiological properties are relatively well characterized

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Bacteria Aquificae *Sulfurihydrogenobium subterraneum*	Japanese gold mine at 320 m below land surface (mbls)	Anaerobic to microaerophilic	Thermophilic (40–70°C)	Chemolithotrophic (H_2, reduced S compounds as electron donors; O_2, nitrate, Fe(III), arsenate, selenite, selenate as electron acceptors	Autotroph	0–4.8%	Takai et al. 2002, 2003
Hydrogenobacter subterranues	Japanese geothermal water pool at 1,500 mbls	Aerobic	Extremely thermophilic (60–85°C)	Chemolithotrophic (reduced S compounds as electron donors; O_2, as an electron acceptor)	Heterotrophic with various substrates	0–2.0%	Takai et al. 2001d
Thermotogae *Thermotoga elfii*	African oil well	Anaerobic	Thermophilic (50–72°C)	Organotorphic with various substrates and thiosulfate reduction	Heterotrophic with various substrates	0–2.8%	Ravot et al. 1995
Thermotoga hypogea	African oil well	Anaerobic	Extremely thermophilic (56–95°C)	Organotorphic with various substrates and thiosulfate reduction	Heterotrophic with various substrates	0–1.5%	Fardeau et al. 1997
Thermotoga subterranea	continental oil reservoir at East Paris Basin	Anaerobic	Thermophilic (50–75°C)	Organotorphic with various substrates and thiosulfate reduction	Heterotrophic with various substrates	0–2.4%	Jeanthon et al. 1995
Thermotoga petrophila	Japanese oil reservoir	Anaerobic	Thermophilic (47–88°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	0.1–5.5%	Takahata et al. 2001
Thermotoga naphthophila	Japanese oil reservoir	Anaerobic	Thermophilic (48–86°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	0.1–6.0%	Takahata et al. 2001

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Thermosipho geolei	Continental oil reservoir at Western Siberia	Anaerobic	Thermophilic (45–75°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–7%	L'Haridon et al. 2001
Geotoga petraea	Brine from oil fields at Texas and Oklahoma	Anaerobic	Thermophilic (30–55°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–10%	Davey et al. 1993
Geotoga subterranea	Brine from oil fields at Texas and Oklahoma	Anaerobic	Thermophilic (30–60°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–10%	Davey et al. 1993
Petrotoga miotherma	Brine from oil fields at Texas and Oklahoma	Anaerobic	Thermophilic (35–65°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–10%	Davey et al. 1993
Petrotoga mobilis	Oil reservoir at the North Sea	Anaerobic	Thermophilic (40–65°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–9%	Lien et al. 1998
Petrotoga olearia	Continental oil reservoir at Western Siberia	Anaerobic	Thermophilic (37–60°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–8%	L'Haridon et al. 2002
Petrotoga sibirica	Continental oil reservoir at Western Siberia	Anaerobic	Thermophilic (37–55°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0.5–7%	L'Haridon et al. 2002
Kosmotoga olearia	Oil production fluid at North Sea	Anaerobic	Thermophilic (20–80°C)	Organotorphic with various substrates and thiosulfate-reduction	Heterotrophic with various substrates	1–6%	DiPippo et al. 2009
Thermococcoides shengliensis	Chinese oil production well	Anaerobic	Thermophilic (45–75°C)	Organotorphic with various substrates and S^0 reduction	Heterotrophic with various substrates	0–4%	Feng et al. 2009

Thermodesulfobacteria *Thermodesulfobacterium thermophilus*	Oil production water at the North Sea (2–4 km below seafloor)	Anaerobic	Extremely thermophilic (45–85°C)	Sulfate-reducing hydrogenotrophic and organotrophic with organic acids	Heterotrophic with various substrates		Christensen et al. 1992
Thermodesulfobacterium commune	Continental oil reservoir at East Paris Basin	Anaerobic	Extremely thermophilic (60–82°C)	Sulfate-reducing organotrophic with organic acids	Heterotrophic with various substrates	0–2%	L'Haridon et al. 1995
Deinococcus-Thermus *Thermus scotoductus*	South Africa gold mine at a 3.2 kmbls	Facultative anaerobic	Thermophilic (55–75°C)	Organotorphic with various substrates and O_2-, nitrate-, Fe(III)-, S^0- and Mn(IV)-reduction. Also capable of reducing Cr(VI), U(VI) and Co(III)	Heterotrophic with various substrates		Kieft et al. 1999; Balkwill et al. 2004
Deferribacteres *Deferribacter thermophilus*	Oil production water at the North Sea	Anaerobic	Thermophilic (50–65°C)	Hydrogenotrophic and organotorphic with various substrates and nitrate-, Fe(III)-, S^0- and Mn(IV)-reduction	Heterotrophic with various substrates	0–5%	Greene et al. 1997
Synergistetes *Anaerobaculum thermoterrenum*	Oil field at Utah	Anaerobic	Thermophilic (28–60°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	0–2%	Rees et al. 1997
Dethiosulfovibrio peptidovorans	African offshore oil field at Congo	Anaerobic	Mesophilic (25–45°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	1–10%	Magot et al. 1997a
Fermicutes *Thermaerobacter subterraneus*	Great Artesian Basin borehole with a depth of 1,613 mbls	Aerobic	Thermophilic (55–80°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0–1%	Spanevello et al. 2002

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Alkaliphilus transvaalensis	South Africa gold mine at a 3.2 kmbls	Anaerobic	Mesophilic (20–50°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0.5–4.0%	Takai et al. 2001c
"Acetobacterium pssamolithicum"	Deep terrestrial Crataceous sediments at New Mexico	Anaerobic	Mesophilic	Homoacetogenesis and heteroacetogenesis	Autotrophic and heterotrophic		Krumholz et al. 1999
Thermoanaerobacter brockii	French oil field at a depth of 2,100 mbls	Anaerobic	Thermophilic (40–75°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	0–4.5%	Cayol et al. 1995
Thermoanaerobacter subterraneus	French oil field at a depth of 645 mbls	Anaerobic	Thermophilic (35–80°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	0–3%	Fardeau et al. 2000
Thermoanaerobacter sp.	Deep subsurface aquifer at Colorado at depths of 860–1,996 mbls	Anaerobic	Thermophilic (55–70°C)	Hydrogenotrophic or organotorphic with various substrates and Fe(III)-, Mn(IV)-, Cr(VI)-, U(VI)- and Co(III)-reduction	Heterotrophic with various substrates		Roh et al. 2002
Haloanaerobium acethylicum	Brines in oil rigs in the Gulf of Mexico	Anaerobic	Mesophilic (15–45°C)	Organotorphic with various substrates	Heterotrophic with various substrates	4.7–22%	Rengpipat et al. 1988
Haloanaerobium congolense	African offshore oil field at Congo	Anaerobic	Mesophilic (20–45°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	1.7–23%	Ravot et al. 1997

Haloanaerobium kushneri	Brines in oil reservoir at Oklahoma	Anaerobic	Mesophilic (20–45°C)	Organotorphic with various substrates	Heterotrophic with various substrates	8.8–18%	Bhupathiraju et al. 1999
Haloanaerobium salsuginis	Brines in oil reservoir at Oklahoma	Anaerobic	Mesophilic (22–51°C)	Organotorphic with various substrates	Heterotrophic with various substrates	5.9–24%	Bhupatiraju et al. 1994
Desulfotomaculum australicum	Great Artesian Basin aquifer at a depth of 1,914 mbls	Anaerobic	Thermophilic (40–74°C)	Sulfate-reducing organotrophic with organic acids	Heterotrophic with various substrates		Love et al. 1993
Desulfotomaculum geothermicum	Terrestrial deep geothermal aquifer at a depth of 2,500 m	Anaerobic	Thermophilic (37–56°C)	Sulfate-reducing hydrogenotrophic and organotrophic with organic acids	Heterotrophic with various substrates	0.2–5%	Daumas et al. 1988
Desulfotomaculum halophilus	Oil production well at Paris Basin at a depth of 1,820 m	Anaerobic	Mesophilic (30–40°C)	Sulfate-reducing organotrophic with organic acids	Heterotrophic with various substrates	1–14%	Tardy-Jacquenod et al. 1998
Desulfotomaculum kuznetsovii	Thermal mineral water at Sukhums	Anaerobic	Thermophilic (50–85°C)	Sulfate-reducing organotrophic with organic acids	Heterotrophic with various substrates	0–3%	Nazina et al. 1989
Desulfotomaculum putei	Terrestrial sedimen core at a depth of 2,700 mbls	Anaerobic	Thermophilic (40–65°C)	Sulfate-reducing hydrogenotrophic and organotrophic with organic acids	Heterotrophic with various substrates	<1–>2%	Liu et al. 1997
Desulfotomaculum thermocisternum	Oil producing well at North Sea at a depth of 2,600 mbsf	Anaerobic	Thermophilic (41–75°C)	Sulfate-reducing hydrogenotrophic and organotrophic with organic acids	Heterotrophic with various substrates	0–7%	Nilsen et al. 1996

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Desulfotomaculum thermosubterraneum	Japanese metal mine at a depth of 250 mbls	Anaerobic	Thermophilc (50–72°C)	Sulfate-reducing hydrogenotrophic and organotrophic with organic acids	Heterotrophic with various substrates	0–1.5%	Kaksonen et al. 2006
Geobacillus uzenensis	Oil field in Kazakhstan	Aerobic	Thermophilic (45–65°C)	Organotorphic with various substrates including hydrocarbons	Heterotrophic with various substrates including hydrocarbons	0–4%	Nazina et al. 2000, 2001
Geobacillus subterraneus	Oil field in China	Aerobic	Thermophilic (45–65°C)	Organotorphic with various substrates including hydrocarbons	Heterotrophic with various substrates including hydrocarbons	0–3%	Nazina et al. 2000, 2001
Bacillus infernus	Terrestrial sedimen core at a depth of 2,700 mbls	Anaerobic	Thermophilic (45–60°C)	Organotrophic with fermentation or with Fe (III)- and Mn(IV)-reduction	Heterotrophic with various substrates	0–3.5%	Boone et al. 1995
Bacillus sp.	Subpermafrost saline fracture water in Canada at a depth of 1,130 mbls	Facultative anaerobic	Mesophilic (10–37°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0.05–7.5%	Onstott et al. 2009

Actinobacteria *Tessaracoccus profundi*	Chesapeake impact crater drill core at a depth of 940 mbls	Facultative anaerobic	Mesophilic (10–45°C)	Organotorphic with various substrates with O_2-respiration and anaerobic fermentation	Heterotrophic with various substrates	0–5%	Finster et al. 2009
Microbacterium sp.	Subpermafrost saline fracture water in Canada at a depth of 1,130 mbls	Aerobic	Psychrophilic to mesophilic (0–30°C)	Oganotorphic with various substrates	Heterotrophic with various substrates	0.05–7.5%	Onstott et al. 2009
CFB Group *carriage return Spirochaeta smaragdinae*	African offshore oil field at Congo	Anaerobic	Mesophilic (20–40°C)	Organotorphic with various substrates and S^0- and thiosulfate-reduction	Heterotrophic with various substrates	1–10%	Magot et al. 1997b
Alphaproteobacteria *Microvirga subterranea*	Terrestrial aquifer water from a depth of 295 mbls	Aerobic	Mesophilic (25–45°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0–1%	Kanso and Patel 2003
Phenylobacterium lituiforme	Terrestrial aquifer water from a depth of 295 mbls	Facultative anaerobic	Mesophilic (25–45°C)	Organotorphic with various substrates with O_2-respiration or fermentation	Heterotrophic with various substrates	0–1%	Kanso and Patel 2004
Sphingopyxis sp.	Subpermafrost saline fracture water in Canada at a depth of 890 mbls	Aerobic	Mesophilic (4–30°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0–0.05%	Onstott et al. 2009

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Brevundimonas spp.	Subpermafrost saline fracture water in Canada at a depth of 890 mbls	Facultative anaerobic	Mesophilic (0–30°C)	Organotorphic with various substrates with O_2-respiration or fermentation	Heterotrophic with various substrates	0.05–5%	Onstott et al. 2009
Methylocystis hirsuta	Ground water at California	Aerobic	Mesophilic	Methanotrophic	Methanotrophic and autotrophic		Lindner et al. 2007
Betaproteobacteria *Thiobacter subterraneus*	Japanese gold mine at 320 m below land surface (mbls)	Microaerophilic	Thermophilic (35–62°C)	Chemolithotrophic (reduced S compounds as electron donors and O_2 as an electron acceptors	Autotrophic	0.1–1.6%	Hirayama et al. 2005
Gammaproteobacteria *Methylomonas scandinavica*	Deep ground water at Sweden at a depth of >400 m	Aerobic	Mesophilic (5–30°C)	Methanotorphic and methylotrophic	Methanotorphic and methylotrophic		Kalyuzhnaya et al. 1999
Arhodomonas aquaeolei	Petroleum reservoir at Oklahoma	Aerobic	Mesophilic	Organotorphic with various substrates with O_2-respiration	Heterotrophic with various substrates	6–20%	Adkin et al. 1993
Shewanella spp.	Subpermafrost saline fracture water in Canada at a depth of 1,130 mbls	Facultative anaerobic	Psychrophilic to mesophilic (0–30°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0.05–5%	Onstott et al. 2009

Shewanella profunda	Subseafloor sediments at Nankai Trough at 4.2 mbsf	Facultative anaerobic	Psychrophilic to mesophilic (4–37°C)	Organotorphic with various substrates with O_2-respiration or anaerobically with nitare-, fumarate-, Fe(III)- and cystine-reduction	Heterotrophic with various substrates	0–6%	Toffin et al. 2004a
Marinobacter aquaeolei	Oil producing well at Vietnam	Facultative anaerobic	Mesophilic (13–50°C)	Organotorphic with various substrates including hydrocarbons with O_2-respiration or anaerobically with nitrate- and fumarate-reduction	Heterotrophic with various substrates	0–20%	Huu et al. 1999
Marinobacter alkaliphilus	Subseafloor serpentinite mud at Mariana Forearc at a depth of 1.5 m	Facultative anaerobic	Mesophilic (10–50°C)	Organotorphic with various substrates including crude oils with O_2-respiration or anaerobically with nitrate-reduction	Heterotrophic with various substrates	0–21%	Takai et al. 2005
Acinetobacter sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobic	Mesophilic (25–45°C)	Organotorphic with various substrates including hydrocarbons	Heterotrophic with various substrates	0–10%	Nazina et al. 2005
Pseudomonas spp.	Subpermafrost saline fracture water in Canada at depths of 890 and 1,130 mbls	Facultative anaerobic	Psychrophilic to mesophilic (0–37°C)	Organotorphic with various substrates with O_2-respiration or nitrate-reduction	Heterotrophic with various substrates	0.05–10%	Onstott et al. 2009

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Deltaproteobacteria *Desulfacinum infernum*	Oil production water at the North Sea	Anaerobic	Thermophilic (40–65°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Autotrophic and mixotrophic with various substrates	0–5%	Rees et al. 1995
Desulfobacter vibrioformis	Oil production platform at the North Sea	Anaerobic	Mesophilic (5–38°C)	Sulfate-reducing organotorphic with various substrates	Heterotrophic with various substrates	1–5%	Lien and Beeder 1997
Desulfosarcina cetonicum	Oil deposits at Azerbaijan	Anaerobic	Mesophilic (20–37°C)	Sulfate-reducing organotorphic with various substrates	Heterotrophic with various substrates	up to 5%	Galushko and Rosanova 1991
Desulfomicrobium apsheronum	Oil deposits at Azerbaijan	Anaerobic	Mesophilic (4–40°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Autotrophic and mixotrophic with various substrates	0–8%	Rozanova et al. 1988
"Desulfomicrobium hypogeium"	Deep terrestrial Crataceous sediments at New Mexico	Anaerobic	Mesophilic	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Autotrophic and mixotrophic with various substrates		Krumholz et al. 1999
Desulfovibrio aespoeensis	Deep subterranean aquifer at a depth of 600 mbls	Anaerobic	Mesophilic (4–35°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates	0–0.7%	Motamedi and Pedersen 1998
Desulfovibrio bastinii	African offshore oil field at Congo	Anaerobic	Mesophilic	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates	0.1–12%	Magot et al. 2004

Desulfovibrio gracilis	African offshore oil field at Congo	Anaerobic	Mesophilic	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates	0.2–12%	Magot et al. 2004
Desulfovibrio cavernae	Deep terrestrial subsurface sandstones at depths of 600–1,060 m	Anaerobic	Mesophilic (20–50°C)	Sulfate-reducing organotorphic with various substrates	Heterotophic with various substrates	0.2–19%	Sass and Cypionka 2004
Desulfovibrio carbinoliphilus	Gas-condensate-aquifer at Colorado	Aanaerobic	Mesophilic (22–36°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates including log chains of alcohols	Autotrophic and mixotrophic with various substrates		Allen et al. 2008
Desulfovibrio longreachensis	Great Artesian Basin aquifer at a depth of 1,914 mbls	Anaerobic	Mesophilic	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates		Redburn and Patel 1994
Desulfovibrio longus	Oil production well at Paris Basin	Anaerobic	Mesophilic (10–40°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates	0–8%	Magot et al. 1992
Desulfovibrio putealis	Deep subsurface aquifer at Paris Basin at a depth of 430 mbls	Anaerobic	Mesophilic (15–45°C)	Sulfate-reducing hydrogenotrophic and organotorphic with various substrates	Heterotophic with various substrates	0–6	Basso et al. 2005

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Desulfovibrio vietnamensis	Vietnam oil production water	Anaerobic	Mesophilic (12–45°C)	Sulfate-reducing organotorphic with various substrates	Heterotrophic with various substrates	0–10%	Nga et al. 1996
Desulfovibrio profundus	Deep subseafloor sediment at Japan Sea at depths of 80–500 mbsf	Aaerobic	Mesophilic to thermophilic (15–65°C)	Sulfate- and Fe(III)-reducing hydrogenotrophic and organotorphic with various substrates	Heterotrophic with various substrates	0.2–10%	Bale et al. 1997
Desulfoglaeba alkanexedens	Oil production water at Oklahoma	Anaerobic	Mesophilic (17–50°C)	Sulfate-reducing organotorphic with various alkanes	Heterotrophic with various alkanes	0–5.5%	Davidova et al. 2006
Desulfocurvus vexinensis	Deep artesian aquifer at Paris Basin	Anerobic	Mesophilic (20–50°C)	Sulfate-reducing organotorphic with various substrates	Heterotrophic with various substrates	0–2%	Klouche et al. 2009
Geoalkalibacter subterraneus	Oil field at Utah at a depth of 1,540 m	Anaerobic	Mesophilic (30–50°C)	Fe(III)-, Mn(IV)-, nitrate- and S^0-reducing organotorphic with various substrates	Heterotrophic with various substrates	0.1–10%	Greene et al. 2009
Geobacter bemidjiensis	Subsurface sediments at New Mexico	Anaerobic	Mesophilic (15–37°C)	Fe(III)-, and Mn(IV)-reducing organotorphic with various substrates	Heterotrophic with various substrates		Nevin et al. 2005
Geobacter psychrophilus	Subsurface groundwater at Massachusetts	Anaerobic	Mesophilic (4–30°C)	Fe(III)-, and Mn(IV)-reducing organotorphic with various substrates	Heterotrophic with various substrates		Nevin et al. 2005

Geobacter chapellei	Deep terrestrial subsurface sediments	Anaerobic	Mesophilic	Fe(III)-, and Mn(IV)-reducing organotorphic with various substrates and also capable of reducing U(VI)	Heterotrophic with various substrates		Coates et al. 2001
Epsilonproteobacteria *Sulfuricurvum kujiense*	Underground oil storage cavity water	Facultative anaerobic	Mesophilic	Hydrogenotrophic and thiotrophic chemolithotrophic with O_2- and nitrate-reduction	Autotrophic		Kodama and Watanabe 2004
Sulfurimonas sp.	Oil production brine fluid at Canada at a depth of 823 mbls	Microaerophilic to anaerobic	Mesophilic (5–35°C)	Hydrogenotrophic and thiotrophic chemolithotrophic with O_2- and nitrate-reduction	Autotrophic	0–7%	Gevertz et al. 2000
Arcobacter sp.	Oil production brine fluid at Canada at a depth of 823 mbls	Microaerophilic to anaerobic	Mesophilic (10–40°C)	Hydrogenotrophic and thiotrophic chemolithotrophic with O_2-, nitrate-, and S^0-reduction	Autotrophic	0–3%	Gevertz et al. 2000
Archaea ***Thermococcales*** *Thermococcus sibiricus*	Western Siberia oil reservoir at depths of 1,800–2,290 mbls	Anaerobic	Extremely thermophilic (40–88°C)	Organotorphic with various substrates and S^0-reduction	Heterotrophic with various substrates	0.5–7%	Miroshnichenko et al. 2001
Thermococcus sp.	Japanese oil reservoir	Anaerobic	Hyperthermophilic (46–95°C)	Organotorphic with various substrates	Heterotrophic with various substrates	0.1–6%	Takahata et al. 2000
Thermococcus sp.	Continental oil reservoir at East Paris Basin	Anaerobic	Hyperthermophilic (50–98°C)	Organotorphic with various substrates and S^0-reduction	Heterotrophic with various substrates	0.3–6.5%	L'Haridon et al. 1995

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Archaeoglobales *Archaeoglobus* sp.	Continental oil reservoir at East Paris Basin	Anaerobic	Hyperthermophilic (64–88°C)	Sulfate-reducing organotrophic with organic acids	Heterotrophic with various substrates	0–3.6%	L'Haridon et al. 1995
Archaeoglobus fulgidus	Geothermal water at Paris Basin at a depth of 1,900 mbls	Anaerobic	Extremely thermophilic (55–85°C)	Sulfate- and Fe(III)-reducing hydrogenotrophic and organotrophic with organic acids	Heterotropic	0–3%	Fardeau et al. 2009
Thermoplasmales *Ferroplasma acidarmanus*	Subsurface acid mine drainage water at California	Aerobic	Mesophilic (23–46°C)	Fe(II)-oxidizing chemolithtrophic and organotorphic with various substrates	Heterotrophic with various substrates	(pH range for growth = pH 0–1.5)	Edwards et al. 2000; Dopson et al. 2004
Methanococcales *Methanothermococcus thermolithotrophicus*	Oil production fluid at North Sea at a depth of 2.6 km	Anaerobic	Thermophilic (17–62°C)	Hydrogenotrophic methanogenesis	Autotrophic	0.6–9.4%	Nilsen and Torsvik 1996
Methanococcus aeolicus	Deep subseafloor sediment at a depth of 247 mbsf	Anaerobic	Mesophilic (10–55°C)	Hydrogenotrophic methanogenesis	Autotrophic	0.5–5.9%	Kendall et al. 2006
Methanobacteriales *Methanobacterium ivanovii*	Oil-bearing sandstone core at Bondyuzshkoe oil field at a depth of 1,650 mbls	Anaerobic	Mesophilic (10–55°C)	Hydrogenotrophic methanogenesis	Autotrophic		Belyaev et al. 1987

Methanobacterium subterraneum	Deep granitic aquifer at depths of 68–420 mbls	Anaerobic	Mesophilic (4–45°C)	Hydrogenotrophic methanogenesis	Autotrophic	1.2–8.2%	Kotelnikova et al. 1998
Methanomicrobiales *Methanoculleus submarinus*	Deep subseafloor sediment at a depth of 247 mbsf	Anaerobic	Mesophilic (15–50°C)	Hydrogenotrophic methanogensis	Mixotrophic	0.6–2.3%	Mikucki et al. 2003
Methanocalculus halotorelans	Oil well at Africa	Anaerobic	Mesophilic (30–45°C)	Hydrogenotrophic methanogensis	Mixotrophic	0–12.5%	Ollivier et al. 1998
Methanosarcinales *Methanolobus zinderi*	Deep subsurface coal seam at Louisiana at a depth of 926 mbls	Anaerobic	Mesophilic (25–50°C)	Methylotrophic methanogenesis	Methylotrophic and hetetrophic	1.2–3.5%	Doerfert et al. 2009
Methanolobus profundi	Gas well water from a depth of 347–795 mbls	Anaerobic	Mesophilic (9–37°C)	Methylotrophic methanogenesis	Methylotrophic and hetetrophic	0.6–5.9%	Mochimaru et al. 2009
Methanosarcina siciliae	Gas and oil well at Gulf of Mexico	Anaerobic	Mesophilic (25–45°C)	Methylotrophic and DMS-utilizing methanogenesis	Methylotrophic and hetetrophic	2.3–3.5%	Ni and Boone 1991
Halobacteriales *Halosimplex carlsbadense*	Rock salt crystals at New Mexico at a depth of 650 mbls	Aerobic	Mesophilic (22–55°C)	Organotorphic with relatively simple organic acids	Heterotrophic with relatively simple organic acids	20–30%	Vreeland et al. 2002

Table 9.2.1 (Continued)

Microorganisms	Source environment	Growth with O_2	Growth temperature	Energy metabolism	Carbon metabolism	Growth with NaCl	References
Halobacterium salinarum	Brine inclusions in halite crystals at depths of 8–85 mbls	Aerobic	Mesophilic	Organotorphic with various substrate	Heterotrophic with various substrate	20% (optimum)	Mormile et al. 2003
Halobacterium noricense	Terrestrial salt crystal core at Austlia at a depth of 470 mbls	Aerobic	Mesophilic (22–50°C)	Organotorphic with various substrate	Heterotrophic with various substrate	12.5-saturation	Gruber et al. 2004

Table 9.2.2

Potential subsurface microorganisms of which physiological properties are characterized in part

Microorganisms	Source environment	Physiological remarks	References
Thermotogae *Petrotoga* sp.	Western Siberia oil reservoir at depths of 1,700–2,500 mbls	Anaerobically grow at 60°C	Slobodkin et al. 1999
Petrotoga spp.	Offshore oil fields at California at depths of 1,500–2,400 mbsf	Anaerobically grow with organic substrates at 60–70°C	Orphan et al. 2000
Thermotoga maritima	Western Siberia oil reservoir at depths of 1,700–2,500 mbls	Anaerobically grow at 75°C with iron-reduction	Slobodkin et al. 1999
Deinococcus-Thermus *Thermus* sp.	Icelandic subterranean hot spring	Aerobically grow at 65°C	Marteinsson et al. 2001
Deferribacteres *Deferribacter* sp.	Offshore oil field at California at depths of 1,500–1,700 mbsf	Anaerobically grow at 80°C	Orphan et al. 2000
Synergistetes *Anaerobaculum* spp.	Offshore oil fields at California at depths of 1,500–2,400 mbsf	Anaerobically grow with organic substrates at 60–80°C	Orphan et al. 2000
Fermicutes *Thermoanaerobacter* spp.	Western Siberia oil reservoir at depths of 1,700–2,450 mbls	Anaerobically grow at 70°C	Bonch-Osmolovskaya et al. 2003
Thermoanaerobacter spp.	Western Siberia oil reservoir at depths of 1,700–2,500 mbls	Anaerobically grow at 60–70°C with iron-reduction	Slobodkin et al. 1999
Thermoanaerobacter sp.	Offshore oil field at California at depths of 1,500–1,700 mbsf	Anaerobically grow with organic substrates at 75°C	Orphan et al. 2000
"Acetobacterium psammolithicum"	Deep terrestrial Crataceous sediments at New Mexico at a depth of 209 mbls	Anaerobically grow at 23°C with homo-acetogenesis	Krumholz et al. 2002
Acetobacterium sp.	Subseafloor sediments at Nankai Trough at 4.2 mbsf	Anaerobically grow at 25°C with homo-acetogenesis	Toffin et al. 2004b
Desulfotomaculum spp.	Deep terrestrial subsurface sandstones at depths of 600–1,060 m	Anaerobically grow with organotrophic sulfate-reduction at 30–65°C and at 0.2–19% NaCl	Sass and Cypionka 2004
Desulfotomaculum spp.	Subseafloor sediments at Cascadia Margin at a depth of 0.5 mbsf	Mesophilic SRB	Barnes et al. 1998
Geobacillus spp.	Western Siberia oil reservoir at depths of 1,700–2,450 mbls	Aerobically grow with hydrocarbons at 60°C	Bonch-Osmolovskaya et al. 2003

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Geobacillus spp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 60°C	Nazina et al. 2005
Geobacillus spp.	Subseafloor sediments from offshore Japan at depths of 0.5–343 m below seafloor (mbsf)	Aerobically grow with organic substrates at 50°C	Kobayashi et al. 2008
Bacillus spp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Bacillus spp.	Core samples from subseafloor oil reservoir at Brazil at depths of 2,608–2,658 m below sea surface	Aerobically grow with hydrocarbons at 30°C	da Cunha et al. 2006
Bacillus spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Bacillus spp.	Subseafloor sediments from offshore Japan at depths of 0.5–343 mbsf	Aerobically grow with organic substrates at 15–50°C	Kobayashi et al. 2008
Bacillus spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–420 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007
Bacillus spp.	Aubseafloor sediments at Sea of Okhotsk at depths of 1.5–31 mbsf	Aerobically grow with organic substrates at 25°C	Inagaki et al. 2003b
Oceanibacillus sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Brevibacillus spp.	Subseafloor sediments from offshore Japan at depths of 0.5–343 mbsf	Aerobically grow with organic substrates at 15–50°C	Kobayashi et al. 2008
Paenibacillus spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Paenibacillus spp.	Subseafloor sediments from offshore Japan at depths of 0.5–343 mbsf	Aerobically grow with organic substrates at 4–15°C	Kobayashi et al. 2008
Paenibacillus spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–420 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Paenibacillus spp.	Subseafloor sediments at Sea of Okhotsk at a depth of 31 mbsf	Aerobically grow with organic substrates at 15°C	Inagaki et al. 2003b
Virgibacillus sp.	Deep terrestrial rock salt crystal core at a depth of 564 mbls	Aerobically grow with organic substrates at 37°C with 8–20% NaCl	Vreeland et al. 2000
Marinolactobacillus spp.	Subseafloor sediments at Sea of Okhotsk at a depth of 31 mbsf	Aerobically grow with organic substrates at 25°C	Inagaki et al. 2003b
Staphylococcus spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Actinobacteria *Micrococcus* sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Micrococcus spp.	Subseafloor sediments from offshore Japan at a depth of 107 mbsf	Aerobically grow with organic substrates at 15–30°C	Kobayashi et al. 2008
Micrococcus spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–381 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007
Dietzia sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 19°C	Fru and Athar 2008
Dietzia sp.	Subseafloor sediments from offshore Japan at a depth of 343 mbsf	Aerobically grow with organic substrates below at 30°C	Kobayashi et al. 2008
Dietzia sp.	Subseafloor sediments at Sea of Okhotsk at a depth of 18 mbsf	Aerobically grow with organic substrates at 35°C	Inagaki et al. 2003b
Rhodococcus spp.	Subseafloor sediments from offshore Japan at depths of 190–343 mbsf	Aerobically grow with organic substrates below at 30°C	Kobayashi et al. 2008
Rhodococcus sp.	Subseafloor sediments at Sea of Okhotsk at a depth of 25 mbsf	Aerobically grow with organic substrates at 25°C	Inagaki et al. 2003b
Aeromicrobium sp.	Subseafloor sediments from offshore Japan at a depth of 8 mbsf	Aerobically grow with organic substrates below at 50°C	Kobayashi et al. 2008
Microbacterium spp.	Deep terrestrial uranium contaminated aquifer at depths of 290–324 mbls	Aerobically grow with organic substrates below at 25°C and is uranium-tolerant	Nedelkova et al. 2007

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Microbacterium sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Microbacterium spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Microbacterium sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 35–55°C	Fru and Athar 2008
Microbacterium sp.	Subseafloor sediments from offshore Japan at a depth of 217 mbsf	Aerobically grow with organic substrates	Kobayashi et al. 2008
Brachybacterium sp.	Subseafloor sediments from offshore Japan at a depth of 343 mbsf	Aerobically grow with organic substrates below at 30°C	Kobayashi et al. 2008
Oerkovia spp.	Subseafloor sediments from ODP Leg#201 at depths of 2–101 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007
Microcella sp.	Subseafloor sediments from offshore Japan at a depth of 48 mbsf	Aerobically grow with organic substrates	Kobayashi et al. 2008
Streptomyces sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 35–55°C	Fru and Athar 2008
Streptomyces sp.	Subseafloor sediments from offshore Japan at a depth of 5 mbsf	Aerobically grow with organic substrates at 15–30°C	Kobayashi et al. 2008
Kocuria sp.	Subseafloor sediments from ODP Leg#201 at depths of 21–40 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007
Kocuria sp.	Subseafloor sediments at Sea of Okhotsk at a depth of 31 mbsf	Aerobically grow with organic substrates at 35°C	Inagaki et al. 2003b
Citrococcus spp.	Subseafloor sediments at Sea of Okhotsk at a depth of 31 mbsf	Aerobically grow with organic substrates at 25°C	Inagaki et al. 2003b
Geogenia spp.	Subseafloor sediments at Sea of Okhotsk at a depth of 31 mbsf	Aerobically grow with organic substrates at 35°C	Inagaki et al. 2003b
Thermoactinomyces spp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 60°C	Nazina et al. 2005

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Mycobacterium sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Cellulomonas sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow with hydrocarbons at 40°C	Nazina et al. 2005
Arthrobacter spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Arthrobacter sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 35–55°C	Fru and Athar 2008
Terrabacter spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Leifsonia spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Rothia sp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Gordonia sp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Ornithinimicrobium sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 35–55°C	Fru and Athar 2008
***CFB* Group** *Flexibacteraceae* sp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Flexibacteraceae sp.	Subseafloor sediments from offshore Japan at a depth of 247 mbsf	Aerobically grow with organic substrates below at 15°C or 30°C	Kobayashi et al. 2008
Flexibacteraceae sp.	Subseafloor sediments from offshore Japan at a depth of 247 mbsf	Aerobically grow with organic substrates below at 15°C or 30°C	Kobayashi et al. 2008
Flexibacteraceae spp.	Subseafloor sediments from offshore Japan at depths of 1–190 mbsf	Aerobically grow with organic substrates below at 15°C or 30°C	Kobayashi et al. 2008
Cytophagaceae sp.	Subseafloor sediments from ODP Leg#201 at a depth of 198 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Alphaproteobacteria *Sphingomonas* spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Sphingomonas sp.	Subseafloor sediments from offshore Japan at a depth of 190 mbsf	Aerobically grow with organic substrates at 15°C or 30°C	Kobayashi et al. 2008
Paracoccus sp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Paracoccus spp.	Subseafloor sediments from offshore Japan at depths of 48–218 mbsf	Aerobically grow with organic substrates at 15°C or 30°C	Kobayashi et al. 2008
Devocia sp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 19°C	Fru and Athar 2008
Sulfitobacter spp.	Subseafloor sediments at Sea of Okhotsk at a depth of 25 mbsf	Aerobically grow with organic substrates at 25°C	Inagaki et al. 2003b
Hyphomonas sp.	Subseafloor sediments from offshore Japan at a depth of 190 mbsf	Aerobically grow with organic substrates at 15°C or 30°C	Kobayashi et al. 2008
Roseinatronobacter sp.	Subseafloor sediments from offshore Japan at a depth of 190 mbsf	Aerobically grow with organic substrates at 15°C or 30°C	Kobayashi et al. 2008
Rhizobium spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–381 mbsf	Aerobically grow with organic substrates below room temperatures	D'Hondt et al. 2004; Batzke et al. 2007
Rhodobacteraceae sp.	Subseafloor sediments from offshore Japan at a depth of 190 mbsf	Aerobically grow with organic substrates at 15°C or 30°C	Kobayashi et al. 2008
Rhodobacteraceae spp.	Subseafloor sediments from ODP Leg#201 at depths of 43–268 mbsf	Aerobically grow with organic substrates below room temperatures and most anaerobically grow with nitrate-reduction	D'Hondt et al. 2004; Batzke et al. 2007
Betaproteobacteria *Hydrogenophilus* sp.	Japanese gold mine at 320 m below land surface (mbls)	Microarophilic S-oxidizing thermophile growing at 55°C	Inagaki et al. 2003b
Azoarcus spp.	Japanese gold mine at 320 m below land surface (mbls)	Anaerobically oligotrophic nitrate-reducer growing at 50–60°C	Inagaki et al. 2003b
Cupriavidus spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Comamonas spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Hydrogenophaga sp.	Subpermafrost saline fracture water in Canada at a depth of 1,130 mbls	Aerobically grow	Onstott et al. 2009
Gammaproteobacteria *Methylothermus* sp.	Japanese gold mine at 320 mbls	Aerobic methanotophic growth at 55–75°C	Inagaki et al. 2003a
Pseudomonas sp.	Formation water from Chinese oil reservoir at depths of 1,206–1,435 mbls	Aerobically grow at 40°C with organic substrates including hydrocarbons	Nazina et al. 2005
Pseudomonas stutzeri	Terrestrial sediment cores at a Japanese oil reservoir at depths of 83–199 mbls	Facultatively anaerobic growth at 25°C with hydrocarbons using O_2 or nitrate as electron acceptors	Nunoura et al. 2006
Pseudomonas spp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Pseudomonas spp.	Deep aquifer at Sweden at a depth of 450 m below sea surface	Aerobically grow at 19–55°C	Fru and Athar 2008
Pseudomonas spp.	Subseafloor sediments from offshore Japan at depths of 13–343 mbsf	Aerobically grow with organic substrates at 4–30°C	Kobayashi et al. 2008
Enterobacter sp.	Terrestrial subsurface sediments at depths of 173–259 mbls in the USA	Resistant to antibiotics	Brown and Balkwill 2009
Enterobacter sp.	Subseafloor sediments from offshore Japan at a depth of 343 mbsf	Aerobically grow with organic substrates at 30°C	Kobayashi et al. 2008
Halomonas spp.	Subseafloor sediments from offshore Japan at depths of 48–217 mbsf	Aerobically grow with organic substrates at 4–30°C	Kobayashi et al. 2008
Acinetobacter spp.	Subseafloor sediments from offshore Japan at depths of 190–213 mbsf	Aerobically grow with organic substrates at 4–30°C	Kobayashi et al. 2008
Pseudoalteromonas spp.	Subseafloor sediments from offshore Japan at depths of 190–217 mbsf	Aerobically grow with organic substrates at 4–30°C	Kobayashi et al. 2008

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Vibrio spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–87 mbsf	Aerobically grow with organic substrates below room temperatures and most anaerobically grow with nitrate-reduction	D'Hondt et al. 2004; Batzke et al. 2007
Marinobacter spp.	Subseafloor sediments from ODP Leg#201 at a depth of 268 mbsf	Aerobically grow with organic substrates below room temperatures and most anaerobically grow with nitrate- or Mn(IV)-reduction	D'Hondt et al. 2004; Batzke et al. 2007
Psychrobacter spp.	Subseafloor sediments from ODP Leg#201 at depths of 1–124 mbsf	Aerobically grow with organic substrates below room temperatures and most anaerobically grow with nitrate- or Mn(IV)-reduction	D'Hondt et al. 2004; Batzke et al. 2007
Deltaproteobacteria *Desulfovibrio profundus*	Subseafloor sediments at Cascadia Margin at a depth of 222 mbsf	Psychrophilic and piezophilic SRB	Barnes et al. 1998
Archaea ***Thermococcales*** *Thermococcus* spp.	Offshore oil fields at California at depths of 1,500–2,400 mbsf	Anaerobically grow with organic substrates and S^0 at 60–85°C	Orphan et al. 2000
Thermococcus spp.	Oil reservoirs at Alaska and North Sea below at a depth of 3,000 m	Anaerobically grow with organic substrates and S^0 at 50–98°C	Stetter et al. 1993
Pyrococcus spp.	Oil reservoirs at Alaska and North Sea below at a depth of 3,000 m	Anaerobically grow with organic substrates and S^0 at 60–103°C	Stetter et al. 1993
Archaeoglobales *Archaeoglobus* spp.	Oil reservoirs at Alaska and North Sea below at a depth of 3,000 m	Anaerobically grow with sulfate-reducing organotrophy or sulfate-reducing chemolithoautotrophy at 60–94°C	Stetter et al. 1993
Methanococcales *Methanothermococcus* spp.	Offshore oil fields at California at depths of 1,500–2,400 mbsf	Anaerobically grow with hydrogentrophic methanogenesis at 60–75°C	Orphan et al. 2000
Methanobacteriales *Methanothermobacter thermautotrophicus*	Offshore oil fields at California at depths of 1,500–2,400 mbsf	Anaerobically grow with hydrogentrophic methanogenesis at 60–70°C	Orphan et al. 2000
Halobacteriales *Halobacteriaceae* spp.	Rock salt crystals at New Mexico at a depth of 650 mbls	Aerobically grow with organic substrates with a 20% NaCl	Vreeland et al. 1998

Table 9.2.2 (Continued)

Microorganisms	Source environment	Physiological remarks	References
Halobacterium spp.	Terrestrial salt deposit core at Brazil at a depth of 768 mbls	Aerobically grow with organic substrates at 30°C at a 20% NaCl	Vreeland et al. 2007
Natronobacterium spp.	Terrestrial salt deposit core at Brazil at a depth of 768 mbls	Aerobically grow with organic substrates at 30°C at a 20% NaCl	Vreeland et al. 2007
Halococcus salifodinae	Terrestrial subsurface salt deposits	Aerobically grow with organic substrates at 37–39°C at a 20–24% NaCl	Stan-Lotter et al. 1999

probably represent the maximal numbers of known microorganisms isolated from the deep biosphere. As shown in *Tables 9.2.1* and *9.2.2*, the potential subsurface microorganisms are phylogenetically diverse and consist of representative species and strains within most of the archaeal and bacterial taxa on the phylum level despite the limited number of successful isolation examples. The phylogenetic diversity of subsurface microbial components would be strongly associated with the environmental (physical, chemical, and biological) variability of their habitats and with their phenotypical and physiological diversity. According to the tables, the phylogenetic and physiological diversity of deep subsurface microorganisms are described in this chapter.

Growth Response to O_2

Since the early studies of subsurface microorganisms focused on the subsurface petroleum reservoir and the associated water samples, a number of potential subsurface microorganisms represented the anaerobic natures for growth (Magot et al. 2000). In addition, a general image of biologically barren subsurface biosphere had been widely accepted according to an assumption that the subsurface environments were physically and chemically static, and thus presenting a very poor energy-yielding state (particularly lack of oxidants such as O_2 and its derivatives). However, it has been becoming evident that both the continental and oceanic subsurface environments are (hydro)geologically and geochemically dynamic and the deep biosphere is not physically, chemically, and (micro)biologically isolated from the surface biosphere in the long and even short time scales. Since the dynamic circulation of O_2-rich and biologically affected surface fluid (gas and liquid) sources such as air, meteoritic water, and seawater into the subsurface environments is driven by geological processes and the recently increasing human activities, oxygenated habitats could be formed and the surface-derived living microbial components could be entrained into many places of the deep biosphere. As shown in *Tables 9.2.1* and *9.2.2*, the subsurface microorganisms isolated so far represent wide spectra of growth and survival response to O_2; from O_2-respiring strict aerobes to extremely O_2-sensitive, strict anaerobes such as thermophilic methanogens. The strictly aerobic O_2-respiring microorganisms are abundantly isolated not only from oligotrophic, relatively shallow terrestrial aquifer waters (relatively oxidative) (Brown and Balkwill 2005) but also organics-rich, deep subseafloor sediment habitats (relatively reductive) (Batzke et al. 2007;

D'Hondt et al. 2004; Kobayashi et al. 2008). Strict aerobes are isolated even from highly reductive subsurface petroleum reservoir habitats probably due to the naturally and artificially introduced oxygenated waters (Nazina et al. 2005). The O_2-sensitive, strictly anaerobic microorganisms such as fermentative organotrophs, sulfate reducers and methanogens are predominantly isolated from the subsurface oil- and gas-associated environments (*Tables 9.2.1* and *9.2.2*).

Growth Response to Temperature

In the deep subsurface environments, many physical and chemical parameters controlling microbial growth and survival can be perceived. Nevertheless, many researchers probably agree that a primary factor is temperature. In the surface environments, liquid water is boiling at up to 100°C, while with an increasing pressure (hydrostatic), liquid water can be present at up to 373°C for pure water and 407°C for seawater (critical points) (Bischoff and Rosenbauer 1988). Indeed, the highest temperature record of liquid water (407°C) was found in a deep-sea hydrothermal vent fluid in the Mid-Atlantic Ridge. In contrast, most of the shallow subseafloor environments are characterized by consistently low temperatures of around 1–4°C, which is not the lowest temperature for life activity but close to a freezing temperature of liquid water. Thus, a great variation of temperature is present in the deep subsurface environments.

Among the potential subsurface microorganisms isolated so far, the highest temperature limit for growth is 103°C, which is the maximal temperature for growth of *Pyrococcus* sp. isolated from an oil-producing well at the North Sea. The in situ temperature of the oil reservoir at a depth of 3,000 m below sea surface is 105°C, corresponding to the growth temperature range of many hyperthermophiles isolated from this habitat and the similar petroleum habitats (Stetter et al. 1993). To date, the upper temperature limit for growth is known to be 122°C of a hyperthermophilic hydrogentrophic methanogen (Takai et al. 2008). In addition, the microorganisms capable of growing above 110°C are all hydrogenotrophic chemolithotrophs within the *Desulfurococcales* or the *Methanopyrus* archaea, living in the deep-sea hydrothermal systems (Blöchl et al. 1997; Kashefi and Lovley 2003; Takai et al. 2008). A hot, deep biosphere associated with terrestrial and oceanic subsurface hydrothermal activities has been proposed (Deming and Baross 1993; Takai et al. 2001a) and suggested (e.g., Takai and Horikoshi 1999; Takai et al. 2001a, 2004, 2006a). If cultivation-dependent microbiological characterization can be successfully conducted in future explorative opportunities of terrestrial and oceanic subsurface hydrothermal systems, extending upper temperature limit for life in this planet may be renewed by the deep subsurface microorganisms.

With decreasing temperatures of the habitats, the growth temperature ranges of the subsurface microorganisms shift to lower values (*Tables 9.2.1* and *9.2.2*). In the subsurface environments of the permafrost in the Siberia, of which in situ temperature is permanently close to the frozen temperature of water, most of the cultivated bacterial components are psychrophiles to psychrotolerant mesophiles (Onstott et al. 2009). From a shallow subseafloor sediment environment closely associated with the cold deep seawater (0–4°C), several psychrophilic marine bacteria are also isolated (Kobayashi et al. 2008; Toffin et al. 2004a). Many of the culture-independent molecular phylogenetic surveys of subsurface microbial communities have suggested the abundant occurrence of thermophilc and hyperthermophilc microbial phylotypes in relatively low temperatures of subsurface environments (e.g., Inagaki et al. 2001; Inagaki et al. 2003a, b; Takai et al. 2001b). Likewise, several thermophiles

have been isolated from consistently low temperatures of surface and near-surface habitats (Takami et al. 1997; Takai et al. 1999). At present, however, none of the living thermophiles are retrieved from low temperatures of subsurface environments by the cultivation-dependent characterization.

Growth Response to Hydrostatic (Lithostatic) Pressure

Hydrostatic pressure is another important physical parameter and is common in the deep subsurface environments. The deepest habitat of the Earth explored by researchers is the Challenger Deep, in the Mariana Trench, at a water depth of ~10,900 m (Kato et al. 1997, 1998), which corresponds to 110 MPa of hydrostatic pressure (*Table 9.2.2*). In addition, a present upper pressure limit for microbial growth is known to be 130 MPa, which was recorded as the highest hydrostatic pressure for growth of deep-sea psychrophilic heterotroph strain MT41 isolated from the Challenger Deep, the Mariana Trench (Yayanos 1986). Thus, the greatest natural environmental pressure that we can now explore in the Earth is apparently not a pressure margin limiting the habitability of life.

There have been very few subsurface microorganisms characterized with respect to their growth and survival responses to pressure. As far as is known, the pressure effect on growth has been investigated in only three species of subsurface microorganisms (Bale et al. 1997; Nilsen and Torsvik 1996; Toffin et al. 2004a). *Desulfovibrio profundus* is a sulfate- and Fe(III)-reducing delta-proteobacterium isolated from deep subseafloor sediments (maximally 500 m below seafloor at a water depth of 900m) in the Japan Sea (Bale et al. 1997). Several strains presented their highest sulfate-reducing activity under the hydrostatic pressures corresponding to the in situ hydrostatic pressures of habitats (Bale et al. 1997). Similarly, *Methanothermococcus thermolithotrophicus* was isolated from a subseafloor oil-producing well at the North Sea (Nilsen and Torsvik 1996). The thermophilic hydrogenotrophic methanogen was likely derived from the oil reservoir at a depth of 2,700 mbsf. The potential in situ hydrostatic pressure of the stratum that the *M. thermolithotrophicus* strain was derived from is about 30 MPa. A deep-sea hydrothermal vent *M. thermolithotrophicus* strain, not the same strain as the subsurface *M. thermolithotrophicus* strain, is known optimally to grow at 30–50 MPa (Bernhardt et al. 1988), which is consistent with the potential in situ pressures of habitats for the subsurface thermophilic methanogen. On the other hand, *Shewanella profunda* was isolated from the shallow subsurface sediments (4.2 mbsf) at a water depth of 4,791 m (Toffin et al. 2004a). Although the in situ hydrostatic pressure was about 48 MPa, the gammaproteobacterium optimally grew at 10 MPa and the sedimentary habitat was located at near the maximum limit of pressure for growth (50 MPa) (Toffin et al. 2004a). These results indicate that the growth response for pressure may be a key physiological index to know the indigenous habitats of the isolated subsurface microorganisms. The consistency between the in situ pressure of isolation environment and the optimal growth pressure of the isolate points that the isolation environment would be the indigenous habitat of the microorganism, while the dissimilarity between the in situ pressure of isolation environment and the optimal growth pressure of the isolate suggests that the microorganism should be originally derived from a spatially different, shallower or deeper, habitat. The growth and survival response for pressure of the subsurface microbial components should be further investigated in the future to understand not only the physiological diversity of subsurface microorganisms but also the propagation and transportation processes and records of subsurface microbial communities.

Growth Response to pH and Salinity

As major chemical parameters affecting microbial growth, variable pH and salinity conditions are found in the deep subsurface environments. The subaerial volcanic and geothermal fields often host extremely acidic environments of which pH values drop close to 0 (e.g., Schleper et al. 1995). This extreme acidity is caused by sulfuric acid and hydrochloric acid originally provided from magmatic volatiles associated with volcanic activity. Since similar volcanisms occur beneath terrestrial and submarine volcanoes, the extremely acidic habitats could be formed in the deep subsurface environments as well. For instance, the lowest pH value of the deep-sea hydrothermal fluid is reported as pH 1.6 in the TOTO caldera field in the Mariana Arc (Nakagawa et al. 2006). The microbiologically habitable environments beneath such deep-sea volcanoes would host a variety of acidic subsurface microorganisms. In addition, mining activities for metal resources are known anthropogenically to create subsurface acidic environments. In a subsurface acid mine drainage environment, extremely acidiphilic microbial communities were found (Edwards et al. 2000). One of the predominant archaeal components in these communities was isolated and was found to grow even at pH 0 (*Table 9.2.1*) (Edwards et al. 2000). This Fe(II)-oxidizing subsurface microorganism was *Ferroplasma acidarmanus*, representing one of the most acidiphilic microorganisms on Earth.

On the other hand, alkaline environments in the deep subsurface are also generated by the water-rock processes. It is well known that the serpentinization reaction of water and olivine, a major mineral of ultramafic rocks abundantly present in the upper mantle, generates highly alkaline water environments (McCollom and Bach 2009; Takai et al. 2006a). The highest pH value reported in the deep subsurface environments is pH 12.5 in the porewater of the South Chamorro Seamount in the Mariana Forearc (Mottl et al. 2003). Although it was not from a deep subsurface environment, an alkaliphilic bacterial species (*Marinobacter alkaliphilus*) was isolated from the serpentine mud core sample at a depth of 1.5 mbsf (*Table 9.2.1*) (Takai et al. 2005). However, the most alkaliphilic life in the Earth was obtained from a man-made alkaline environment (cement container of mining water) in an ultradeep South African gold mine environment at a depth of 3.2 kmbls (Takai et al. 2001c). *Alkaliphilus transvaalensis* is a strictly anaerobic organotrophic bacterium growing at up to pH 12.4 (*Table 9.2.1*) (Takai et al. 2001c). It is a spore-forming member of the *Firmicutes* and is able to form an endospore if the habitat condition become beyond the range of growth. Hence, the dormant cells and spores would be widely distributed in the non-alkaline deep subsurface environments and may reproduce the sizable population in the spatially discrete alkaline habitats encountered. However, as shown in *Tables 9.2.1* and *9.2.2*, most of the subsurface microorganisms grow in the neutrophic range of pH. Indeed, most of the subsurface environments, microbiologically surveyed so far, represent the relatively moderate pH condition.

The salinity of water in the subsurface environments is for the most part controlled by the primary water sources. If the subsurface water source is meteoric water, the salinity would not so much differ from that of the source meteoric water even after the water-rocks (-sediments or -minerals) reaction. In a similar manner, the salinity in most of the subseafloor environments is similar with the seawater even after the geochemical and microbiological alteration processes. Thus, the growth response to salinity in most of the potentially subsurface microorganisms is comparable with those in the surface terrestrial and marine microorganisms (*Tables 9.2.1* and *9.2.2*).

From the subsurface petroleum reservoir environments, halophilic and halotolerant microorganisms have been frequently isolated (*Tables 9.2.1* and *9.2.2*). This is related to the brine and salt-deposit formations often associated with the geological settings and the generation mechanisms of the subsurface petroleum reservoirs. In particular, subsurface salt deposits are quite unique habitats for the subsurface microorganisms. Hypersaline environments such as the subaerial soda lakes and drying inland sea are ubiquitous in the terrestrial surface. In these hypersaline habitats, halophilic and extremely halophilic microbial communities are prosperously present (Oren 2000). The precipitated salts have been accumulated and buried in the geologic time scale, in some cases together with abundant organic compounds. These past dried-up events of lake- and seawater induced by the paleoenvironmental changes are preserved in the subsurface environments and provide unique habitats for the present subsurface microorganisms and even excellent preservative media for the ancient microbial and genetic components (McGenity et al. 2000). Despite the controversy of antiquity for halophilic microorganisms from these ancient salt deposits, it is evident that a diversity of extremely halophilic bacteria and archaea have been isolated from the subsurface salt deposits (*Tables 9.2.1* and *9.2.2*) (Vreeland et al. 2000, 2002; Mormile et al. 2003; Gruber et al. 2004). These bacteria and archaea are able to grow at nearly and over saturation concentrations of NaCl and to survive in the rock salts and salt crystals.

Energy and Carbon Metabolisms

As already described above, the deep biosphere is characterized by a great physical, chemical, and environmental variability. Hence, the microorganisms living there should have a great adaptation potential to the habitational variation. Energy and carbon metabolisms are the energetic and biochemical basis of habitability and adaptation of the living subsurface microorganisms. Many of the recent biogeochemical investigation of the deep biosphere have challenged the crucial energy and carbon sources supporting active microbial communities in representative subsurface regions (Biddle et al. 2008; Blair et al. 2007; Lipp et al. 2008; Lin et al. 2005, 2006). A fundamental question is how much the subsurface microbial communities are sustained by chemolithoautotrophy and/or organoheterotrophy. This question can be translated as how the subsurface microbial activities are primarily driven by interior earth energy (chemosynthesis with geofuels) and/or sunlight (photosynthesis).

The energy and carbon metabolisms frequently identified in the subsurface isolates are organoheterotrophy via fermentation, O_2-respiration, nitrate-reduction, Fe(III)-reduction, S^0-reduction and sulfate-reduction (*Tables 9.2.1* and *9.2.2*). Interestingly, most of the terminal oxidation reactions of inorganic electron acceptors in the subsurface microorganisms are coupled with the organic but not the inorganic energy sources. This is sharply contrary to the case of chemolithotrophic microbial components isolated from the terrestrial geothermal and deep-sea hydrothermal environments (Stetter 2006; Takai et al. 2006b). Most of the hot spring and deep-sea hydrothermal vent chemolithotrophs are strictly autotrophic (Stetter 2006; Nakagawa and Takai 2006, 2008). Many of the subsurface isolates are obtained from the relatively organic compounds-enriched habitats such as the petroleum reservoirs, sediments, and contaminated aquifers, while the terrestrial hot springs and deep-sea hydrothermal vents are highly enriched with the reductive geofuels and quite depleted with organic

compounds. Thus, the organoheterotrophy may somewhat represent the sampling and cultivation bias of the subsurface microorganisms isolated so far.

The major chemolithoautotrophic metabolisms among the potentially subsurface microorganisms are hydrogenotrophic methanogenesis, and hydrogeotrophic and thiotrophic NO_3^- and O_2-reduction (*Tables 9.2.1* and *9.2.2*). In addition to the hydrogenotrophic methanogens, the methylotrophic methanogens are often isolated from the subsurface petroleum and natural gas environments containing metabolic substrates such as methanol, methylamines, and methylsulfides and the product (CH_4) (Doerfert et al. 2009; Mochimaru et al. 2009; Ni and Boone 1991). It is also well known that the subsurface environments serve as enormous pools not only for petroleum but also natural gas and gas hydrate (mainly CH_4) in the Earth (Kvenbolden 1995; Milkov 2004). Not so many but several aerobic methanotrophic bacteria have been cultivated and isolated from the terrestrial, CH_4-rich subsurface habitats (Inagaki et al. 2003a; Kalyuzhnaya et al. 1999; Lindner et al. 2007). Although the significant biomass and biogeochemical function of the anoxic methane-oxidizing (AMO) archaea have been long perceived in the subseafloor sediments (Jorgensen and Boetius 2007), none of the AMO archaea have been successfully isolated from the subsurface and even the surface environments. It is also noteworthy that none of the strictly phototrophic microorganisms has been yet isolated from the deep biosphere.

Other Physiology and Concluding Remarks

The isolation and subsequent physiological characterization of subsurface microorganisms are in many cases related to known taxonomic groups or having known biogeochemical and ecophysiological properties. However, several novel physiological characteristics are of interest from the biochemical and biotechnological points of view. Brown and Balkwill (2009) investigated the antibiotic resistance capability of many terrestrial subsurface isolates. More than half of the subsurface microorganisms represented the multi-antibiotics resistance of growth although it was expected that most of the isolates did not have strong interaction with the antibiotics (Brown and Balkwill 2009). The authors pointed out that the muti-drugs resistance potential of the subsurface isolates was influenced by the connectivity between the surface and subsurface environments (Brown and Balkwill 2009). As already described above, this finding is also a good evidence that the deep biosphere is not physically, chemically, and (micro)biologically isolated from the surface biosphere in the long and even short time scales.

As indicated in *Tables 9.2.1* and *9.2.2*, many of the potential subsurface microorganisms have been obtained from petroleum reservoirs and aquifers contaminated with toxic and harmful substances. These subsurface microorganisms are found to have capability of degrading recalcitrant or toxic hydrocarbons and concentrating disseminated radioactive compounds and toxic elements (*Tables 9.2.1* and *9.2.2*). It is still premature to elucidate these useful functions of the subsurface microorganisms and to apply them to practical environmental remediation and augmentation. However, it is also true that the presently isolated and even the yet uncultivated subsurface microorganisms have great potentials for the future applications. Thus, the cultivation-based investigation of the microbial components in the deep biosphere should be further advanced in order to understand the phenotypic and physiological diversity of subsurface microorganisms and to explore the previously unknown functions and potentials concealed in the deep biosphere.

Cross-References

- 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)
- 9.3 Biochemistry
- 9.4 Genetics, Genomics, Evolution

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9.3 Biochemistry

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_9.3,

Prokaryotes in Deep Biosphere

The prokaryotes in deep biosphere are estimated to constitute 10–33% of total living biomass in the Earth (Parkes et al. 2000; Whitman et al. 1998). Quite a few bacterial and archaeal genera and species have been isolated and described from the terrestrial and oceanic subsurface environments. For example, the following isolates are representative of the taxonomically described prokaryotes obtained from the subsurface environments: a Fe (III)- and Mn (IV)-reducing anaerobe, *Bacillus infernus*, was isolated from 2,700 m below the land surface (mbls) of Tarlorsville Triassic Basin in Virginia (Boone et al. 1995); a chemolithoautotrophic methanogen, *Methanobacterium subterraneum*, and a sulfate-reducing bacterium, *Desulfovibrio aespoeensis*, were isolated from deep granitic groundwater at depths of ~420 m (Kotelnikova et al. 1998) and 600 m (Motamedi and Pedersen 1998), respectively; a thermophilic bacterium, *Thermus scotoductus* (Kieft et al. 1999; Moller and van Heerden 2006), and an extremely alkaliphilic bacterium, *Alkaliphilus transvaalensis* (Takai et al. 2001a), were isolated from the groundwater sampled at about 3,200 m depth in the South African gold mines; furthermore, a thermophilic bacterium, *Hydrogenobacter subterraneus*, was isolated from a water sample of the deep subsurface geothermal water pool at a depth of 1,500 m in the Hacchoubaru geothermal plant in Oita prefecture, Japan (Takai et al. 2001b), and chemolithoautotrophic thermophiles, *Sulfurihydrogenobium subterraneum* and *Thiobacter subterraneus*, were from the subsurface hot aquifer water at more than 300 m depth in Hishikari gold mine, Japan (Takai et al. 2003; Hirayama et al. 2005). Other examples are mainly isolates from deep oil reservoirs, such as the members of *Archaeoglobus* (Stetter et al. 1993), *Thermotoga* (Jeanthon et al. 1995; Slobodkin et al. 1999; Takahata et al. 2000), *Fusibacter* (Ravot et al. 1999), *Mahella* (Salinas et al. 2004), *Petrimonas* (Grabowski et al. 2005), *Bacillus* (da Cunha et al. 2006), and *Sulfuricurvum* (Kodama and Watanabe 2004). On the other hand, from the oceanic subsurface, only three anaerobic isolates (a sulfate-reducing bacterium and methanogens) have been characterized as novel, indigenous subseafloor microorganisms, namely, *Desulfovibrio profundus* from 518 m below seafloor surface (mbsf) in the Sea of Japan (Bale et al. 1997), *Methanoculleus submarines* and *Methanococcus aeolicus* from 247 mbsf in Nankai Trough, Japan (Mikuchi et al. 2003; Kendall et al. 2006).

These subsurface microorganisms are of great interest in terms of their potentially unique biochemistry that would establish the molecular and physiological basis of adaptation to the deep subsurface environments. Nevertheless, there has been little investigation of the biochemistry of subsurface. In this chapter, a few examples of enzymological studies from the subsurface extremophiles are reviewed and recent environmental enzymatic characterizations, "meta-enzymatic analyses," are introduced.

Alkaliphilic Protease from a Subsurface Extremophile

Alkaliphilus transvaalensis SAGM1 was isolated from a mine-water containment dam at 3,200 mbls in an ultra-deep gold mine near Carletonville, South Africa (Takai et al. 2001a). The isolate was a strictly anaerobic chemoorganotroph. It grew over the temperature range from 20°C to 50°C with an optimum temperature of 40°C and pH range from 8.5 to 12.5 with an optimum pH of 10.0. The isolate is the most alkaliphilic life reported so far. The strain utilizes proteinaceous substrates such as yeast extract, peptone, tryptone, and casein as the sole energy

and carbon sources. Thus it seemed likely that the strain extracellularly produced protease(s). Indeed, an extremely alkaliphilic protease (ALTP) was produced by the isolate.

Purification procedures of the protease were very simple. The spent media were centrifuged, and the supernatant was dialyzed against tap water at 4°C for 24 h. To the retentate, a DEAE-Toyopearl 650M resin equilibrated with 10 mM phosphate buffer (pH 7) was added. The filtrate was applied to a CM-Toyopearl column equilibrated with the same buffer, and ALTP was passed through the column. The elutant was concentrated by ultrafiltration to prepare the purified enzyme (Kobayashi et al. 2007).

Properties of Purified ALTP

The molecular mass of ALTP was approximately 30 kDa in SDS-PAGE. ALTP showed the maximal activity with casein at a pH of higher than 12.6 (actual pH of the reaction mixture) in 50 mM KCl-NaOH buffer (*Fig. 9.3.1a*). The optimal pH value was one of the highest values among the known alkaline proteases. The enzyme was stable over a pH range of 5–11, when preincubated at 50°C for 10 min in various pH buffers. The optimal temperature of ALTP was around 70°C at pH 10 in 50 mM borate buffer (*Fig. 9.3.1b*). The enzyme was stable up to 65°C after heating at various temperatures for 10 min. Calcium ions did not affect either the enzyme activity or stability (Kobayashi et al. 2007). These properties are similar to those of other alkaline proteases reported so far (Horikoshi 1971, 1999; Takami et al. 1989; Kobayashi et al. 1995), with the exception of lack of Ca^{2+} ion effects. Casein was the preferred substrate for ALTP among proteinaceous materials tested. The distinct substrate specificity of ALTP was the great number of cleavage sites to the oxidized insulin B-chain. The greatest number of

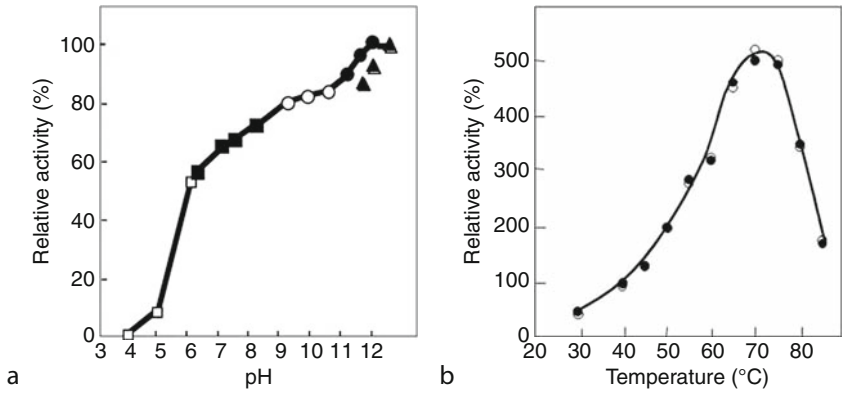

Fig. 9.3.1

Effects of pH and temperature. (a) Effects of pH on the activity. The activity was measured at 40°C for 15 min in the following buffers: □, acetate (pH 3.5–6.0); ■, phosphate (pH 6.5–8.1); ○, carbonate (pH 9.0–11.0); ●, phosphate-NaOH (pH 11.0–12.2); ▲, KCl-NaOH (pH 11.5–12.6). (b) Effects of temperature on the activity. Reactions were carried out for 15 min at various temperatures in 50 mM borate-NaOH buffer (pH 10). The relative activities were expressed as the percentages of the activity at 40°C in the absence of $CaCl_2$. ●, in the absence of $CaCl_2$; ○, in the presence of 5 mM $CaCl_2$

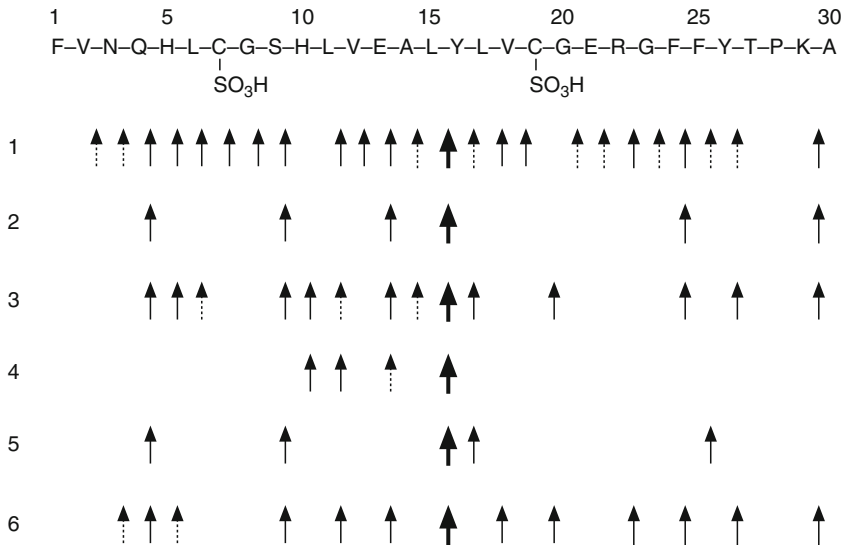

Fig. 9.3.2

Pattern of cleavage by ALTP and other subtilisins of the oxidized insulin B-chain. The arrows indicate the unambiguous LC-MS/MS identification of the fragments generated by ALTP digestion. The initial cleavage sites (*thick arrow*) were determined after incubation for 5 min. The other cleavage sites (*arrows*, major sites; *dashed arrows*, minor sites) were determined after various periods of incubation up to 20 h. 1, ALTP; 2, Sendai (Yamagata et al. 1995); 3, M-protease (Kobayashi et al. 1995); 4, AH101 (Takami et al. 1992a); 5, subtilisin BPN′ (Morihara et al. 1969); 6, subtilisin Carlsberg (0.2 U of commercial enzyme)

cleavage sites previously reported on the oxidized insulin B-chain was 21 sites by an alkaline protease from alkaliphilic *Streptomyces* sp. (Nakanishi and Yamamoto 1974), whereas ALTP showed 24 cleavage sites on the substrate (*Fig. 9.3.2*). The two major peaks, which appeared after 5-min incubation with ATLP, were identified as fragments of Phe^{1}-Leu^{15} and Tyr^{16}-Ala^{30}, indicating that the preferentially initial cleavage site was between Leu^{15} and Tyr^{16}, which was similar with the preferential cleavage site of other subtilisins. Several small fragments were detected after 5- to 60-min incubation, and then most of the peptides detected after complete hydrolysis (20 h) were composed of 4 to 8 amino acid residues. These results suggest that ATLP cleaves the oxidized insulin B-chain in a block-cutting manner. Moreover, ALTP instinctively cleaves Val and Gly at the P1 position in the oxidized insulin B-chain, as compared with the cleavage sites of other subtilisins reported to date (*Fig. 9.3.2*). ALTP activity was inhibited by 1 mM Hg^{2+} ions (85% inhibition) and 1 mM phenylmethylsulfonyl fluoride (100% inhibition). ALTP activity was not affected by other metal ions and representative chemicals including surfactants and protease-specific inhibitors.

Nucleotide and Deduced Amino Acid Sequences of ALTP

A gene encoding ALTP was amplified by PCR using the mixed primers 5′-GCNCARWS-NACNCCNTGGGG-3′ and 5′-CCNGCNACRTGNGTNCCRTG-3′ designed from the *N*-terminal amino acid sequence of ALTP, Ala-Gln-Ser-Thr-Pro-Trp-Gly-Val-Thr-Arg,

purified from the culture of *A. transvaalensis* and the amino acid sequence of His-Gly-Thr-His-Val-Ala-Gly conserved in known subtilisins, respectively, and by using the genomic DNA as the template. The open reading frame (G + C content: 40.0 mol%) encodes 376 amino acids, and the determined *N*-terminal amino acid sequence of the purified ALTP was found in the internal Ala^{98} to Arg^{107}. Therefore, the sequence Met^{1} through Leu^{97} was its prepropeptide. The amino acid sequence of the prepropeptide of ALTP was distantly related with those of the other known subtilisins with 23–42% identity. The calculated molecular mass and p*I* value of mature ALTP (279 amino acids, Ala^{98}–Tyr^{376}) were 28,307 Da and 9.61, respectively.

When suitably aligned with other mature subtilisins, ALTP was the most closely related with subtilisin LD-1 (Saeki et al. 2003) with 64% identity and with subtilisin Sendai (Yamagata et al. 1995) with 61% identity. However, the enzymatic properties of ALTP are not similar to those of subtilisins LD-1 and Sendai. ALTP showed 53–59% identities in amino acid sequence level with other subtilisins, such as high-alkaline M-protease (Hakamada et al. 1994), true subtilisins BPN' (Q44684) and Carlsberg (P00780). The possible catalytic triads were conserved as Asp^{129}, His^{162}, and Ser^{317} in the mature enzyme. Generally, high-alkaline proteases such as M-protease (Hakamada et al. 1994) and no. 221 (Takami et al. 1992b) have the four amino acid deletion around the 160th amino acid of true subtilisin BPN', which corresponds to the P1 binding site (pocket). ALTP had no deletion of the binding site, like LD1 (Saeki et al. 2003), SprC, and SprD (Schmidt et al. 1995). Many anaerobic, proteolytic bacteria have been described, but the properties of the purified proteases from them have been rarely reported (Jang et al. 2002a, b). Most of the proteases from the anaerobes belong to subtilase family B (Siezen and Leunissen 1997), although ALTP belongs to subtilase family A. ALTP was the first subtilisin-like protease obtained from the strictly anaerobic bacteria. An unrooted phylogenetic tree of subtilisins based on their amino acid sequences is shown in *Fig. 9.3.3*. A new clan of ALTP and LD-1 has been proposed to designate phylogenetically intermediate subtilisins between true subtilisins and high-alkaline protease (PIS) (Saeki et al. 2003; Kobayashi et al. 2007; Takimura et al. 2007). The phylogenetic clusters of true subtilisins (I), high-alkaline proteases (II), oxidatively stable proteases (IV) (Saeki et al 2000), high-molecular-mass subtilisins (V) (Okuda et al. 2004), intracellular proteases (III), PIS (VI) (Saeki et al. 2003; Kobayashi et al. 2007; Takimura et al. 2007), and other five clans (VII to XI) are clearly separated in the tree. Furthermore, clans I, II, III, VI, and VII comprise a larger cluster 1, clans V, IX, and X comprise a cluster 2, clan IV forms a cluster 3, and clans XI and VIII form a cluster 4. There is a wide diversity of subtilisin-like proteases even in subtilase family A, one of the most subdivided families.

As a conclusion, ALTP has the very unique characteristics representing the highest optimal pH value for the activity and the greatest number of cleavage sites for the oxidized insulin B-chain among the known proteases. ALTP is obtained from *A. transvaalensis* SAGM1, the most alkaliphilic microorganism isolated from one of the deepest underground sites (3.2 km) reported so far. This is an excellent example of the potential of deep subsurface extremophiles as previously unidentified molecular resources such as novel and/or superior enzymes that may have industrial applications.

Deep Subseafloor Enzymes from Aerobic Bacteria

Deep subseafloor sediments harbor an enormous microbial biomass (Parkes et al. 1994, 2000; Whitman et al. 1998). Despite significant potentials in the prokaryotic biomass and functions, net microbial activity and culturability demonstrated so far are very low (D'Hondt et al. 2002, 2004).

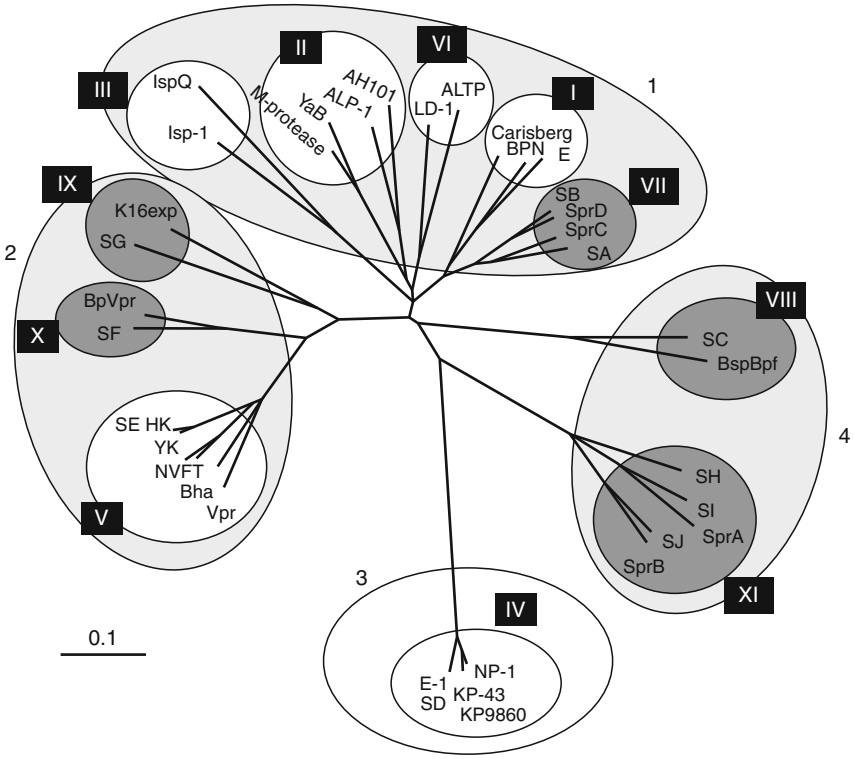

Fig. 9.3.3

Unrooted phylogenetic tree of subtilisins. Clan I, true subtilisins; clan II, high-alkaline proteases; clan III, intracellular subtilisins; clan IV, oxidatively stable subtilisins (OSP) (Saeki et al., 2000, 2002); clan V, high-molecular-mass subtilisins (HMS) (Ogawa et al. 2003; Okuda et al. 2004); clan VI, phylogenetically intermediate subtilisins between true subtilisins and high-alkaline proteases (PIS) (Saeki et al. 2003; Kobayashi et al. 2007; Takimura et al. 2007). Clans VII to XI are identified as new clans of subtilisin-like serine proteases in this study and shown as *circles* filled with light gray. Clans 1, 2, 3, and 4 were proposed as new subgroups in subtilase family A (Takimura et al. 2007), which are drawn as ovals with *dotted lines*. Bar represents *knuc* value. Sources of sequences aligned: subtilisin E (P04189) from *B. subtilis* 168; BPN′ (Q44684) from *B. amyloliquefaciens*; Carlsberg (P00780) from *B. licheniformis*; AH101 (D13158) from *Bacillus* sp. AH-101; ALP-1 (Q45523) from *Bacillus* sp. strain NKS-21; YaB (P20724) from *Bacillus* sp. strain YaB; M-protease (Q99405) from *B. clausii* KSM-K16; IspQ (Q45621) from *Bacillus* sp. strain NKS-21; Isp-1 (P08750) from *B. subtilis* IFO3013; E-1 (AB046402) from *B. cohnii* D-6; KP-43 (AB051423) from *Bacillus* sp. strain KSM-KP43; KP9860 (AB046403) from *Bacillus* sp. strain KSM-KP9860; NP-1 (AB046406) from *Bacillus* sp. strain NCIB12289; HK (AB100357) from *B. cohnii* D-6; YK (AB100359) from *Bacillus* sp. strain Y; NV (AB096097) from *Bacillus* sp. strain NCIB12289; FT (AB096094) from *Bacillus* sp. strain KSM-KP43; Bha (G83753) from *B. halodurans* C-125; Vpr (M76590) from *B. subtilis*; LD-1 (AB085752) from *Bacillus* sp. strain KSM-LD1; ALTP (AB266094) from *Alkaliphilus transvaalensis* SAGM1; SprC and SprD (U39230) from *Bacillus* sp. strain LG12; BspBpf (AAOX01000022.1) of *Bacillus* sp. strain NRRL-14991; K16exp (AP006627-748) of *B. clausii* KSM-K16; BpVpr (AF330160) of *B. pseudofirmus*; SprA and SprB (U39230) from *Bacillus* sp. strain LG12

Although the phylogenetic and functional diversity of subseafloor microbial components in the sediments have been revealed by culture-independent molecular approaches using PCR-amplified 16S rRNA genes (Biddle et al. 2005; Fry et al. 2008; Inagaki et al. 2003, 2006; Parkes et al. 2005; Biddle et al. 2006; Sørensen and Teske 2006; Teske and Sørensen 2008), there is little known about the compositional and functional characteristics of the culturable subseafloor microorganisms (Batzke et al. 2007; Süß et al. 2004) (see Chap. 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)).

During August–October in 2006, the newly constructed deep-sea drilling vessel "Chikyu" Shakedown Expedition CK06-06 was conducted off Shimokita forearc basin at a water depth of 1,180 m, northwestern Japan, where the presence of free hydrocarbon gases and methane hydrates had been predicted by preliminary seismic survey (Taira 2005). Extra-sediment enzyme activities were detected successfully in the supernatants of core sediment suspensions and subsequently numerous aerobic bacteria were detected in the core sediments (Kobayashi et al. 2008).

Extracellular Enzyme Activity in Pelagic Sediments

There have been several reports of extracellular enzyme activity (EEA) in deep-sea sediment samples, such as aminopeptidase, esterase, chitinase, chitobiase, α-glucosidase, β-glucosidase, pullulanase, and laminarinase in sediment surfaces at a depth of shallower than 15 cm (Meyer-Reil and Köster 1992; Poremba 1995; Vetter and Deming 1994; Poremba and Hoppe 1995; Arnosti 1995, 1996; Fabiano and Danovaro 1998). In marine sediments, decomposition of organic matters is dependent on microbial activity (Meyer-Reil 1990). EEA is generally thought to serve as the first step in degradation of organic matters in the marine sedimentary environment and subsequently utilization of their products by heterotrophic microorganisms (Hoppe 1991). Recently, hydrogenase activity was determined in coastal marine sediments (Soffientino et al. 2006) as well as deep subseafloor sediments at depth of ~600 mbsf (Nunoura et al. 2009). This means EEA could be detected in not only shallow marine sediments but also deep subseafloor core sediments. In addition to EEA, a "meta-enzyme" was proposed as an assemblage of both extra- and intracellular enzymes of a microbial community in deep subseafloor sediment cores (Kobayashi et al. 2008).

Meta-enzyme Activity in Deep Subseafloor Sediments off Shimokita Peninsula

The cored sediments were mainly composed of diatom-rich hemipelagic clay and silt, intercalated commonly with volcanic ash and sand layers (*Table 9.3.1*). Preliminary biostratigraphic age models indicate very high sedimentation rates, ranging from 54 to 95 cm kyr^{-1} (kilo-years) and an approximate core-bottom age of 640 Ka (kilo-ages). The sediments contain abundant organics (an average total organic carbon content through the depth of 1.35 wt%) and high concentrations of methane gas (1,000–3,000 μM in porewater). The existence of methane hydrates was observed in several porous layers (ca. 190, 217, 247, 275, and 343 mbsf) using the Thermo-View camera onboard.

Table 9.3.1

Characterization of deep subseafloor sediments off Shimokita Peninsula

Core section	Depth (mbsf)	Total organic carbon (wt%)	Methane concentration (μM)	Remarks
1-1	0.5	1.62	11.2	Sulfate reduction zone, silty clay
1-4	4.8	1.32	1,000.4	Silty clay
2-1	8.0	1.30	3,010.7	Silty clay
3-2	13.4	1.24	2,095.3	Silty clay
6-3	48.2	1.79	1,375.9	Silty clay
12-4	106.7	1.91	2,071.5	Silty clay
21-4	190.4	nd[a]	nd[a]	Hydrate-bearing ash layer
24-4	212.5	1.13	1,658.9	Silty clay
24-4′	216.9	1.13	nd[a]	Hydrate-bearing silt and sand
27-6	247.1	0.99	1,744.6	Hydrate-bearing silt and sand
38-7	342.5	1.02	2,866.0	Hydrate-bearing sand

[a]nd: not determined.

A portion of each sediment suspension was centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatants were passed through a 0.22 μm pore size of filter, and the filtrates were then diluted with an equal volume of sterilized artificial seawater (Nissui, Japan). The resultant solutions were subjected to a kit for detection of enzyme activities (API ZYM, Bio Merieux, France), and then incubated at 30°C for 48 h. Acid phosphatase, C4-esterase activities were also quantified using *p*-nitrophenyl phosphate (Sigma) and *p*-nitrophenyl butyrate (Sigma) as the substrates, respectively. Catalase activity was quantified by the degradation rate of H_2O_2. Protein contents were measured in all extra-sediments by a protein assay kit (Bio-Rad) using bovine serum albumin as the standard.

Almost all supernatants showed naphthol-AS-BI-phosphate (7-bromo-3-hydroxy-2-naphthoic-*o*-anisidide phosphate) hydrolysis, indicating the presence of acid phosphatase activities in situ. 2-Naphthyl phosphate hydrolyzing activity was detected in many horizons but not in cores 1-4, 2-1, and 38-7. Only one supernatant of core 6-3 showed esterase (2-naphthyl butyrate as the substrate) activity. Cytochrome oxidase activity was qualitatively detected in cores 21-4 and 38-7. Furthermore, acid phosphatase, C-4 esterase, and catalase activities were quantitatively detected in almost all samples, as shown in *Table 9.3.2*. A variety of levels of phosphatase activity (0.08 to 390 nmol PO_4^{3-} cm^{-3} h^{-1}) have been reported from various marine environments (such as the Baltic Sea and Indian Ocean) (Hoppe 2003). The acid phosphatase activities in the core sediments at different depths were within this range. In water column environments, extracellular phosphatases are recognized to be derived from alga and marine bacteria, which play an important role in the recycling of organic phosphorus to avoid phosphorus limitations in marine ecosystem (Hoppe 2003). Therefore, the detectable activities of extracellular acid phosphatase would also play a role in taking up, resolving, or recycling biological phosphates and/or phosphorous in deep subseafloor microbial ecosystems. Esterase activity is a good index for estimation of microbial biomass and/or organic matter content in

Table 9.3.2

Enzyme activities in the supernatant of core sediments off Shimokita Peninsula

Core section (mbsf)	Protein (mg cm^{-3})	Acid phosphatase (mU cm^{-3})	C4-esterase (mU cm^{-3})	Catalase (mU cm^{-3})
1-1 (0.5)	0.122	0.05	<0.01	<10
1-4 (4.8)	0.632	0.072	0.178	52
2-1 (8.0)	0.788	<0.01	0.644	46
3-2 (13.4)	0.57	0.12	0.128	126
6-3 (48.2)	0.652	0.042	0.758	<10
12-4 (106.7)	0.62	0.068	0.87	480
21-4 (190.4)	0.15	0.042	0.064	<10
24-4 (212.5)	0.212	<0.01	0.66	60
24-4′ (216.9)	0.464	0.038	0.322	776
38-7 (342.5)	0.22	0.072	1.02	58

sediments of seas, lakes, and reservoirs (Poremba and Hoppe 1995; Boschker and Cappenberg 1998; Wobus et al. 2003). Catalase is generally involved in removal of hydrogen peroxide by aerobic microorganisms. One of the unexpected findings was the observation of significantly high catalase activities even in deep pelagic clay samples such as a core sample of 12-4, as well as in hydrate-bearing porous layers. The detection of high catalase activity throughout the core suggests the presence of microbes that can alternatively grow on oxygen as an electron donor. A "meta-enzyme analysis" is proposed as an ecological enzymatic method to explore the potential functions of microbial communities in extreme environments such as the deep marine subsurface.

Potential Ability to Produce Extracellular Enzymes by Deep Subseafloor Bacteria

The highest population size of viable cells in the deep subseafloor sediments (366 mbsf) has been reported to be 1.3×10^6 cells cm^{-3} by Wellsbury et al. (2002). The other results of viable cells population have been in the order of 10^4–10^5 cells cm^{-3} at 200, 395, and 500 mbsf, respectively (D'Hondt et al. 2002; Cragg et al 1996). In the offshore case, the Shimokita Peninsula, cultivable populations were obtained from all core sections; the maximum viable population was found to be 2.4×10^8 cells cm^{-3} at cores 24-4 or 24-4′ (210–220 mbsf). Even in the deepest core sample of 38-7 (342.53 mbsf), the viable cells population was to be 4.4×10^7 cells cm^{-3} (Kobayashi et al. 2007). Based on the sequence analysis of nearly complete 16S rRNA genes of 552 isolates that were obtained from the aerobic heterotrophic solid media, the isolates were classified into 9, 5, 31, 5, and 12 phylotypes within the *Actinobacteria, Bacteroidetes/Chlorobi, Firmicutes, Alphaproteobacteria*, and *Gammaproteobacteria*, respectively. With regard to the cultivated population of each strain, the emerging patterns varied with sediment depth or lithostratigraphic characteristics. More specifically, the population of *Gammaproteobacteria*

increased with depth. Otherwise, the populations of *Firmicutes* and *Actinobacteria* occupied relatively shallower core sediments. This suggested that core sediments off Shimokita had gradually piled up for a long time with slipping down of organic-rich stratum around Shimokita Peninsula.

The potentials of the isolates for extracellular enzyme production were characterized. Most of the aerobic, heterotrophic strains from different core sections appeared to have a variety of extracellular enzyme activities (Table 9.3.3). Protease activity was mainly identified in the members of *Pseudoalteromonas* and *Bacillus* (14.8% of total isolates). α-Amylase-producing strains (42.5%) were also predominantly found in the genus *Pseudomonas,* and lipase-producing strains (54.3%) were members of the genera *Pseudoalteromonas*, *Pseudomonas,* and *Aeromicrobium*. More than 60% of the isolates produced phosphatase. DNase activity was also detected in many isolates, dominantly from the genera *Pseudoalteromonas* and *Microcella*. In contrast, around 10% or less of the isolates produced carboxymethyl cellulase (CMCase), xylanase, and chitinase, and these enzyme activities were detected mainly in members of the genera *Pseudomonas*, *Microcella*, and *Pseudoalteromonas*. Most of the isolates produced catalase (98.2%) and cytochrome oxidase (79.4%). Interestingly, no isolate showed agarose- or carrageenan-degrading activities although these polysaccharides were expected to be buried in the sediments, probably derived from marine algae. In addition, only *Shewanella* sp. strain JAM-GA0101, which was isolated from a near-seafloor section (1-1), produced an alginate lyase. No chondroitinase, ligninase, or pectinase activity was found among the isolates.

The meta-enzyme assay of sediment slurry samples and isolates from the deep subseafloor off Shimokita Peninsula strongly suggested that the subseafloor environment could be an active microbial habitat and that their extracellular enzymes would play important roles in degrading or recycling the consumable substrates in a microbial ecosystem. The meta-enzyme approach provides new insights into the functioning of the least-explored subseafloor microbial communities. Additionally, the genetic, molecular, and functional resources of these subseafloor microorganisms may have potential for industrial application.

Concluding Remarks

As already described above, the prokaryotes in deep biosphere are proposed to have the greatest physiological, biochemical, and genetic potentials for diversity based on the existent biomass potentials in the Earth (Parkes et al. 2000; Whitman et al. 1998). Nevertheless, due to their strong resistance to cultivation, a very limited number of isolates have been identified and available for the subsequent physiological and biochemical characterizations. Since it is evident that the biochemical investigation is strongly inhibited by the unsuccessful cultivation of indigenous subsurface microorganisms, even of aerobic heterotrophic components, an increasing number of cultured surface microorganisms should be explored from a variety of subsurface environments. In addition, not only the culture-dependent biochemical investigation but also the culture-independent approaches by means of metagenomic and environmental transcriptomic, proteomic, and metabolomic techniques should be encouraged. Here, we present an excellent example of a novel alkaliphilic protease produced from the most alkaliphilic microorganism living in the deep subsurface.

Table 9.3.3

Number of extracellular enzyme-producing bacteria in the deep subseafloor sediments off Shimokita Peninsula

Core	Number of isolates	Number of enzyme-producing isolates								
		Protease	α-Amylase	CMCase	Xylanase	Alginase	Phosphatase	Chitinase	Lipase	DNase
1-1	69	35	26	5	2	4	56	20	41	44
1-4	4	3	3	1	0	0	3	1	3	1
2-1	9	0	4	0	0	0	1	0	4	0
3-2	16	1	2	0	1	0	15	0	11	1
6-3	54	2	13	3	5	0	27	3	18	15
12-4	5	5	5	1	0	0	5	2	2	4
21-4	94	13	34	3	0	0	58	7	54	8
24-4	89	2	30	1	1	0	54	0	41	5
24-4′	68	13	32	0	0	0	47	18	40	18
27-6	15	0	13	3	3	0	12	0	10	1
38-7	76	0	50	9	0	0	48	1	47	1
Total	499	74	212	26	12	4	326	52	271	98

Cross-References

- 2.2 Distribution and Diversity of Soda Lake Alkaliphiles
- 2.3 Environmental Distribution and Taxonomic Diversity of Alkaliphiles
- 2.8 Enzymes Isolated from Alkaliphiles
- 6.6 Genetics, Genomics, Evolution
- 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)
- 9.2 Physiology

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9.4 Genetics, Genomics, Evolution

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_9.4,

Extent of Deep Biosphere as Extreme Environment

The deep biosphere is generally considered an extreme environment for organisms because of the living space limitation in sediments and rocks and the physical-chemical constraints of energy generation for life. In most parts of the biosphere, microbial growth and maintenance are sluggishly sustained by very low supplies of long-dead organic matters, reduced mineral substrates, thermally released gasses from organic and/or inorganic sources, or possible radiolytic molecular hydrogen (Fredrickson and Fletcher 2001; Colwell and Smith 2004; Blair et al. 2007). In contrast, some subsurface environments in and around fracture zones, petroleum reservoirs and subseafloor environments associated with hydrothermal activity, could provide both livable space and energy demand for prosperous formation of microbial communities. Especially in some fracture systems and the sediment-basaltic basement interface, it has been suggested that molecular oxygen and other oxidative ions are likely present and available for indigenous microbial activity (Colwell and Smith 2004; D'Hondt et al 2004). On the other hand, in any deep subsurface environment, physical-chemical extremes also constrain the boundary of the biosphere. The most evident parameter is temperature. According to the presently known temperature range of growth and maintenance for life, the extent of the deep biosphere may be shaped by a subsurface temperature zone ranging from subzero at permafrost area to the upper temperature limit of life (currently 122°C) (Takai et al. 2008), corresponding to the depth of more than 10 km from the surface in some locations (Colwell and Smith 2004). Thus, as combined with other physical-chemical parameters, the deep biosphere harbors a great phylogenetic, physiological, metabolic, and biochemical diversity of microorganisms.

Modern subsurface microbiology started primarily for investigating the relationship between ground water quality and microbial community structures and functions living there in the late 1970s and early 1980s (Fredrickson and Fletcher 2001; Colwell and Smith 2004). In the early phase, a number of microorganisms were isolated from petroleum reservoirs and terrestrial deep (> approx. 50 m) subsurface sediments, rocks, and aquifers (Balkwill et al. 1997c; Magot et al. 2000). However, only few model isolates from the deep biosphere have been developed for further biochemical and molecular genetic investigations, e.g., *Desulfovibrio desulfuricans* G20i (Weimer et al. 1988). Only 11 complete genome sequences from deep subsurface organisms are publically available and only a limited number of reports have been published until the end of 2009 (*Table 9.4.1*). Accordingly, we do not have enough genetic and genomic knowledge to understand potential metabolisms and machineries for the adaptation of microorganisms to the deep biosphere. Currently, several metagenomic studies targeting specific subsurface environments have been reported (*Table 9.4.2*), and have provided important insights into the genetic and genomic aspects of the microbial ecosystem in deep biosphere. This chapter reviews the features of the deep subsurface microbes of which genome sequences have been completely analyzed and the knowledge from each of the metagenomic projects.

Genomes of Deep Subsurface Microorganisms

Genomic analyses of deep subsurface microorganisms are expected to give the genetic and genomic basis of understanding the metabolisms and machineries for adaptation to deep subsurface extreme environments and of exploration of novel genetic resources (Feng et al. 2007; Mardanov et al. 2009). In the deep biosphere, some microorganisms are utilizing toxic

Table 9.4.1

Completed genome sequences of deep subsurface microorganisms at the end of 2009

Organism	Genome size (Mbp)	Site of isolate	References
Bacteria			
Thermotogae			
Thermotoga petrophila RKU-1	1.82 (plasmid: 0.85 kb)	Production fluid of Japanese oil reservoir	Zhaxybayeva et al. 2009
Petrotoga mobilis SJ95	2.17	Production water of North Sea oil reservoir	Lien et al. 1998
Kosmotoga olearia	2.30	Production water of North Sea oil reservoir	Dipippo et al. 2009
Firmicutes			
Exiguobacterium sibiricum 255-15	3.03 (plasmids: 4.9 and 1.8 kb)	Russian permafrost (a depth of 43.6 m)	Rodrigues et al. 2008
Geobacillus thermodenitirificans NG80-2	3.5 (plasmid: 58kb)	Chinese oil reservoir (formation water taken at a depth of 2,000 m and a temperature of 73°C)	Feng et al. 2007
Thermoanaerobacter ethanolicus X514	2.46	Drilling fluid circulated to a depth of 2,000 m, Colorado, US	Roh et al. 2002
Alphaproteobacteria			
Novosphingobium aromaticivorans DSM12444	3.56 (plasmids: 48.7 and 18.4 kb)	American subsurface Cretaceous formation at 410 m depth	Balkwill et al. 1997b
Deltaproteobacteria			
Desulfovibrio desulfuricans G20 (derived from strain G100A)	3.73	American producing oil well	Luo et al. 2007
Gammaproteobacteria			
Shewanella putrefaciens CN32	4.66	Core sample (shale-sandstone) at a depth of 250 m below land surface (mbls)	Fredrickson et al. 1998
Archaea			
Euryarchaeota			
Thermococcus sibiricus MM739	1.85	Siberian oil reservoir (stratal fluid from a depth of 2,350 mbls and a temperature of 84°C)	Mardanov et al. 2009
Methanococcus aeolicus Nankai-3	1.60	Subseafloor sediment from a depth of 247 m below seafloor (Nankai Trough)	Kendall et al. 2006

Table 9.4.2

Metagenomic studies in subsurface environments at the end of 2009

Targeted habitat	Dominant organisms	Potential energy metabolisms	Sequencing strategy	References
Terrestrial				
Acid mine drainage in California	*Leptospirillum* sp. (Nitrospirae) *Ferroplasma* sp. (Euryarchaeota)	Iron-oxidation Iron-oxidation	Shotgun sequencing by Sanger sequencing	Tyson et al. 2004
Fracture water at 2.8 km depth in a South African gold mine	Candidatus "*Desulforudis audaxviator*" (Firmicutes)	Sulfate-reduction	Shotgun sequencing by Sanger sequencing and pyrosequencing	Chivian et al. 2008
Geothermal water stream at a depth of 320 mbls in a Japanese gold mine	HWCG I & III (*Crenarchaeota*) *Sulfurihydrogenibium* sp. (*Aquificae*) *Methylothermus* sp. (Gammaproteobacteria)	Unknown Ammonia-oxidation Hydrogen-oxidation Methane-oxidation	Shotgun sequencing for fosmid pools by pyrosequencing, and Sanger sequencing for individual fosmid clone	Nunoura et al. 2005
Subseafloor				
Subseafloor sediments (Peru Margin)	Diverse *Bacteria* and *Archaea*	Mainly organotrophy	Shotgun sequencing by pyrosequencing	Biddle et al. 2008

metals as the electron donors or acceptors, and/or hydrocarbons as the carbon and energy sources (Colwell and Smith 2004). Thus, the genetic and molecular components related to these metabolisms have a great potential for bioremediation and degrading persistent substrates. This section focuses on characteristics of the deep subsurface microorganisms from petroleum reservoirs, terrestrial subsurface environments, and subseafloor sediments revealed by the genome analyses.

Genomic Analysis for Microbes from Petroleum Reservoirs

Petroleum reservoirs possess enough space for microbial colonization and propagation, and provide enough energy and carbon sources for the growth and maintenance. In fact, microbial degradation of hydrocarbons has significant implications for oil quality and production while temperature of deep reservoirs sometimes exceeds 130°C (Magot et al. 2000; Head et al. 2003). The first deep subsurface microorganism was isolated from an oil-producing well in 1920s (Bastin 1926), and to date, a variety of microbes have been obtained from petroleum reservoirs (Magot et al. 2000). In the list of microbes from the petroleum reservoirs, we can find mesophiles to thermophiles such as sulfate-reducers, methanogens, fermenters, iron-reducers, and aerobes (Magot et al. 2000). Among these microbes, complete genome sequences were

obtained from thermophilic to hyperthermophilic *Thermotogales* bacteria, *Thermotoga petrophila* RKU-1, *Petrotoga mobilis* SJ95 and *Kosmotoga olearia* TBF 19.5.1 (Zhaxybayeva et al. 2009; Lien et al. 1998; Dipippo et al. 2009), hyperthermophilic archaeon *Thermococcus sibilicus* MM739 (Mardanov et al. 2009), a sulfate-reducing bacterium *Desulfovibrio desulfuricans* G20 (Luo et al. 2007), and an alkane-degrading bacterium *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007) (*Table 9.4.1*).

Thermococcus Sibilicus

Thermococcales is a cosmopolitan hyperthermophilic archaeal group in global hydrothermal vent environments and deep petroleum reservoir environments (Takai et al. 2006; Magot et al. 2000). In addition, *Thermococcales*-related 16S rRNA gene sequences are also found in many of the deep hot subseafloor sediments (Roussel et al. 2008), the low temperature subseafloor sediments, and the fissure waters in subsurface mines (Inagaki et al. 2006; Takai et al. 2004). Thus, *Thermococcales* could be distributed ubiquitously in the deep hot biosphere in the earth (Takai et al. 2004) and their substrates predicted to be buried persistent organic matters. However, no strains have been reported growing with hydrocarbons or lipids as sole energy and carbon sources (Magot et al. 2000; Zillig and Reysenbach 2001).

T. sibilicus was originally characterized to grow exclusively with peptides but not with polysaccharides and hydrocarbons (Mardanov et al. 2009). As opposed to the results of previous cultivation experiments, the genome encodes a total of 281 proteins carrying N-terminal signal sequences that include transporters, proteases, glycoside hydrolases, and lipase/esterase (Mardanov et al. 2009). According to the genomic information, the substrate utilization was reexamined, and then the stimulation of growth with cellulose, agarose, hexadecane, acetone, glycerol, and olive oil were observed (Mardanov et al. 2009). In fact, the genes for each of the degradation pathways for cellulose, agarose, and lipids were found in the genome. The strain is not able to grow with chitin, xylan, pectin, starch, and aromatics, which is consistent with the absence of genes that encode chitinases, xylanases, pectinases, extracellular amyloritic enzymes, and key enzymes for aromatics degradation. Interestingly, *T. sibilicus* grows with olive oil and glycerol but not with long-chain fatty acids, and it is also well explained by the absence of β-oxidation pathway of fatty acid degradation. Therefore, the growth on lipids is probably explained by the utilization of glycerol liberated after hydrolysis of triglycerides by extracellular lipase(s) (Mardanov et al. 2009). On the other hand, the complete known alkane degradation pathway is not observed in the genome, and *T. sibiricus* probably possesses a new mechanism for utilization of alkanes. The growth of *T. sibiricus* with agarose, cellulose, alkane, and triglycerides and the substrate utilization patterns revealed by a combination of genomic and cultivation analyses provide very important clues to address why *Thermococcales* members are so ubiquitously distributed in the global deep subsurface biosphere.

Thermotogales Species

Thermotogales is a representative thermophilic to hyperthermophilic bacterial group often found in petroleum reservoirs (Magot et al. 2000) and the members are also distributed in the global hydrothermal systems (Reysenbach 2001). It has been known that *Thermotogales* species isolated from the petroleum reservoirs do not utilize hydrocarbon, aromatics, and alkanes as

carbon sources. Although a genome sequence of *Thermotoga petrophila* RKU-1 has been determined among *Thermotogales* recovered from the deep subsurface environment, the genome-based adaptation mechanisms to petroleum reservoir have not been discussed in detail (Zhaxybayeva et al. 2009).

As the genetic components related with the utilization of persistent substrates in the genome sequences of the *Thermotogales* members studied so far, xylan and/or other carbohydrate degradation genes were found in *Thermotoga maritima* isolated from geothermal heated seafloor and *P. mobilis* (Conners et al. 2006; Lien et al. 1998), but no component of the degradation pathways for the aromatics and hydrocarbons were identified (Conners et al. 2006). Thus, the *Thermotogales* species of which genome sequences are already determined do not have the genetic potentials to utilize alkanes, aromatics, and other hydrocarbons as shown by previous cultivation-based experiments. Nevertheless, these results do not rule out the possibility that deep subsurface *Thermotogales* members inhabiting the petroleum reservoirs have the genetic and molecular components of the previously unknown hydrocarbon utilization pathways.

Geobacillus Thermodenitirificans NG80-2

Geobacillus thermodenitirificans NG80-2 is a facultatively aerobic bacterium that degrades long-chain alkanes (Feng et al. 2007). Aerobic organisms from the deep petroleum reservoirs may have been introduced by water injection and oxygen is only transiently available after the injection of oxygenated water (Magot et al. 2000; Feng et al. 2007). The genome of this strain encodes genes for a typical pyruvate fermentation pathway, uptake transporters for organic acids including benzoate, propionate and butyrate, uptake transporters for fatty acids, and detoxification systems for heavy metals that may be required for growth and survival in the petroleum reservoir environments (Feng et al. 2007). Interestingly, a gene for alkane monooxygenase potentially involved in the degradation of long-chain alkanes is found in a large plasmid (Feng et al. 2007). This implies that the alkane degradation capability may be horizontally transferred from other microbial components.

Feng et al. (2007) hypothesized that *G. thermodenitirificans* NG80-2 originated from the external soil environment and already obtained the genetic capability of alkane degradation before the entrainment into the oil reservoir by injection water. However, as the genes for lipid degradation pathway are encoded in the chromosome, it still seems debatable whether *G. thermodenitirificans* NG80-2 and its hydrocarbon metabolizing capability are of indigenous or exotic origin.

Desulfovibrio Desulfuricans G20

Desulfovibrio desulfuricans is a sulfate-reducing bacterium (SRB) and is also known as a uranium (VI)-reducer (Payne et al. 2002). *D. desulfuricans* G20 is a spontaneously nalidixic acid-resistant derivative of the wild-type strain G100A that is also cured of the endogenous cryptic plasmid and is originally isolated from an oil-producing well. *D. desulfuricans* G20 serves as a model organism for the study of survival in the sediments and for the molecular genetics and the proteomics in SRB (Luo et al. 2007), but the genome sequence has not yet been published. The transformation and gene disruption using shuttle plasmid vector can be applied to this strain by conjugation with *E. coli* (Payne et al. 2002). The Transposon mutagenesis and

signature-tagged mutagenesis technologies also work in the strain G20 (Wall et al. 1996; Groh et al. 2005). Using these molecular genetic tools, not only the potential mechanisms for uranium-reduction and other energy metabolisms but also the adaptation in the sedimentary habitats have been extensively studied (Payne et al. 2002; Luo et al. 2007).

Genomic Analyses for Microbes from Terrestrial Subsurface Environments

Complete genome sequences of four terrestrial deep subsurface microorganisms such as *Exiguobacterium sibiricum* 255-15, *Thermoanaerobacter ethanolicus* X514, *Novosphingobium aromaticivorans* DSM12444, and *Shewanella putrefaciens* CN32 are available in public database. Among these genome sequences, only the genome of *Exiguobacterium sibiricum* 255-15 (Rodrigues et al. 2008) is published, and a genome sequence of *Shewanella putrefaciens* CN32 is used for comparative proteogenomics among the genus *Shewanella* (Gupta et al. 2008) (*Table 9.4.1*).

Exiguobacterium Sibiricum 255-15

Exiguobacterium sibiricum 255-15, a member of *Bacillaceae*, was isolated from Siberian permafrost (Rodrigues et al. 2008). The strain is a facultatively aerobe and grows with fermentation under anaerobic condition. Distinct features of this bacterium are the wide growth temperature range (−5°C to 40°C) and a biphasic growth response to temperature (Rodrigues et al. 2008). Transcriptome analysis combined with the genome sequence analysis revealed that unique gene regulations occurred at subzero temperature and maximal temperature range for the growth. The change in gene expression was observed in the transcripts associated with carbohydrate metabolism, energy metabolism, amino acid biosynthesis and catabolism, and membrane and cell wall adaptation as well as DNA replication, transcription, and translation. For example, a gene for glycerol 3-phosphate dehydrogenase (*glpA*), a component in the glycerol metabolism, was up regulated at 39°C and down regulated at 10°C, however, at −2.5°C the glycerol degradation pathway was again up regulated. Furthermore, the differential gene expression of *glpKFA* was highest at −2.5°C than at all the other temperatures. As another example, two amylases (isozymes) were differentially expressed at different temperatures: one was upregulated at −2.5°C and the other was down regulated at −2.5°C but slightly upregulated at 10°C and 40°C (Rodrigues et al. 2008). In many previous studies, it has been considered that these gene expression changes at different temperatures would be transient as the initial response to the temperature-shock. The results shown in *E. sibiricum* 255-15 strongly suggested that the shift in gene expression could be a somewhat specific mechanism for the growth and maintenance in the subsurface permafrost environments.

Novosphingobium Aromaticivorans DSM12444

Novosphingobium aromaticivorans DSM12444 (formerly *Sphingomonas aromaticivorans*) is a mesophilic and aerobic alpha-proteobacterium that degrades aromatic hydrocarbons such as benzoate, p-cresol, naphthalene, and m-xylene. Unfortunately, the genome sequence analysis has been not published, but a previous study revealed that plasmids encode the genes for

aromatic hydrocarbon catabolism (Balkwill et al. 1997a, b). Interestingly, another *Novosphingobium* species isolated from a subsurface environment capable of degradation of aromatic hydrocarbons also possesses the similar genes for the aromatic hydrocarbon catabolism in its plasmid (Balkwill et al. 1997a). Since the aromatic hydrocarbons are likely dominating the organic compounds buried in the deep subsurface environments throughout the thermal degeneration and the microbial consumption during the long deposition period, the aromatic carbon catabolism may be a great advantage for subsurface microbial components to survive in the deep biosphere. If these plasmids are kinds of selfish gene fragments, the genes for aromatic hydrocarbon catabolism in the deep biosphere may be equivalent for the antibiotic resistance genes.

Metal Reducers

Thermoanaerobacter ethanolicus X514 (Roh et al. 2002) is a thermophilic low G+C gram-positive bacterium isolated from a deep subsurface environment in Colorado. The strain utilizes both molecular hydrogen and organic carbons as the energy sources and Fe (III), Co (III), Cr (VI), Mn (IV), and U (VI) as the electron acceptors. In addition, the strain is capable of producing ethanol from glucose (Roh et al. 2002). *Shewanella putrefaciens* CN32, isolated from deep shale-sand stone environment, is a member of the *Gammaproteobacteria*. The *Shewanella* strain heterotrophically grows with Fe (III), Co (III), Cr (VI), Mn (IV), and U (VI) as the electron acceptors (Liu et al. 2002). Both species are the candidates for the bioremediation and the genetic resources of novel enzymes, but the genome sequences have not yet been reported.

Genome Sequences of Subseafloor Microorganisms

Differently from the terrestrial deep subsurface environments, very limited numbers of isolates have been obtained from the oceanic subsurface environments (subseafloor sedimentary environments) more than 10 m below seafloor (mbsf) (Bale et al. 1997; Kendall et al. 2006; Kobayashi et al. 2008; Mikuchi et al. 2003) (see Chap. 9.2 Physiology). At present, *Methanococcus aeolicus* Nankai-3 is the only deep subseafloor microorganism whose genome sequence was completely sequenced although it has been not yet published. Methanogenesis in subsurface biosphere is a very important microbial process in global carbon circulation, but only *M. aeolicus* and *Methanoculleus submarinus* were isolated and characterized from the subseafloor sediments (Kendall et al. 2006). The metabolic activity of subseafloor biosphere is enormously lower than that of terrestrial environments. Comparative genomics and other omics studies between the subseafloor and terrestrial methanogens would be very useful how the subseafloor methanogens grow and maintain in such quite low energy-supplying habitats.

Metagenomics

Metagenomics and environmental "community-omics" are now keys to the current renaissance in microbial ecology as well as in biotechnology. To date, four unique metagenome projects related to the deep biosphere have been reported. Three targeted terrestrial mine environments such as acid mine drainage, deep fracture water and geothermal aquifer stream

(Tyson et al. 2004; Chivian et al. 2008; Nunoura et al. 2005), and the other was for subseafloor sediments (Biddle et al. 2008). From the technical point of view, all the projects adopt a whole genome shotgun sequencing technique but each project took a different sequencing strategy using Sanger sequencing and/or pyrosequencing (*Table 9.4.2*).

Metagenomics in Californian Acid Mine Drainage (AMD)

The AMD is located in Richmond mine at Iron Mountain, California, and at a depth greater than 100 m from ground surface. The AMD results from biotic and abiotic oxidations of sulfide ores. For the aqueous dissolution of sulfide ores dominated by pyrite (FeS_2) at low pH, ferric ion is the predominant oxidant in the abiotic oxidation; $FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4{}^{2-} + 16H^+$. At pH of less than 3, the microbial oxidation by iron-oxidizers exceeds the abiotic oxidation process. The metagenome of microbial mat analyzed by shotgun sequencing was dominated by bacterial and archaeal iron-oxidizers such as *Leptospirillum* sp. group II and III, *Sulfobacillus* and *Ferroplasma* sp. I and II (Tyson et al. 2004). Whole genome structures of *Leptospirillum* group II and *Ferroplasma* sp. I were reconstructed from the AMD microbial mat metagenome (Tyson et al. 2004). This project further revealed the genomic dynamics in *Ferroplasma* sp. I, namely the genetic exchange across a species boundary between *Ferroplasma* sp. I and II, and the virus–host relationship in the microbial community naturally occurring in the terrestrial subsurface (Allen et al. 2007; Eppley et al. 2007; Andersson and Banfield 2008).

Following the metagenomic analyses, proteomics using 2D shotgun mass spectrometry was applied to the same microbial community, and presented clear relation between the genetic and protein components in the microbial community (Lo et al. 2007; VerBerkmoes et al. 2009). This approach was further applied to different AMD biofilms, and mapped each peptide in two types of *Leptospirillum* group II genomes. By mapping the peptides into a genome structure, it was possible to predict the genotype of *Leptospirillum* group II in the proteomically characterized samples without genome sequencing. This process was called as proteomics inferred genome typing (PIGT). The combination of metagenomics and PIGT is very powerful tool for assessing genetic diversity and community and for monitoring the specific microbial community. The relatively low diversity in the AMD microbial community brought great success in a series of environmental-omics studies.

Metagenomics for Deep Fracture Water in a South African Gold Mine

The Mponeng gold mine is located in the Witwatersrand basin in South Africa, and the fracture fluid for metageomic analysis was collected from a borehole at a depth of 2.8 km from ground surface. Before the metagenomic analysis, intensive geochemical and microbiological studies were conducted. Fractures were found in the 2.7-billion-year-old Vntersdorp Supergroup metabasalt and the age of fracture water was tens of millions of years. Abundant potential electron donors for microbial life in the water were the abiotically supplied hydrocarbons and the radiolitically produced H_2 (Lin et al. 2006). The temperature and pH were ~60°C and 9.3, and the microbial population density was $\sim 5 \times 10^4$ cells/mL. The 16S rRNA gene clone libraries of fracture water microbial communities were always dominated by one bacterial phylotype affiliated within the *Firmicutes*. However, cultivation of the predominant

bacterium has been unsuccessful although the sulfate-reducing metabolism was predicted from the phylogenetic relatedness of the phylotype to the 16S rRNA gene sequences of *Desulfotomaculum* and other sulfate-reducers.

The microbial community DNA for metegenomic libraries was extracted from ~5,400 L of fracture water, and analyzed by a combination of 454 pyrosequencing and Sanger sequencing (Chivian et al. 2008). As a result, a 2.35-Mbp of a single complete genome of the *Firmicutes Candidatus* "*Desulforudis audaxviator*" was assembled. Interestingly, heterogeneity in the population of the dominant species as measured with single nucleotide polymorphisms (SNP) was quite low, showing only 32 positions with an SNP observed more than once (Chivian et al. 2008).

It seems very likely that the predominant single species in such an isolated fracture water environment should possess all the genetic components such as energy metabolism, carbon fixation, and nitrogen fixation necessary for the growth and maintenance within its genome. As predicted from the geochemical analyses in this environment, the genes for H_2 uptaking multiple hydrogenases and dissimilatory sulfate-reducing pathway are present. For carbon assimilation, all the machineries of reductive acetyl-CoA pathway, which uses carbon monoxide dehydrogenase (CODH) for the assimilation of inorganic carbon, are found. Thus, CO and formate may serve as alternative carbon sources for CO_2. For adapting to the environment depleted with the inorganic and organic nitrogen compounds, the strain had the archaeal type nitrogenase genes in addition to the ammonium uptake pathways. All of the amino acid synthesis pathways are included in the genome. These abilities enable "*D. audaxviator*" to colonize the deep subsurface nutrient limited environment.

Metagenomics in Japanese Gold Mine Aquifer Stream

The project aimed to obtain the genetic information of specific uncultivated lineages of archaea in a geothermal aquifer stream in a Japanese gold mine located at a depth of 320 m from ground surface (Nunoura et al. 2005). At the discharge point (69°C) of the geothermal aquifer system, uncultivated lineages of *Crenarchaeota*; Hot Water Crearchaeotaic Group (HWCG) I and III (Fig. 9.4.1), dominated in microbial mat formation with thermophilic methanotrophic and chemolithoautotrophic bacteria. Despite numerous experiments, the HWCG archaea remain uncultivated. A metagenomic fosmid library was constructed for recovering the genomic fragments from these creanrchaeotes (Nunoura et al. 2005). The HWCG I does not belong to any of the previously cultivated lineages of hyperthermophilic crenarchaeota and marine group crenarchaeota "*Nitrosopumilales*" (Fig. 9.4.1). On the other hand, the HWCG III is now recognized as one of the subgroups of marine crenarchaeota, of which a member was enriched under ammonia-oxidation cultivation condition at 72°C, and is named as the order Candidatus "*Nitrosocaldales*" (de la Torre et al. 2008).

Five crenarchaeotic clones were identified by PCR amplification targeting the archaeal 16S rRNA gene from a metagenomic fosmid library; clones of HWCG I and III 16S rRNA genes, were sequenced and analyzed. Similarity of the overlapping region (approx. 31 kb) between two HWCG III clones was more than 99 %. In the fragment from the HWCG I clone, the genes for potential aerobic type carbon monoxide dehydrogenase (CODH) were found, suggesting the presence of CO-dependent metabolism in the HWCG I. The distribution of molybdenum hydroxylase family including aerobic type CODH is restricted to the aerobic microbes with few

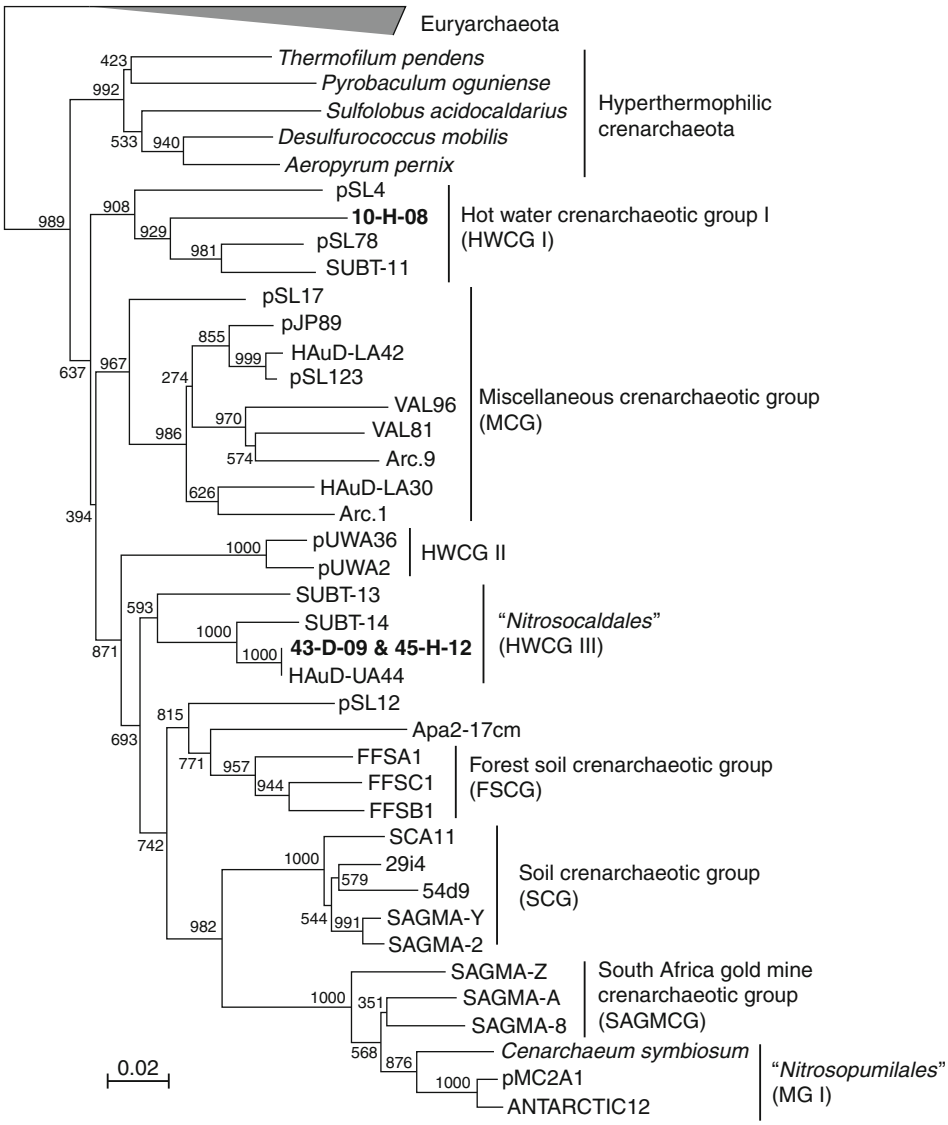

Fig. 9.4.1

A 16S rRNA gene phylogenetic tree of *Crenarchaeota* including HWCG I and III. *Bold* indicates the sequences obtained in metagenomic library from a microbial mat formation in a Japanese gold mine

exceptions. Taken into consideration together with the environmental conditions of the habitat, the incidence of the putative CODH in the HWCG I would be an important signature of the facultative or obligate aerophily of the archaeon.

Metagenomics for Subseafloor Biosphere

Diverse uncultivated lineages of both bacteria and archaea dominate in microbial communities in subseafloor sediments. Thus, the metagenomic analysis of the subseafloor microbial communities is expected to provide important insights into the energy and carbon metabolisms of these uncultivated microbial communities. Currently, only one metagenomic study targeting the deep subseafloor sediments was reported (Biddle et al. 2008). Over 60 Mbp of sequences were retrieved from the subseafloor sediment metagenomes at 1, 16, 32, and 50 m below seafloor (mbsf) from ODP site 1229 in the Peru Margin using 454 pyrosequencing. However, this study provided only short sequences (average 100 bp).

The most frequently detected sequences were related to genes for methyl-viologen-reducing hydrogenases that may be involved in methanogenesis pathway as well as other anaerobic processes (Teske and Biddle 2008). Among the essential genes in the known methanogenesis pathway, the gene for methylene-5,6,7,8-tetrahydromethanopterin dehydrogenase was detected and the frequency increased at greater depths. In addition, the gene for methyl-CoM reductase was not detected in all the depths. On the other hand, the metagenome analysis yielded only two genes related with the sulfate-reduction at relatively shallower depths (1 and 16 mbsf).

In contrast to the poor recovery of key functional genes for estimating the potential metabolic traits in the subseafloor biosphere, the phylogenetic analyses of protein-encoding sequences, 16S rRNA genes, and ribosomal proteins gave important insight into the diversity and abundance of bacterial and archaeal components in subseafloor sediments. In all the phylogenetic trees, the abundance of archaeal genes was more than 25 % of whole homologous gene assemblages at depths of 16, 32 and 50 mbsf (Biddle et al. 2008).

This metagenomic analysis for the deep subseafloor microbial communities analyzed only 60 Mbp. Regarding the high microbial diversity and low recovery of key functional genes in this analysis, many more sequences should be analyzed to establish the genetic and genomic basis of the metabolic and ecophysiological functions of the subseafloor microbial communities.

Low Genomic Heterogeneity in Deep Biosphere: Effect of Population Bottleneck?

Among three terrestrial subsurface metagenome projects described above, low microbial diversity and low genomic heterogeneity at species and at genotype (ecotype) levels were observed. In the AMD metagenome project, the relatively low heterogeneity in *Leptospirillum* and *Ferroplasma* were coupled with the genotype- and species-level heterogeneity, and clarified the inter-species genomic recombination. The species- and ecotype-level low heterogenity in the HWCG I crenarchaeote will also lead to reconstruction of the whole genome from the metagenomic fosmid library constructed from the Japanese terrestrial subsurface mine. The fracture water microbial ecosystem in the South African gold mine was dominated by one species of sulfate-reducing bacterium, and the ecotype-level diversity was also very low. Chivian et al. (2008) mentioned that the extraordinary simple biosphere representing the remarkable low genomic heterogeneity might be created by a recent selective sweep or other population bottleneck. One possibility is the population bottleneck in early stage of development of microbial community in the terrestrial deep subsurface environments. The deep subsurface prepares the isolated and stable microbial habitat that spatially and temporally

segregated from the surface, and the microorganisms living there seem fully to adapt the physical-chemical conditions and the energy state of the habitat on a geologic time scale (Colwell and Smith 2004). However, episodic or periodical geologic events definitely destroy the stablity regionally. Fracturing and associating fluid flows and inputs from the interior and/or the surface environments provide drastic environmental changes of the habitat and catastrophic effects on the residential microbial communities. In the cases of mine environments, mining activity can act like a geologic event. These drastic environmental changes would cause an extremely selective sweep to the previously existing microbial communities. Similar selection would be operative for the exotic microbial components entrained by the fluid input along the fractures. Consequently, the microbial species or ecotypes capable of quickly adapting the newly generated conditions would dominate in the microbial communities of the novel subsurface habitats. The population bottleneck hypothesis will be tested by future comparative metagenomic investigations of the relatively recently altered and stable subsurface environments.

Cross-References

- 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)
- 9.2 Physiology

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Part 10

New Frontiers: Radiation Resistant Organisms

10.1 *Deinococcus radiodurans*: Revising the Molecular Basis for Radiation Effects on Cells

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_10.1,

Abstract: The field of radiobiology was built on the premise that radiation is dangerous because of its damaging effects on DNA, where only a few events, or even a single event, at the molecular level can inactivate cells (Hutchinson 1966). The discordance of modern radiation toxicity models with results spanning nearly 5 decades of research on the extremely radiation-resistant bacterium *Deinococcus radiodurans* is reviewed. Much of the early data implicating DNA itself were for bacterial systems. However, recent studies show that extreme resistance to gamma radiation among bacteria consistently coincides with a greatly diminished susceptibility to protein oxidation but with similar DNA lesion-yields as other organisms. A growing body of experimental evidence now supports that naturally sensitive bacteria are killed by radiation mainly owing to protein oxidation, whereas extreme resistance in bacteria is achieved by protecting enzymes and the repair functions they catalyze. Based on new insights, the prospects for exporting the radioprotective mechanisms outside of *D. radiodurans* for practical purposes are considered.

Introduction

At the end of the nineteenth century, the greater scientific community had little faith in the existence of entities that could not be seen. All this began to change on November 8, 1895 when Wilhelm C. Röntgen was studying the passage of an electric current through a vacuum tube. At the Physical Institute of the University of Würzburg, Germany, he noticed that, if the Crookes tube was wrapped in black cardboard in a dark room, a barium platinocyanide screen located a few feet away glowed softly (Glasser 1993). Because the nature of the invisible light emanating from the Crookes tube was then unknown, Röntgen gave them the name X-rays. Within weeks, his discovery was an international news story; within months, Röntgen's original experiment was being treated as a novelty (Kevles 1997). Thomas A. Edison arranged a special exhibit on Röntgen rays at the annual Electrical Exhibition in New York City's Grand Central Palace in May 1896. This exhibit was a public sensation, mainly due to his demonstrating on a fluorescent screen the shadows of the bones of the hands of visitors. Thousands accessed the fluoroscopic room circuitously along galleries, which contrived to establish a supernatural atmosphere and which ended in a restaurant where the visitors could discuss their experiences (Kevles 1997). The early success and acceptance in the practical use of the X-ray in medicine was facilitated by such public displays. Unfortunately, the dangers of X-rays were not recognized until too late.

Radiation burns were recorded within a month of Röntgen's announcement of his discovery of X-rays (Glasser 1993). Edison had noticed some reddening around his own eyes and stopped experimenting with X-rays himself. About the same time, Marie and Pierre Curie found that pitchblende, a common ore of uranium, also emitted invisible light, gamma rays, even more penetrating than X-rays (Glasser 1993). From 1896 to 1903, hundreds reported slowly developing burns, and discharge tube operators in both medical and novelty settings died from overexposure. Reports on the lethal effects of X-rays and gamma rays followed, with safety guidelines for medical workers finally published by Britain's Röntgen Society in 1913. In 1928, 5 years after Röntgen's death, scientific units to quantify ionizing radiation (IR) were defined as the roentgen (R), the amount of energy deposited in air (Mould 1993). In 1953, a more practical unit of radiation was introduced. Named the "rad," it was an abbreviation for "radiation absorbed dose," the amount of radiation received by a person or object exposed to X-rays, gamma rays, alpha particles (helium nuclei), or beta particles (electrons) (Mould 1993). In 1975, the standard dose unit was modified once again, with the adoption of the

"gray," this time named after the British radiobiologist Louis Harold Gray, where 100 rad is the same as 1 gray (Gy) (Mould 1993).

Just 10 Gy of radiation will kill most vertebrate animals including humans, a dose delivered by conventional ^{60}Co irradiators in a few seconds. In general, the vast majority of bacteria will not survive 500 Gy (Thornley 1963), which is key to the widespread success of industrial IR-based sterilization technologies. Yet, a few representatives from the three domains of life display remarkably high levels of resistance. For instance, baker's yeast can survive 300 Gy; many insects, which are largely post-mitotic, can survive 500 Gy; the roundworm *Caenorhabditis elegans*, the water bear *Milnesium tardigradum*, and the freshwater invertebrate animal *Philodina roseola* can tolerate 1,000–3,000 Gy but are rendered sterile (Gladyshev and Meselson 2008), and as a diploid, the basidiomycete fungus *Ustilago maydis* can withstand 6,000 Gy (Holloman et al. 2007). The archaeon *Halobacterium* sp. NRC-1 can resist 5,000 Gy (Kottemann et al. 2005), and a few spore-forming bacteria (e.g., *Bacillus megaterium*) and vegetative cyanobacteria (e.g., *Chroococcidiopsis*) can withstand 10,000 Gy (Levinson and Hyatt 1960; Thornley 1963; Billi et al. 2000; Daly 2009).

By far, the most radiation-resistant group of organisms yet discovered belongs to the bacterial family *Deinococcaceae*, which typically displays near 100% survival following acute exposures to 12,000 Gy or 1,000 J/m^2 (254 nm) ultraviolet (UV) light (Battista 1997; Makarova et al. 2001) and can grow under extremely harsh conditions of chronic irradiation (60 Gy/h) (Daly 2000). The most characterized member of this group of extremophiles is *Deinococcus*

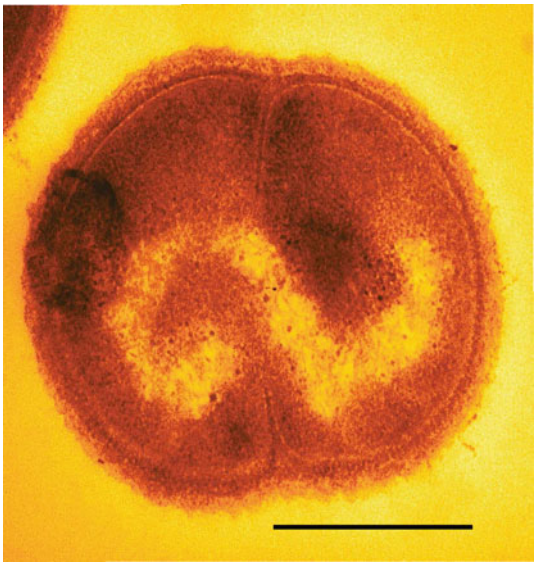

Fig. 10.1.1

Transmission electron micrograph of *D. radiodurans*. The cell was harvested from a mid-logarithmic culture in rich medium (Daly et al. 2004). The cell envelope consists of the plasma and outer membranes, which are separated by a 14- to 20-nm peptidoglycan layer (Makarova et al. 2001). The central, light-staining (∞-shaped) structure is the genome-containing nucleoid, which is being replicated and partitioned into two daughter cells. When division is complete, the diplococcus contains approximately four haploid genome copies per cell. Scale bar 0.5 μm

radiodurans (Fig. 10.1.1), first isolated from irradiated meat in 1956 by Arthur (Andy) Anderson and colleagues at the Oregon Agricultural Experimental Station in Corvalis, Oregon, USA (Anderson et al. 1956). The picture that has emerged for the life cycle of most *Deinococcus* species is one comprised of a cell-replication phase that requires nutrient-rich conditions, such as in the gut of an animal, followed by release, drying, and dispersal (Makarova et al. 2001; Daly 2009). Desiccated deinococci can endure for years, and if blown by winds through the atmosphere would be expected to survive and land worldwide. As reported, some become encased in ice (Zhang et al. 2009), and some entombed in dry desert soils (de Groot et al. 2005). High temperatures also are not an obstacle to the survival of some deinococcal species. *Deinococcus geothermalis* and *Deinococcus murrayi* were originally isolated from hot springs in Italy and Portugal, respectively (Ferreira et al. 1997). The prospects of harnessing the protective systems of *D. radiodurans* for practical purposes have captured the imagination of four generations of scientists. Perhaps if we understood why *D. radiodurans* is so resistant, we could find ways to protect people from atomic radiation, clean up radioactive waste sites, and live longer.

Cellular Targets of Ionizing Radiation

Classical models of radiation toxicity are built on the tacit assumption that ionizing radiation (IR) indiscriminately damages cellular macromolecules (Scott 1937; Hutchinson 1966). As individual proteins in a cell typically exist at much higher levels than their corresponding genes, IR-induced cell death has been attributed mainly to DNA damage (Hutchinson 1966; Daly 2009); long before the structure of DNA was solved, the genetic effects of IR were attributed directly to nuclear injury and chromosomal damage (Scott 1937). However, extreme IR resistance among bacteria consistently coincides with a greatly diminished susceptibility to IR-induced protein oxidation but with similar IR-induced DNA lesion-yields as other organisms (Daly et al. 2007). It has been proposed recently that naturally sensitive bacteria are killed by IR mainly owing to protein oxidation, whereas manganese complexes in extremely resistant bacteria protect enzymes needed to repair DNA and allow survival (Daly 2009; Daly et al. 2010).

The argument that damaged proteins may be responsible for the lethal action of IR was first developed by Walter M. Dale in the early 1940s (Dale 1940, 1942, 1943). This early view of IR toxicity was based on findings that enzymes in aqueous solution could be inactivated by relatively small doses of X-rays (less than 50 Gy). The possibility that resistance to IR could be increased was supported by studies which showed that the radiosensitivity of an enzyme is not a fixed entity but a variable, where inactivation could be prevented by the addition of nucleotides, sugars, amino acids, and a variety of other organic compounds. Dale's lasting contribution to radiobiology was to establish that the major route to radiation injury was reactive molecular species derived from the ionization of water (Barron et al. 1949), which generates hydroxyl radicals ($HO^{\bullet}$), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) as the major products (von Sonntag 1987); In contrast, in the 1930s, the lethal action of IR had been attributed mainly to direct damage (Scott 1937). Dale's idea that damaged proteins might be most responsible for toxicity in irradiated cells, however, was supplanted in the 1960s by radiobiology's dogmatic theme "death by DNA damage" (Hutchinson 1966).

The discordance of modern radiation toxicity models with results spanning nearly 5 decades of *Deinococcus* research illustrates the extent to which entrenched ideas have stymied progress. Experimental evidence favoring a transition away from DNA-centric models of

radiation toxicity has been mounting since the 1960s. Numerous lines of evidence have converged on the conclusion that damage to DNA and lipids is a secondary process, and that proteins are more probable initial targets of cellular radiation damage and should be placed at the top in the hierarchy of radiation-induced lesions most responsible for cell death (Du and Gebicki 2004; Daly et al. 2007; Krisko and Radman 2010). Certainly, DNA is a critical target in all irradiated cells (Scott 1937; Hutchinson 1966), but their survival ultimately rests on the ability of proteins to repair the damage (Daly et al. 2010).

When You Have Eliminated the Impossible, Whatever Remains, However Improbable, Must Be the Truth

The nature of the "target" molecules in cells – the alteration of which by ionizing radiation (IR) leads to the eventual damage – was first studied in bacteria, and the implications were broadly applied to formulating models of radiation toxicity (Hutchinson 1966; Blok and Loman 1973). However, it was evident to a few scientists in the 1930s that many improbable assumptions and false implications had been applied to the biological actions of IR (Scott 1937). Yet, many radiation biologists today remain beholden to the idea that only a few primary IR-induced events are required to inactivate a cell. By postulating a clear-cut all-or-none effect instead of a gradual build-up of toxic radiation products, target theory was able to explain the exponential (or nearly exponential) survival curves of the cells being studied (Blok and Loman 1973). Typical survival curves for *Escherichia coli*, and other IR-sensitive bacteria, seemed to correspond to the requirement that only a few events are necessary to produce inactivation (*Fig. 10.1.2*). Because the survival of an irradiated cell ultimately depends on whether its genome is repaired, the inactivation events were ascribed to single genes as IR damage to a few proteins was not likely to be a lethal event (Daly 2009). In contrast, survival curves for *D. radiodurans*, and other extremely IR-resistant bacteria, display very large shoulders in their dependence on dose, which are attributed to vey efficient DNA repair (*Fig. 10.1.2*) (Daly et al. 2004). Neither DNA protection, unusual DNA repair proteins nor genome multiplicity, alignment, and structure provide an explanation for the extended shoulders in *Deinococcus* dose–response relations that distinguish them from radiosensitive organisms (*Fig. 10.1.2*) (Daly 2009). The picture that emerges from the considerations listed below is that the major defense against radiation damage in *Deinococcus* bacteria is an enhanced capacity for scavenging the reactive molecular species generated by IR. The fate of irradiated bacteria that contain multiple genomes appears to reside not on the level of DNA damage but instead on their capacity to protect proteins (Daly et al. 2007).

DNA Protection

Early on, it was shown that the number of IR-induced double strand breaks (DSBs) inflicted per unit length of DNA in diverse organisms was similar and increased linearly with dose (Blok and Loman 1973; Gérard et al. 2001; Daly et al. 2004; Rothkamm and Löbrich 2003); DSBs are the most severe form of DNA damage (Daly 2009). The yield of IR-induced DSBs in a wide variety of cell-types was originally determined by sedimentation distances after ultracentrifugation of their DNA through sucrose gradients (Blok and Loman 1973). Later, IR-induced DSB

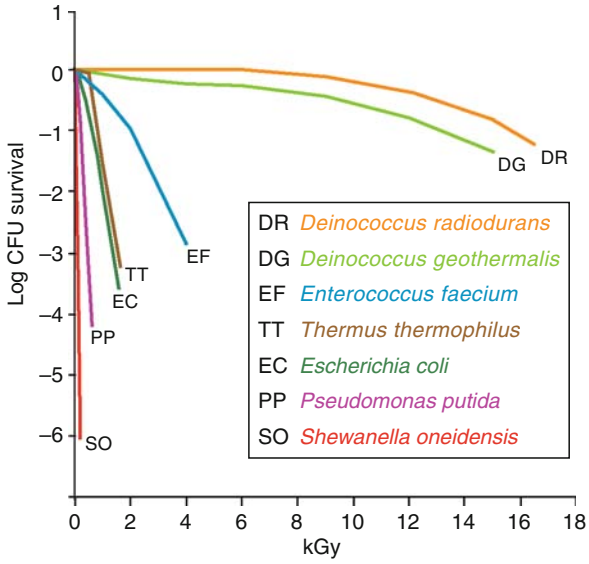

Fig. 10.1.2

Gamma-radiation survival curves for multi-genomic bacterial species that encode a similar repertoire of DNA-repair proteins (Daly et al. 2004). Bacteria were grown in rich medium to the late-logarithmic phase and irradiated (^{60}Co) on ice. Survival frequencies were determined by dilution of irradiated cultures and colony forming unit (CFU) assays on nutrient agar plates (Daly et al. 2004; Omelchenko et al. 2005). 1 kGy = 1,000 Gy = 100,000 rad

studies on bacteria used pulsed field gel electrophoresis (PFGE) (Daly et al. 2004), and studies on irradiated mammalian cells used immunofluorescence techniques based on quantifying DSB-dependent gamma-H2AX foci (Rothkamm and Löbrich 2003; Goodarzi et al. 2009). Values approximating 0.005 ± 0.002 DSB Gy^{-1} Mbp^{-1} were reported for IR-sensitive bacteria and *D. radiodurans*. Most bacteria can tolerate only 1–5 IR-induced DSBs per haploid genome, whereas *D. radiodurans* can survive nearly 200 IR-induced DSBs per haploid genome (Daly and Minton 1995; Lin et al. 1999). The value of 0.005 ± 0.002 DSB Gy^{-1} Mbp^{-1} is similar to those for IR-resistant archaea (e.g., *Pyrococcus* sp.), for simple eukaryotes (e.g., yeast), for simple animals (e.g., rotifers), human cells in liquid culture (e.g., NIH 3T3), and for viruses (e.g., SV40) (Blok and Loman 1973; Krisch et al. 1991; Gérard et al. 2001; Gladyshev and Meselson 2008; Rothkamm and Löbrich 2003; Daly 2009). Thus, the level of DNA protection in irradiated *D. radiodurans* is not appreciably different than in other organisms.

DNA Repair Proteins

The first clue that a highly specialized set of DNA repair proteins was not needed for extreme IR resistance, surprisingly, came from *E. coli*. In 1961, Erdman and colleagues reported the directed evolution of IR-resistant *E. coli* by the repeated passage of survivors through successive sublethal doses of ^{60}Co irradiation (Erdman et al. 1961). This work was followed in 1973 by

similar studies and results published by Davies and Sinskey for *Salmonella typhimurium* (Davies and Sinskey 1973) and then in 1974, by Parisi and Antoine for *Bacillus pumilus* (Parisi and Antoine 1974). The stepwise approach to selecting exceptionally high levels of radioresistance in *E. coli* was validated once again in 2009 by John R. Battista and colleagues, followed by genome sequencing and analysis of the most radiation-resistant mutants, which revealed surprisingly few mutations (Harris et al. 2009). A subsequent study showed that the radiation-resistant *E. coli* mutants were significantly less susceptible to IR- and UV-induced protein oxidation than the wild-type parent strain (Kriško and Radman 2010). Collectively, these experimental results support that a relatively conventional set of DNA repair genes is sufficient for extreme radiation resistance (Daly 2009).

Over the years, genetic and molecular studies reinforced this conclusion. In 1971, Moseley and Mattingly reported the first mutant analyses for *D. radiodurans* which showed that its recovery from radiation is dependent on DNA repair (Moseley and Mattingly 1971). Subsequent research confirmed that DNA repair enzymes, which are central to recovery of irradiated bacteria in general, were key to *D. radiodurans* survival. However, several highly radiation-sensitive *D. radiodurans* DNA repair mutants were fully complemented by the expression of orthologous genes from *E. coli* (Daly 2009). For many IR-sensitive cell-types, the frequency of the induction of a given mutation is directly proportional to the dose of IR, but not for *D. radiodurans*, which displays the same low levels of IR-induced mutation irrespective of dose (Minton 1994). Thus, the extreme resistance phenotype appears to be dependent, at least in part, on a conventional set of DNA repair proteins, which *D. radiodurans* uses extremely efficiently (Daly 2009).

The whole-genome sequence of *D. radiodurans* was one of the first to be published. The international news stories covering this milestone accurately reported the most significant finding: *D. radiodurans* encoded just about the same number and types of DNA repair proteins as radiation-sensitive bacteria. *Deinococcus* researchers were flummoxed. Perhaps there were novel DNA repair genes lurking unrecognized within the 3,284,156 base pairs of its genome (Makarova et al. 2001). This possibility was addressed systematically using functional genomic techniques based on whole-genome microarrays and whole-proteome approaches to study RNA and protein expression patterns in *D. radiodurans* cells recovering from high-dose irradiation (Daly 2009). Alas, the mystery deepened further with the finding that hundreds of genes were induced in *D. radiodurans* recovering from IR, and most of the upregulated novel genes had no effect on resistance when disrupted. Since then, two other *Deinococcus* species have been sequenced, *Deinococcus geothermalis* and *Deinococcus deserti*, which share only approximately 150 uncharacterized genes with *D. radiodurans* (Makarova and Daly 2010). Among the shared novel genes induced in irradiated *D. radiodurans*, only a few have a discernible functional relevance to the preservation of genome integrity. One moderately IR-sensitive *D. radiodurans* mutant which has been constructed is *pprA*$^{-}$, which is a putative DNA-binding protein (Kota and Misra 2006). Another is a moderately IR-sensitive *D. radiodurans* mutant *ddrB*$^{-}$, which encodes an extremely diverged single-strand DNA-binding protein (Norais et al. 2009). However, for most of the mutants derived from this subset of novel deinococcal genes, there was no significant change in the level of IR resistance, indicating that few of the putative resistance proteins, at least individually, contribute to recovery (Daly 2009). This large body of functional genomics research was conducted in numerous laboratories and funded mainly by the US Department of Energy (DOE) from 1997 to 2007. Thus, genetic evidence supporting the existence of novel genes responsible for extreme IR resistance in deinococci has grown progressively weaker.

Genome Multiplicity, Alignment, and Structure

Until the 1970s, bacteria were generally considered haploid organisms with only one copy of their genome in resting cells. This possibility might have explained the great sensitivity to IR of most bacteria as one DSB in a bacterial genome would have been lethal. However, subsequent studies revealed that IR-resistant and IR-sensitive bacteria are multi-genomic. For example, *D. radiodurans* and *E. coli* have four to eight haploid genomes per cell during logarithmic growth (Hansen 1978; Akerlund et al. 1995), but only *D. radiodurans* is IR-resistant (*Fig. 10.1.2*). Other notable examples are *Micrococcus luteus*, *Micrococcus sodonensis*, and *Azotobacter vinelandii*, which contain at least ten haploid genomes per cell but are IR-sensitive (Minton 1996).

In an early repair model, the alignment of *D. radiodurans*' four to eight genomes per cell was taken as the launching point for DSB repair (Minton and Daly 1995). This model made two major predictions: first, *recA*-dependent recombination between homologous DSB fragments originating from widely separated genomic locations should show strong positional effects upon irradiation, and second, transmission electron microscopy (TEM) of chromosomal DNA in *D. radiodurans* should reveal evidence of structures linking chromosomes. Both predictions were tested and refuted: molecular studies showed high levels of recombination between homologous DSB fragments irrespective of their genomic origin (Daly and Minton 1996, 1997), and no linking structures were observed by TEM-based optical mapping (Lin et al. 1999). Another model proposed that high levels of chromosomal condensation observed in *D. radiodurans* grown in rich medium (*Fig. 10.1.1*) facilitated repair by holding proximal DSB ends together, and that manganese promoted the condensation of its nucleoids into ring-like structures. This model is also generally discounted: *D. radiodurans* grown in defined minimal medium did not display condensed nucleoids but remained extremely IR-resistant, and *D. radiodurans* which was depleted in manganese displayed condensed ring-like nucleoids but was rendered IR-sensitive (Daly et al. 2004; Ghosal et al. 2005). Thus, IR-induced DSB fragments in irradiated *D. radiodurans* are not immobilized, and the structural form of its nucleoids does not play a deciding role in radioresistance. *D. radiodurans* contains numerous, unusual, mosaic-type small nuclear repeats (SNRs) and G-quadruplex sequences (Makarova et al. 2001); both types of sequence potentially could contribute to genome structure and reassembly. However, SNRs and G-quadruplex sequences were not identified in the genomes of *D. geothermalis* or *D. deserti* (Makarova and Daly 2010). In summary, no distinctly unusual features based on genome structure, sequence or multiplicity are shared between *D. radiodurans*, *D. geothermalis*, and *D. deserti*, to the exclusion of IR-sensitive bacteria, which establish an unequivocal molecular basis of radioresistance.

A Recipe for Radiation Resistance?

Manganese in *D. radiodurans*

Defined minimal media compositions for *D. radiodurans*, all list Mn as an essential ingredient. In 1976, Alan K. Bruce and colleagues first reported a large depot of Mn in *D. radiodurans*, which contained approximately 100 times more Mn than *E. coli*, and that Mn depletion decreased the UV resistance of *D. radiodurans* (Leibowitz et al. 1976). Using neutron activation analysis (NAA), they showed that *D. radiodurans* normally accumulated about 5 mM Mn. In

2004, the NAA results were corroborated using inductively coupled plasma mass spectrometry (Daly et al. 2004), and other studies showed that when *D. radiodurans* was incubated in defined minimal medium containing the radioisotope ^{54}Mn, the cells accumulated about 2 mM Mn (Daly et al. 2004). More recently, X-ray fluorescence (XRF) microspectroscopy revealed that Mn is distributed throughout *D. radiodurans* cells but with regional intracellular Mn concentrations ranging from 0.3 to 3 mM (Fig. 10.1.3) (Daly et al. 2007). In contrast, most of the Fe in *D. radiodurans* was sequestered outside of the cytosol, in the septa of dividing cells (Fig. 10.1.3). Based on electron paramagnetic resonance (EPR) spectroscopy and X-ray-absorption near-edge structure (XANES) analyses, the dominant form of manganese in normal *D. radiodurans* cells is Mn^{2+}, with no significant levels of Mn^{3+} detected (Daly et al. 2004, 2007). When *D. radiodurans* cells were grown in conditions that limited Mn accumulation, their cellular Mn concentration decreased together with their IR resistance (Daly et al. 2010). Since the concentration of Mn in bacteria does not affect the level of IR-induced DSBs, this left the question, what does Mn protect in *D. radiodurans*? It has been proposed that Mn^{2+} ions functionally replace Fe^{2+} and Mg^{2+} ions as mononuclear cofactors in enzymes, thereby protecting the active sites from oxidative damage (Daly 2009), and that Mn complexes are

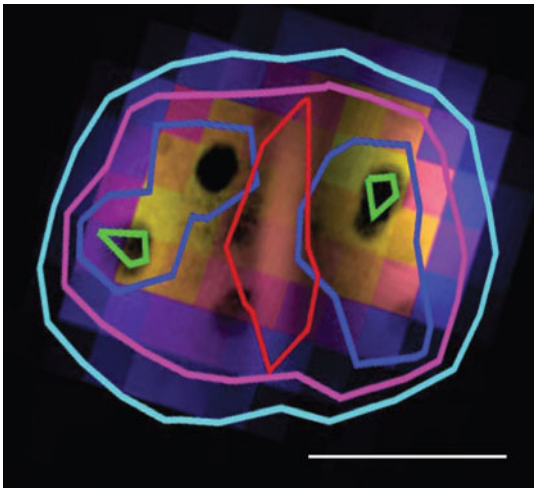

Fig. 10.1.3

X-ray fluorescence maps of the qualitative distribution and concentration gradients of manganese and iron in *D. radiodurans*. Transparent image overlay assembled from transmission electron microscopy, light microscopy, and X-ray fluorescence (XRF) microspectroscopy analyses of a single desiccated *D. radiodurans* diplococcus (Daly et al. 2007). Approximate depth-average abundance of Mn (*green*, 3 mM; *dark blue*, 2 mM; *mauve*, 1 mM; *light blue*, 0.3 mM) and Fe (*red*, 0.5 mM) are shown. A mathematical model of the original morphology of the diplococcus was constructed to determine the distribution and approximate concentration of Mn and Fe. As the diplococcus was dried out during preparation for XRF, the regional concentrations of Mn and Fe are predicted to be lower in hydrated *D. radiodurans* cells. XRF analysis measurements were made using the hard X-ray microprobe beamline 2ID-D at the Advanced Photon source, Argonne National Laboratory, Chicago, USA, under previously described conditions (Daly et al. 2007). Scale bar 0.5 μm

formed which defend against $O_2^{\bullet -}$ and H_2O_2 (Ghosal et al. 2005; Daly et al. 2010). The most consequential damage by $O_2^{\bullet -}$ and H_2O_2 in cells is to proteins which contain exposed iron-sulfur or heme groups, to proteins which contain cysteine residues, and to proteins containing cation-binding sites, where an iron-catalyzed site-specific oxidation occurs (Ghosal et al. 2005; Daly 2009).

Proteins as Targets of Radiation

The effect of IR on enzyme systems was the subject of in vitro investigations in the 1930s (Scott 1937). In those experiments, the amount of radiation necessary to produce inhibition was so high (>400 Gy) that researchers quite reasonably concluded that X-rays and gamma rays only influence enzymes when the dose is enormous. Failure to see enzyme inactivation at high doses turned out to be an artifact of using large amounts of enzyme and impure preparations (Dale 1940). In the 1940s, Walter M. Dale and his small group at the Holt Radium Institute in Manchester, England were the first to study the in vitro effects of IR on purified enzymes, which were inactivated by doses of less than 20 Gy (Dale 1940, 1942, 1943). He noted that when the purified enzymes were irradiated at low concentrations, their activity was lost at doses below those used in radiotherapy. Dale went on to show that the addition of an enzyme's substrate or other small organic compounds (e.g., nucleotides, amino acids, and sugars) greatly increased their IR resistance. It seemed that radiation acted indirectly on the protein moiety of the enzymes and on their prosthetic groups. Within several years, Thomas P. Singer's group demonstrated that sulfhydryl enzymes were even more sensitive than the enzymes studied by Dale, inactivated by just 5 Gy (Barron et al. 1949), which is in the same range as doses which kill most organisms. It took another 65 years before the dose-dependent relationship between protein oxidation and survival was first examined in vivo. In 2007, Michael J. Daly and colleagues showed that for a given dose of IR, the level of protein oxidation in IR-resistant and IR-sensitive bacteria was very different and quantitatively related to their survival (*Fig. 10.1.4*) (Daly et al. 2007). For a group of well-characterized bacteria at the opposite ends of IR resistance, when intracellular Mn to Fe concentration ratios were high, bacteria were extremely resistant to protein oxidation and IR; when intracellular Mn to Fe concentration ratios were low, bacteria were hypersensitive to protein oxidation and IR (*Fig. 10.1.4*) (Daly et al. 2004; Daly 2009). A mathematical model of radiogenic oxidative stress by Igor Shuryak and David J. Brenner is consistent with those data and can potentially be generalized to other organisms and lower radiation doses (Shuryak and Brenner 2009).

Manganese Complexes

The idea that Mn might somehow impart protection to proteins exposed to reactive oxygen species (ROS) is not new. Irwin Fridowich and colleagues discovered that Mn^{2+} and orthophosphate spontaneously form complexes, which catalytically remove $O_2^{\bullet -}$ from solutions via a disproportionation mechanism (Archibald and Fridovich 1982), and Earl Stadtman and colleagues discovered an unexpected property of complexes consisting of Mn^{2+} and amino acids or peptides, namely their ability to catalytically decompose H_2O_2 (Berlett et al. 1990). Since $O_2^{\bullet -}$ and H_2O_2 do not react with purified DNA (Blok and Loman 1973; Daly 2009), proteins were the putative targets of protection by Mn complexes (Daly et al. 2004). The first

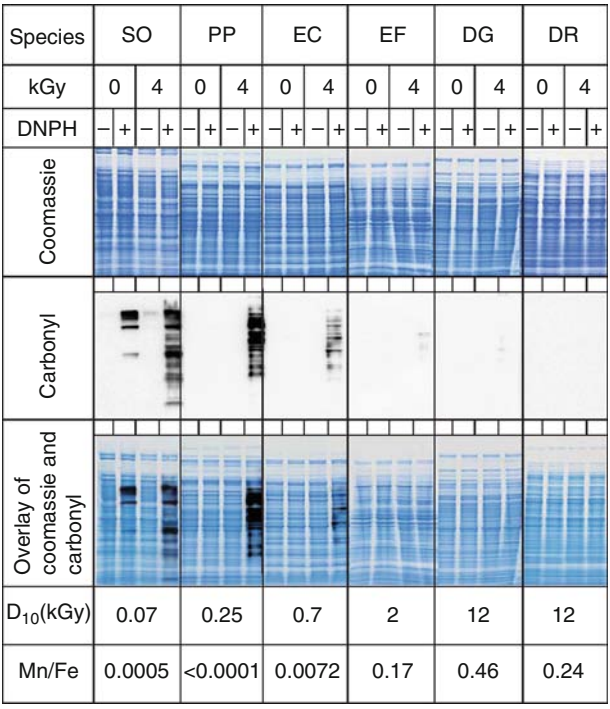

Fig. 10.1.4

Western blot immunoassay of protein-bound carbonyl groups in cell extracts prepared from bacteria irradiated to 4,000 Gy (^{60}Co at 0°C); 20 μg of a protein sample (soluble fraction) was loaded per lane and assayed for oxidation using 2,4-dinitrophenylhydrazine (DNPH) under previously described conditions (Daly et al. 2007). Coomassie represents a Coomassie-stained polyacrylamide denaturing gel of the soluble proteins, whereas carbonyl represents the corresponding western blot, which reveals the presence (*black*) or absence of protein oxidation (*no signal*). Carbonyl groups (aldehydes and ketones) are widely used as markers of irreversible protein damage. The transparent overlay of the Coomassie and carbonyl images shows that not every protein in IR-sensitive bacteria is oxidized during irradiation. Abbreviations – bacterial species, as listed in *Fig. 10.1.2*. D_{10}, the ionizing radiation dose that reduces the number of viable cells by 90%; note, the D_{10} cell survival value for *D. radiodurans* is slightly less than the D_{10} CFU survival value (*Fig. 10.1.2*) because *D. radiodurans* typically clusters as groups of two cells (diplococci) or four cells (tetracocci) (Daly et al. 2004). Mn/Fe, intracellular Mn to Fe concentration ratios, was determined by inductively coupled plasma mass spectrometry as described previously (Daly et al. 2004). For all data sets, bacteria were grown in the same medium to the same stage of growth (late-logarithmic phase).

evidence that small radioprotective molecules existed in *D. radiodurans* was published by Alan K. Bruce in 1964 (Bruce 1964). He reported that low molecular weight agents (<15 kDa) in protein-free extracts prepared from *D. radiodurans* protected sensitive bacteria against the lethal effects of IR. Based on whole-genome comparisons, there is a remarkable abundance in *Deinococcus* species of genes encoding phosphatases, proteases, and peptide transporters, which might give rise to precursors for ROS-scavenging Mn^{2+} complexes needed for recovery

(Makarova et al. 2001; Ghosal et al. 2005). In 2010, such Mn complexes were identified and characterized in *D. radiodurans* (Daly et al. 2010), and protein damage in *E. coli* exposed to IR or UV was shown to be causative and not merely correlative in radiation toxicity (Križko and Radman, 2010).

Deinococcus Prospects

Since the 1960s, the goal of exporting the radioprotective processes of *D. radiodurans* outside of the host cell for practical purposes has eluded researchers. The conceptual framework presented here, that protein oxidation in irradiated cells is not the consequence of cell death but its major probable cause, carries with it important theoretical and practical implications (Daly et al. 2010).

Mn-Dependent Chemical Antioxidants

Based on experimental analyses in *D. radiodurans*, Mn^{2+} accumulation trumps enzymatic ROS defense systems by far; the genes that encode the constitutively and highly expressed ROS-scavenging enzymes SodA (superoxide dismutase) and KatA (catalase) can be knocked out in *D. radiodurans* with almost no loss in IR resistance, but its Mn^{2+} transporter gene (*nramp*, DR1709) is essential (Daly 2009). Given that protein-free cell extracts of *D. radiodurans* are highly protective of irradiated *E. coli* and human cells in liquid culture (Bruce 1964; Daly et al. 2010), this bodes well that reconstituted chemical antioxidants of *D. radiodurans* could be delivered to widely differing cell-types without toxicity. In diverse practical settings, this could facilitate the development of bioremediation strategies aimed at the cleanup of radioactive mixed wastes, or sites containing other strong oxidants (Daly 2000), and approaches to protect humans from radiation (Daly et al. 2010). For example, several naturally IR-sensitive bacteria are known to express a suite of functions that can deal with toxic organic mixtures and heavy metals but are not an option at radioactive sites. Biostimulation with Mn complexes may present an opportunity to increase the resistance of bacteria without the need for genetic engineering (Daly 2000). In another setting, targeted delivery of Mn complexes to patients undergoing radiotherapy may help prevent side effects such as alopecia.

Metabolic Routes to Radiation Resistance

Survival of *D. radiodurans* exposed to extreme doses of ionizing radiation (IR) is highly dependent on a rich source of peptides and amino acids during recovery (Ghosal et al. 2005). The possibility that Mn-dependent chemical antioxidants in *D. radiodurans* are based on common metabolites raises the possibility that equivalent synergistic processes promoted by Mn^{2+} may be acting similarly in other organisms (Daly 2009). Over the last 2 decades, dozens of genes from IR-sensitive bacteria have been cloned and functionally expressed in *D. radiodurans* growing under high-level chronic irradiation or recovering from high-dose acute irradiation (Daly 2000; Daly et al. 2004). Similarly, many *D. radiodurans* proteins have been functionally expressed in IR-sensitive bacteria without problems. When purified from *D. radiodurans*, proteins are neither inherently radiation-resistant nor do they have unusual requirements for activity (Daly et al. 2007). A direct route to IR resistance appears to be via

metabolite regulation. For example, the development of exceptional radioresistance in naturally sensitive bacteria is typically accompanied by a progressive loss of metabolic functions, which appear to promote the accumulation of secondary metabolites. The heterotrophic nutritional modes reported in IR-resistant mutants of *E. coli*, *S. typhimurium*, and *B. pumilus* resemble those of wild-type *Deinococcus* species (Davies and Sinskey 1973; Parisi and Antoine 1974; Ghosal et al. 2005). The radioprotective benefits of Mn accumulation in cells which are unable to amass small organic molecules together with orthophosphate may be limited, and similarly for cells which accumulate small molecules without Mn. Several extremely IR-resistant cell-types are known to accumulate precursors of Mn complexes. For example, dormant spores of *Bacillus* species, both monogenomic (*Bacillus subtilis*) and digenomic (*Bacillus megaterium*), are IR-resistant and accumulate high levels of Mn and dipicolinic acid as well as a large depot of small, acid-soluble proteins (SASP) (Levinson and Hyatt 1960; Eisenstadt et al. 1973; Setlow 2007), Mn-rich cyanobacteria stockpile trehalose (Shirkey et al. 2003; Billi et al. 2000), and radiation resistant fungi accumulate melanin (Chapter 10.3, Melanin and Resistance to Ionizing Radiation in Fungi). It is conceivable that metabolic interventions in mammalian cells in G2 (tetraploid) could facilitate the accumulation of small organic compounds, Mn^{2+}, and orthophosphate, and thereby prevent oxygen radical-mediated protein damage during irradiation or aging (Levine and Stadtman 2001).

Irradiated Vaccines

All proteins in *D. radiodurans* cells are enormously resistant to IR-induced oxidation (*Fig. 10.1.4*) (Daly et al. 2007). In contrast, many proteins in IR-sensitive bacteria are readily and irreversibly oxidized (Daly et al. 2007) (*Fig. 10.1.4*). One tangible application of Mn-dependent chemical antioxidants of *D. radiodurans* could be the preparation of IR-sterilized whole bacteria, whole virus, or protein vaccines with only nominal loss in immunogenicity. Others have shown that bacteria attenuated by exposure to 8,000 Gy are able to trigger long-lasting immunity (Datta et al. 2006). However, the anticipated levels of IR required to inactivate bacteria without any risk of infection would be massive and render vaccines with greatly diminished or no immunogenicity due to oxidation of their antigenic determinants. Similar drawbacks apply to viruses, which require even higher IR doses than bacteria for inactivation because of the small size of their genomes. It is tantalizing to consider that the epitopes of cells or viruses irradiated in the presence of reconstituted deinococcal Mn complexes might survive IR doses which obliterate their genomes. This could expedite vaccine production and the deployment during epidemic outbreaks, bioterrorist attacks, and other biothreats (Datta et al. 2006; Daly et al. 2010).

Summary

The vast majority of known organisms are extremely sensitive to the cytotoxic effects of X-rays and gamma rays, killed by doses far below 200 Gy; hence, the global concerns over the impact of environmental radiation on human health, including from medical devices, and growing concerns over the emerging threat of radiological terrorism acts involving potential mass casualty incidents. There are, however, bacteria which are remarkably resistant, capable of surviving immense doses of acute IR (12,000 Gy), and growing under high-level chronic IR (60 Gy/h) (Daly et al. 2004). The most characterized member of this group of extremophiles is

D. radiodurans (Makarova et al. 2001). As the lethal effects of radiation are mediated principally through reactive oxygen species (ROS) generated from water in irradiated cells, the essential protective role of accumulated manganese in *D. radiodurans* has been attributed to the formation of small ROS-scavenging Mn complexes (Ghosal et al. 2005; Daly et al. 2007; Daly 2009; Daly et al. 2010) and the replacement of Fe^{2+} and other divalent cations with Mn^{2+} as mononuclear cofactors in enzymes, thereby preventing metal-catalyzed reactions which proliferate ROS (Anjem et al. 2009; Daly 2009). Based on the insights from physiology, comparative genomics, and biochemical studies on *D. radiodurans*, the prime candidates for the complexes include orthophosphate and Mn^{2+}, which together catalytically remove $O_2^{\bullet -}$ (Barnese et al. 2008; Daly et al. 2010) and amino acids and peptides bound to Mn^{2+}, which catalytically decompose H_2O_2 and stoichiometrically scavenge hydroxyl radicals (Berlett et al. 1990; Daly et al. 2010).

During the Duck-and-Cover Times of the Cold War, the overriding goal of the field radiobiology was to develop medical countermeasures against atomic radiation released from nuclear bombs. Yet, 50 years later, almost no clinically relevant radioprotective pharmaceuticals have been developed. Early studies on radiation-sensitive bacteria implicated DNA as the principal radiosensitive target, which became a working hypothesis around which scientists began searching for a unified theory of radiation toxicity (Hutchinson 1966). Unfortunately, the DNA double strand break (DSB) assumed the role of the most lethal event in irradiated cells (Blok and Loman 1973). As a result, early studies on the effects of irradiated proteins (Dale 1942) were largely ignored, and the phenomenal ability of *D. radiodurans* to survive hundreds of IR-induced DSBs was dismissed as a biological curiosity. Today, *D. radiodurans* stands poised to reform approaches for radioprotection based on preventing protein oxidation, ranging from pre-exposure prophylactic interventions to post-exposure therapeutics (Daly et al. 2010). It has been 115 years since Röntgen's discovery of X-rays, and it is sobering to think that the main target of IR in cells remains ambiguous. Given the delayed, but profound impact, *D. radiodurans* could have in revising the molecular basis for radiation effects on cells (Daly 2009; Daly et al. 2010; Križko and Radman 2010), the immediate lesson to the greater scientific community may be a warning – ignore extremophiles at your peril.

Acknowledgment

The work of M.J. Daly is supported by the Air Force Office of Scientific Research.

Cross-References

10.2 Ecological Stress: Melanization as a Response in Fungi to Radiation
10.3 Melanin and Resistance to Ionizing Radiation in Fungi

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10.2 Ecological Stress: Melanization as a Response in Fungi to Radiation

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_10.2,

Introduction

Fungi are known as organisms highly adapted to their environment. Among various extremes they need to cope with when colonizing natural and man-made substrates is a high level of radiation of different kinds – solar (UV) or ionizing. In the last decades, a significant reduction in the stratospheric ozone layer has been registered, with ozone loss between 1979 and 1991 averaged around 4 ± 6% per decade at northern temperate latitudes (Hollandsworth et al. 1995). As a result, the amount of more harmful UV-B radiation (280–315 nm) reaching the Earth's surface has increased (e.g., Kerr and McElroy 1993). In parallel, contamination of the environment by radionuclides, a source for the ionizing radiation, is also enhanced. All these man-induced environmental changes increase the necessity to withstand high radiation levels, and the protective role of dark pigmentation mainly of melanin nature in surviving and exploiting highly radiated environments is confirmed to be crucial.

What is Fungal Melanin?

Melanin (Greek *melas*) is a pigment of high molecular weight composed of various types of phenolic or indolic monomers usually associated with protein, and often with carbohydrates. Detailed description of fungal melanin types, their chemical structure, and pathway is given in the comprehensive reviews of Bell and Wheller (1986) and Butler and Day (1998). Numerous studies have shown that fungal melanin is always located in the cell wall, either entangled within the structure of the wall, or as its outermost layer, and may be granular or fibrillar (Butler and Day 1998 and references therein).

UV-Protective Functions of Fungal Melanin

Melanin pigments are found in all kingdoms of living organisms thus revealing their ancient origin. Melanized fungal spores in large quantities have been discovered in the deposits of early Cretaceous period when many animal and plant species died out (Dadachova and Casadevall 2008). In that period, Earth crossed the "magnetic zero" causing the loss of its protective "screen" against cosmic radiation (Hulot and Gallet 2003). And so far, the science has accumulated a large body of knowledge, both from nature and laboratory experiments, evidencing on the radioprotective role of melanin pigments in interactions of fungi with their environment.

Air

The atmosphere usually contains fungal spores in high concentrations reaching 10^4 spores per m^3, which is much higher than concentrations of bacterial cells – of the order of 10^3 cells per m^3 in the air of a large town and 10^2 cells per m^3 in the countryside (Yanagita 1990). Dominance or frequent occurrence of dematiaceous airborne fungal species has been well documented all over the world – for example, in Spain (dominance of the genera *Cladosporium*, *Ustilago*, *Pleospora* – Herrero et al. 2006), in Israel and Turkey (*Cladosporium*, *Alternaria* – Waisel et al. 1997; Sen and Asan 2001, respectively), in Lithuania (*Cladosporium*, *Alternaria*, *Aspergillus niger* – Lugauskas et al. 2003), in Brazil (*Cladosporium*, *Leptosphaeria*, *Alternaria* – Zoppas et al.

2006), and in Africa (*Cladosporium*, *Alternaria*, *Arthrinium*, *A. niger* – Prospero et al. 2005). Even some frequently recorded in the air spores of light-colored fungi from the genera *Penicillium* and *Aspergillus* are known to contain greenish pigments of a melanin nature (e.g., Coelho et al. 1997; Cuadros et al. 1999; Youngchim et al. 2004). Field study of the responses of airborne fungi to UV-B radiation in the coastal area of Lithuania has shown that predominantly dark-colored sporulated and non-sporulated species were documented after exposure to the radiation (Ulevicius et al. 2004). Protective pigmentation of cell walls with melanin and melanin-like pigments is considered to make airborne fungal propagules less vulnerable to the UV radiation damage compared to bacterial cells.

Leaf Surface

The plant leaf surface, or phyllosphere (phylloplane), supports the growth of diverse fungal biota. Just simply counting the percentage of melanin-containing species in such phyllosphere mycobiota from the British Isles containing near 4,300 species described in the classic book of Ellis and Ellis (1997) shows overwhelming dominance of melanized species – more than 95% of species composition. Most of them have double (dark color of fruit bodies and ascospores or conidia and mycelia) or even triple (dark color of fruit bodies, spores, and mycelia) defense against direct exposure to UV radiation. Microfungal communities from plant leaves collected in the Mediterranean area in Portugal (Pereira et al. 2002) provides another characteristic example from the numerous on this topic. Here, melanin-containing *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *C. spherospermum*, and *Alternaria alternata* comprised more than 80% of total fungal isolates. Laboratory experiments on survival rates of some dark-colored phylloplane-inhabiting fungi from the genera *Alternaria*, *Ulocladium*, and *Epicoccum* under short wavelength UV radiation (250–270 nm) demonstrated their much higher resistance to the treatment in comparison with lighter pigmented *Botrytis cinerea*, which had also conidia with thinner cell walls (English and Gerhardt 1946; Boyd-Wilson et al. 1998). Such UV-resistant leaf surface fungi, mainly from the genera *Alternaria*, *Cladosporium*, and *Phoma* were found to be considerable contributors to the air spora (Hameed and Awad 2005; Levetin and Dorsey 2006).

Rock Surface

Bare rock surfaces in hot and cold deserts as well as in high mountains represent terrestrial environments most hostile and extreme for any kind of life (e.g., Shilo 1978). But in spite of the hostility, rock surfaces and more specifically, rock varnish (Perry et al. 2003) are persistently inhabited by a very specialized group of free-living microscopic fungi (e.g., Staley et al. 1982; Sterflinger and Krumbien 1995; Gorbushina 2003). These rock-dwelling fungi develop very small colonies both in situ and in vitro and are called microcolonial fungi (MCF). MCF not only survive but grow under extremely stressful environmental conditions including high UV radiation and, again, black pigments highly concentrated in their cell walls serve as UV-protective substances (e.g., Urzi et al. 1995). Melanin is located in the cell walls of black fungi and yeasts as an external electron-dense granular layer as well as in the matrix between the cells forming an extracellular intracolonial structure (Gorbushina 2003). Along with melanin pigments, other groups of UV-absorbing compounds of low molecular weight, mycosporines, have been found in MCF (Gorbushina et al. 2003). Mycosporines have a unique absorption spectrum of

310–320 nm (Bandaranayake 1998) and can serve as specialized structures enhancing the survival potential and longevity of rock-inhabiting microfungi (Gorbushina et al. 2003).

Specific fungal life has been discovered in the lichen-dominated cryptoendolithic communities colonizing rocks in the Antarctic ice-free desert. Two new genera and four new species of black MCF, probably endemic, have been described using morphological and molecular analyses (Selbmann et al. 2005). Strains of *Cryomyces minteri* and *C. antarcticus* tested for their UV resistance were highly resistant to UV-B irradiation showing long-time survival and no morphological modifications at both colony and cellular levels after the treatment (Onofri et al. 2007). The species were found to express melanized thick walls as a stable characteristic, allowing them to tolerate dryness and the UV irradiation that reaches them through the translucent crystals of orthoquarzite in sandstone (Selbmann et al. 2005). The tested strains of *Cryomyces* have been considered as good candidates to be exposed to the space and simulated Mars conditions, including space vacuum, solar UV, and cosmic radiation (Onofri et al. 2007).

Not only specific microcolonial fungi, but also common black fungi from the genera *Phoma* and *Alternaria* were found to cause physical and chemical damage (blackening and brown patinas) of sandstones, limestones, and marbles collected from monuments and rock outcrops throughout Europe and Africa (Diakumaku et al. 1995). Most of the strains examined produced pigments of a melanin nature responsible for staining of the rocks under extreme environmental conditions including high persistent irradiation.

Soil and Litter

UV radiation is known to penetrate only up to about 100 microns into soil depth (Johnson 2003) but in the uppermost layers of sun-exposed soils, this environmental factor plays an important role in the organization of microfungal communities. Non-surprisingly, dominance of dark-colored microfungi is characteristic for almost all mycologically studied desert soils (e.g., Ranzoni 1968; Christensen 1981; Halwagy et al. 1982; Skujins 1984; Abdullah et al. 1986; Hashem 1991; Ciccarone and Rambelli 1998; Mulder and El-Hendawy 1999, Zak 2005). In the Negev desert, Israel, melanin-containing microfungi comprised 55% of general species composition and strongly prevailed in relative abundance (58–77% in different localities). Importantly, melanized fungi with large thick-walled multicelled conidia increased their abundance southward in open localities of northern and central Negev (Grishkan et al. 2006) and overwhelmingly dominated all microfungal communities in southern Negev (Grishkan et al. 2007). These fungi were mainly represented by *Ulocladium atrum*, *U. botrytis*, *Alternaria alternata*, and *A. chlamydospora*. The first three species are cosmopolitan, the latter one a desert fungus, which also produces large multicelled chlamydospores (Ellis 1971, 1976). The multicellular spore morphology, together with melanin pigmentation, should be considered as an important adaptive feature of desert soil mycobiota. Spores of such morphology can successfully survive under UV radiation, extreme temperatures, and drought. In nature, species with dark, multicelled conidia isolated from the Negev uppermost soil layer are also phylloplane inhabiting (Ellis 1971, 1976; Ellis and Ellis 1997). Under laboratory conditions, Durrell and Shields (1960) showed that the survival time of thick-walled multicellular conidia of *Stemphylium ilicis* under the same irradiation was 30-fold longer than for thin-walled one-celled conidia of *A. niger*. These authors also revealed that melanized fungi could absorb heat from solar radiation in winter. Thus, dark-colored, many-celled conidia carry out both dispersal and resting functions, which is crucial in climatically and microclimatically stressful desert habitats.

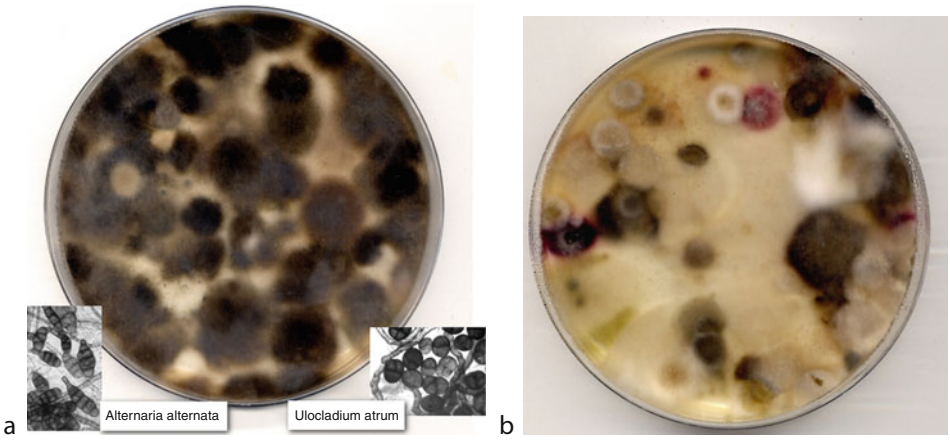

Fig. 10.2.1

Microfungal communities from crust (0–0.2 cm; a) and below soil layer (0.2–5 cm; b) in Nahal Nizana, the central Negev desert, Israel

Vertical distribution of microfungi through soil depth in a desert can efficiently illustrate the association between solar radiation and structure of microfungal communities. *Fig. 10.2.1* shows Petri dish cultures of microfungal communities from the soil layers 0–0.2 cm and 0.2–5 cm (a and b, respectively) sampled in Nahal Nizana, the central Negev desert. Communities from the uppermost crust layer are overwhelmingly dominated by melanin-containing fungi with many-celled conidia (*U. atrum*, *A. alternata*, *Embellisia phragmospora*, and *Stemphyllium* state of *Pleospora tarda*), while the layer only 0.2 cm below is mainly inhabited by light-colored species from the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Mortierella*.

Melanin-containing fungi prevailed also in the uppermost soil layer of the sunny-exposed south-facing slopes of the canyons located in northern Israel (Grishkan et al. 2003a) but quantitatively, this prevalence was almost two times less pronounced than in the desert and was subjected to seasonal changes (did not appear in the autumn and winter). Qualitatively, the group of melanized fungi in the northern canyons was mainly composed of species with one-celled, comparatively small conidia such as *A. niger*, *C. cladosporioides*, and *Humicola fuscoatra*. For *A. niger* from the Mount Carmel canyon, the concentration of melanin in conidia and their survival after UV-A exposure were examined in comparison between strains collected from the south-facing (SFS) and north-facing (NFS) slopes (Singaravelan et al. 2008). Conidia of the SFS strains contained melanin concentrations more than three-fold higher compared with conidia of the NFS strains. Such remarkable difference between slopes in melanin concentration was accompanied by significant difference in conidial culturability after UV-A irradiation and corresponded to the interslope microclimatic divergence caused by much higher solar radiation (200–800%; Pavlicek et al. 2003), which the SFS received in comparison with the NFS.

The effect of UV radiation has been also tested on the growth of Antarctic litter fungi (Hughes et al. 2003). Expectedly, hyphal extension rate of the melanin-containing *Phoma herbarum* was 2.5- to sixfold less reduced after the UV-B exposure than that of the light-colored species. *P. herbarum* produced a brown pigment within 24 h of the artificial UV treatment

accompanied by production of fruit bodies and conidia. Key confirmation of the UV-protective role of black pigmentation has been obtained in studies that compared sensitivity of melanized and non-melanized strains of the same fungal species (e.g., Lamb et al. 1992; Rehnstrom and Free 1997). One of these studies was done on the coprophilous fungi *Ascobolus immersus*, *Sordaria brevicaullis*, and *S. fimicola* (Lamb et al. 1992). The authors tested UV-C sensitivity of the wild-type red or black pigmented ascospores of the fungi versus mutants with pale or no visible ascospore pigmentation thus avoiding complications associated with coevolutionary changes in efficiencies of repair mechanisms when comparing wild-types of different species. The experiment demonstrated direct association of UV-tolerance of the ascospores with the intensity of their dark pigmentation. It also revealed the importance of pigment distribution because the concentration of most of the red pigment into large granules in the *Ascobulus* mutant increased its UV-sensitivity although the total amount of pigment per ascospore was approximately the same as in wild-type.

Response to Ionizing Radiation

Radionuclide contamination as a source of ionizing radiation coming from exploitation of nuclear power plants, nuclear weapons, and uses of radioisotopes has become a reality nowadays and recent studies have demonstrated that fungi could be remarkably resistant to such kind of radiation. Resistance of many fungi, especially of melanized ones to gamma radiation was found to be higher than resistance of bacteria (*Table 10.2.1*). Some melanin-containing fungi

Table 10.2.1

Comparative sensitivity of bacteria and fungi to external gamma radiation (adapted from Dadachova and Casadevall 2008)

Species	LD_{10} (kGy)[a]	Source
Bacteria		
Thermus thermophilis	0.8	Sghaier et al. 2008
Escherichia coli	0.7	Sghaier et al. 2008
Kineococcus radiotolerans	2	Sghaier et al. 2008
Rubrobacter xylanophilus	5.5	Sghaier et al. 2008
Deinococcus radiodurans	2–15	Sghaier et al. 2008
Fungi		
Penicillium lutum 352	0.4	Mirchink et al. 1972
Fusarium sp. 117	0.45	Mirchink et al. 1972
Stemphylium botryosum	>5	Mirchink et al. 1972
Alternaria tenuis	>5	Mirchink et al. 1972
Cladosporium cladosporioides	>5	Mirchink et al. 1972
Cryptococcus neoformans	4.3	Dadachova et al. 2004
Histoplasma capsulatum	6.7	Dadachova et al. 2004

[a]Dose (kilogray) required achieving 90% cell killing.

Note: ionizing radiation resistant bacteria (IRRB) are underlined; melanized fungi are in bold.

causing spoilage of food products (*A. alternata*, *Curvularia lunata*, and *C. genticulata*) were shown to survive under doses of gamma radiation exceeding the international standard for food (Saleh et al. 1988). Resistance of these fungi to the irradiation from a ^{137}Cs source was two- to eightfold higher than of the light-colored species involved in the experiment.

Measuring the rate of radionuclide immobilization by fungi indicated that dark-colored species had some advantage in this ability in comparison with light-colored ones. For example, mycelia of light pigmented fungi accumulated 1.1–4.7 less ^{90}Sr than mycelia of dematiaceous fungi (Zhdanova et al. 1990). Fully melanized *A. alternata* showed a higher capacity in uptake of both ^{60}Co and ^{137}Cs than *A. pulverulens* or *Fusarium verticilloides* (Mahmoud 2004). According to this author, melanin accounted for 45–60% of the incorporation of these isotopes into fungal hyphae.

Special attention was drawn to the resistance of fungi to ionizing radiation after the nuclear reactor accident at Chernobyl in 1986. Before the accident, soils in the Kiev region were dominated by non-melanized fungal genera, but after the radionuclide contamination in the vicinity of the reactor, these genera were replaced by melanin-containing genera such as *Stachybotrys*, *Ulocladium*, *Sporormiella*, *Humicola*, *Aureobasidium*, and *Alternaria* (Zhdanova et al. 2005). Comparison of the species growing under severe and comparatively weak radioactive pollution in the inner locations of the fourth unit of the Chernobyl nuclear power plant showed dominance of melanized fungi (mostly *Cladosporium* spp., *A. alternata*, and *A. pululans*) in heavily contaminated sites (Zhdanova et al. 2000). Some strains of *C. cladosporioides* and *C. sphaerospermum*, together with *Penicillium roseopurpureum* and *P. hirsutum*, isolated from the inner and outer locations of the reactor, displayed positive radiotropism, that is, statistically significant directed growth to the ^{109}Cd source of radiation (Zhdanova et al. 2004). The authors consider such fungi as promising bioremediation agents in radio-contaminated environments (Zhdanova et al. 2005).

Mechanism of Radioprotective Ability of Fungal Melanins

Amorphous semiconductivity of melanin (dihydroxyphenylalanine – DOPA – melanin), which forms free radicals and is itself a stable free radical, is considered to be the fundamental molecular property associated with the melanin radioprotective capacity (Hill 1992). DOPA melanin is able to convert different kinds of energy into vibrational and rotational activity in the melanin molecular structure dispersing it as heat and thus protecting a cell from the damaging effects of radiation (Butler and Day 1998). This energy absorbing and dispersing property accompanied with an ability to act as a sponge for cytotoxic free radicals was stated to be the fundamental explanation of melanin-protective functions.

The above explanations have been successfully confirmed in the experimental studies conducted by Dadachova et al. (2007a, b) that are discussed in detail by Dadachova and Casadell in Chap. 10.3 Melanin and Resistance to Ionizing Radiation in Fungi.

Other Functions of Fungal Melanins

While this chapter is devoted to the radioprotective function of fungal melanins, there are several other functions that are necessary for fungi in coping with environmental stress and thus are worth mentioning. Melanin can protect fungi against temperature extremes as it was

shown on conidia of *Monilia fruticola* (heat) (Rehnstrom and Free 1997) and on *Cryptococcus neoformans* (both heat and cold) (Rosas and Casadevall 1997). Melanin probably prevents fungal cells from dehydration (Zhdanova and Pokhodenko 1973; Rehnstrom and Free 1997) because of its water-binding capacity (Prota 1992). Both these protective functions participate in overwhelming dominance of melanin-containing fungi on leaf surfaces and in the upper layers of desert soils as well as in highly saline environments (e.g., Gunde-Cimerman et al. 2004) even if they are slightly or non-UV-radiated (Grishkan et al. 2003b, 2004). Melanin is known to protect fungal cells not only from abiotic stress factors but also against enzymatic lysis by antagonistic microorganisms (e.g., Bull 1970; Old and Robertson 1970; Rehnstrom and Free 1997; Butler and Day 1998). Recently in the Netherlands, the proportion of melanized hyphae in different soil types was found to average 61% with highest values in non-disturbed forest soils where melanized hyphae actively participated in the degradation of recalcitrant organic substrates (van de Wal et al. 2009).

It has been found that melanin-containing microfungi increase their contribution to soil mycobiota in the response to chemical pollution (Marfenina 1998), especially near the roadsides both in soil and in winter snow cover (Kul'ko and Marfenina 2001). There is also evidence that melanin serves as a virulence factor contributing to fungal pathogenicity both for plants and animals (e.g., reviewed in Butler and Day 1998). Regarding the human pathogens *C. neoformans* and *Histoplasma capsulatum*, it has been shown that melanin protected fungal cells against microbicidal agents produced by immune effector cells (Wang and Casadevall 1994) as well as reduced the susceptibility of pigmented cells to antifungal drugs (Van Diun et al. 2002). In some plant pathogens, melanin is involved in penetration of appressoria into host cells by developing sufficient turgor pressure (Money and Howard 1996).

Fungal melanins have a high biosorptive capacity for a variety of metal ions (Fogarty and Tobin 1996, and references therein). The pigments can prevent toxic metals entering into the fungal cell. Adsorption of metal ions by melanized outer surfaces may also protect fungal propagules from antagonistic microorganisms (Rizzo et al. 1992). On the other hand, melanin was found to play a major role in the metal ion absorption in rock-inhabiting fungal microcolonies (Gadd and Mowll 1985). Such binding capacity might be especially important on rock surfaces in providing fungi with necessary ions and stimulating their growth and survival (Gorbushina et al. 2002).

Conclusion

Melanin is not a necessary compound for fungal growth because both pigmented and non-pigmented strains of the same fungus can exist. But as a huge quantity of evidence from nature and laboratory experiments testify, this pigment is highly advantageous for fungi in withstanding various environmental extremes. The ability to absorb all types of electromagnetic radiation is an important feature of melanin which bestows the pigment with the capacity for both energy transformation and shielding. The well-established fact that melanin-containing fungi as well as other melanized organisms inhabit highly radiated environments all over the world, together with phenomenon of "radiotropism" has prompted the conclusion that melanin probably has functions analogous to other energy-harvesting pigments such as chlorophyll (Dadachova and Casadevall 2008).

Cross-References

- 3.3 Osmoadaptation in Methanogenic Archaea: Physiology, Genetics, and Regulation in *Methanosarcina mazei* Gö1
- 6.4 Adaptation Mechanisms of Psychrotolerant Bacterial Pathogens
- 10.1 *Deinococcus radiodurans*: Revising the Molecular Basis for Radiation Effects on Cells

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10.3 Melanin and Resistance to Ionizing Radiation in Fungi

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_10.3,

Introduction

Constant exposure to ionizing radiation is a "fact of life" for all life forms on Earth – for example, 90% of the annual radiation dose for a person living in the US comes from natural sources such as cosmic radiation and radioactive rocks (Early and Sodee 1995). However, fungi seem to interact with the ionizing radiation in a manner that may differ from other life forms. Large quantities of highly melanized fungal spores have been found in early Cretaceous period deposits when many species of animals and plants died out. This time period coincides with Earth's crossing the "magnetic zero" resulting in the loss of its "shield" against cosmic radiation (Hulot and Gallet 2003). When exposed to high doses of ionizing radiation under experimental conditions, fungi in general, and especially melanized ones, are highly radioresistant (Saleh et al. 1988; Dembitzer et al. 1972; Dadachova et al. 2004; Mirchink et al. 1972). As ionizing radiation is perceived to be harmful to life, this unusual ability of eukaryotes to survive or maybe even benefit from exposure to ionizing radiation is in contrast to such perception. Recently, there has been considerable interest in the interaction of fungal cells with radionuclides in the environment (reviewed in Dighton et al. 2008), since this phenomenon could be potentially useful for environmental remediation. However, the nature of this interaction is almost certainly chemical, involving chelation and binding of isotopes by melanin and thus is different from interaction of fungi with ionizing radiation, which more properly belongs to biophysics domain. In this chapter we will summarize the recent findings on the possible mechanisms involved in melanin radioprotection and ionizing energy utilization in fungi, and will describe various examples of such radioprotection and energy utilization by melanized fungi on Earth, in stratosphere, and in open space.

Mechanism of Radioprotection by Melanin in Fungi

What is Melanin?

Melanin is a pigment which is ubiquitous in nature. It is generally accepted that there are two major types of melanin: eumelanin and pheomelanin. Eumelanin is a dark-brown to black pigment composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomer units with 6–9% nitrogen (*Fig. 10.3.1a*) while pheomelanin is a reddish-brown pigment composed of benzothiazine monomer units with 8–11% nitrogen and 9–12% sulfur (*Fig. 10.3.1b*) (Ito and Fujita 1985; Wakamatsu and Ito 2002). The majority of naturally occurring melanins, however, constitute a mixture of eumelanin and pheomelanin subunits which explains the fact that up to 1% of sulfur is found in predominantly eumelanin samples. Structures of both types of melanin are characterized by the presence of multiple conjugated aromatic rings with π-electrons (*Fig. 10.3.1a*, *b*).

Radioresistance of Fungi

Radioresistance of fungi. Fungi in general are very radioresistant (Saleh et al. 1988; Dembitzer et al. 1972; Dadachova et al. 2004; Mirchink et al. 1972). The bacterium Deinococcus

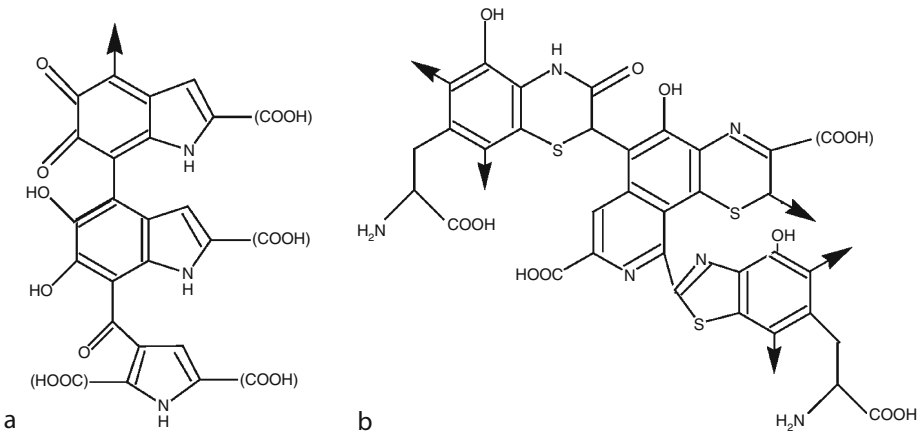

Fig. 10.3.1

Chemical structure of melanin: (a) structure of eumelanin oligomer; (b) structure of pheomelanin oligomer

Table 10.3.1

Radiosensitivity of melanized and non-melanized fungi to external gamma radiation

Species	LD_{10} (kGy)	Source
Penicillum lutum 352	0.4	Mirchink et al. (1972)
Fusarium sp. 117	0.45	Mirchink et al. (1972)
Stemphylium botryosum[a]	> 5	Mirchink et al. (1972)
Alternaria tenuis[a]	> 5	Mirchink et al. (1972)
Cladosporium cladosporioides[a]	> 5	Mirchink et al. (1972)
Cryptococcus neoformans[a]	4.3	Dadachova et al. (2004, 2008)
Histoplasma capsulatum[a]	6.7	Dadachova et al. (2004, 2008)

[a]Melanized fungi

radiodurans is considered the most radioresistant microorganism known with LD10 for some of its strains approaching 15 kGy (Sghaier et al. 2008; Daly, Chap. 10.1 Deinococcus radiodurans: Revising the Molecular Basis for Radiation Effects on Cells). The standard dose for food irradiation in the US is 1 kGy which is considered sufficient to kill the bulk of the food-contaminating microorganisms since only a few strains of bacteria have LD_{10} values higher than 1 kGy. However, many fungi, especially melanized ones, are very radioresistant with LD_{10} values approaching or exceeding 1 kGy (*Table 10.3.1*). This radioresistance of fungi is not widely appreciated and should be taken into consideration when gamma radiation is used for sterilization of food or medical supplies. In contrast, the lethal whole body dose for a human is approximately 5 Gy, making humans more than 1,500 times more susceptible to ionizing radiation than fungi.

Melanin Contribution to Fungal Radioresistance

Melanin in fungi appears to have significant radioprotective properties (Mirchink et al. 1972; Dadachova et al. 2008). To investigate the ability of melanin to protect fungi against radiation damage we analyzed two fungi capable of melanogenesis, *Cryptococcus neoformans* (Cn) and *Histoplasma capsulatum* (Hc) (Dadachova et al. 2008). These fungi were selected for study because they can be evaluated in their non-melanized or melanized states depending on whether they are grown with substrate for melanization. Furthermore, Cn and Hc are representatives of basidiomycetes and ascomycetes, respectively. When Cn and Hc are grown with L-dopa (3,4-dihydroxyphenylalanin), melanin is concentrated in the cell wall (*Fig. 10.3.2a*). Hollow melanin particles with a roughly spherical shape can be isolated from melanized cells digested in concentrated acid and have been dubbed "ghosts" because they retain the shape and dimensions of the parent cell (Wang et al. 1996). Melanin in these ghosts is assembled into multiple concentric layers of approximately 100 nm thickness consisting of closely packed much smaller particles (Eisenman et al. 2005). Late stationary phase melanized and non-melanized Cn and Hc cells were subjected to very high doses of radiation – up to 8 kGy. Since the LD_{10} for these organisms in their non-melanized form is around 0.05–0.1 kGy (Dadachova et al. 2004), high doses were needed to ascertain the radioprotective effect of melanin. Melanized Cn cells were significantly less susceptible than non-melanized cells to external gamma radiation ($p =$

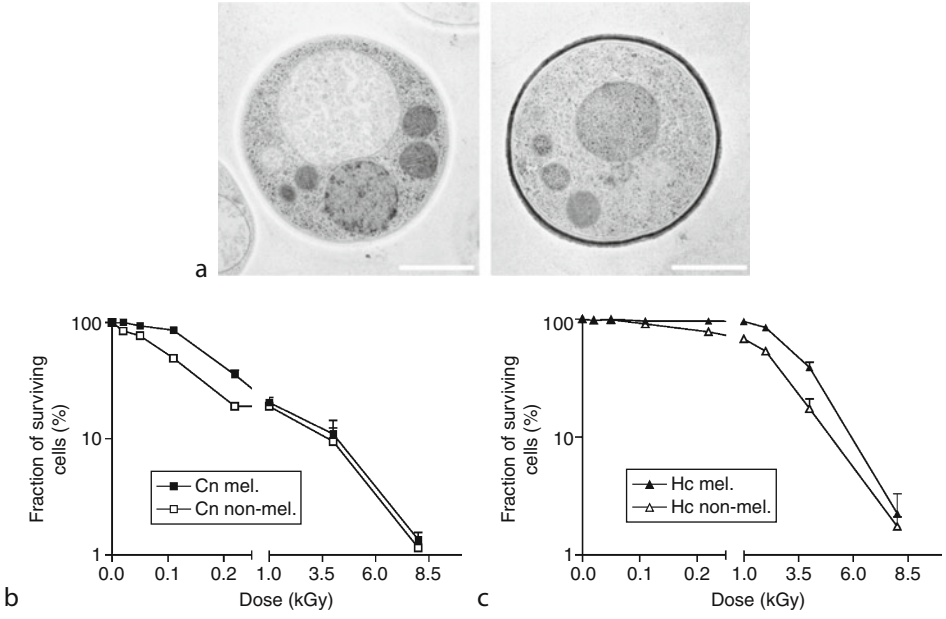

Fig. 10.3.2

Survival of non-melanized and melanized fungal cells following exposure to external gamma rays: (a) TEM micrographs of non-melanized (*left panel*) and melanized (*right panel*) *C. neoformans* cells; (b) *C. neoformans* in PBS up to 0.22 kGy at 0.014 kGy/min and up to 8 kGy at 0.030 kGy/min; (c) *H. capsulatum* in PBS up to 0.22 kGy at 0.014 kGy/min and up to 8 kGy at 0.030 kGy/min. Bar in (a) is 2 μm

0.01) in the dose range of 0–0.22 kGy (Fig. 10.3.2b) with the dose reduction factor (DRF) attributable to melanin calculated to be 1.6. DRF is a measure of the radioprotective properties of a substance when applied to a biological system with a range of $1 \leq \text{DRF} \leq 2.2$ (Hall 2000). At 1.0–8.0 kGy, the protective effects were not statistically significant ($p = 0.4$). For Hc cells melanin provided protection up to 8 kGy ($p < 0.01$) with DRF of 1.6 (Fig. 10.3.2c). Thus, for both Cn and Hc, melanin provided significant protection against very high doses of radiation, especially when taking into consideration that the doses were delivered at a very high dose rate of 0.014 kGy/min and that the damage to the cells is directly proportional to the dose rate at which radiation is being delivered. From these studies we concluded that melanin composition and melanin particle spatial arrangement were important contributing factors towards melanin radioprotective properties in fungi.

Mechanism of Radioprotection by Melanin

Meredith and Sarna in 2006 reviewed the relationship between melanin (eumelanin) structure and its optical properties, condensed phase electric properties, electron exchange, paramagnetic properties, ion exchange capacity; and ability to absorb UV and visible radiation (Meredith and Sarna 2006). However, in contrast to the large body of knowledge accumulated over the past 50 years in establishing the structure-property relationship for melanin, there were no attempts to give a physico-chemical explanation of radioprotective properties of melanin. In fact, the mechanism of melanin radioprotection has been assumed to be free radical scavenging, primarily of the highly destructive, short-lived radicals generated by the radiolysis of water (Mosse et al. 2000).

We hypothesized that the physical interaction between melanin and the recoil electrons generated by Compton scattering of incident photons in melanin itself or transferred to melanin by other molecules and radicals was another significant contributor to the mechanism of radioprotection. A single Compton recoil electron traveling through tissue can result in multiple points of damage to DNA or other cell structures via the generation and propagation of free radical species (Bomford and Kunkler 2002). To test this hypothesis we synthesized chemically diverse melanins using precursors with different functional groups. To select melanins with the best radioprotective properties from the panel of synthesized melanins, we determined their physico-chemical characteristics to identify those that correlated with favorable radioprotective properties. Consequently, the melanins were subjected to: (1) elemental analysis, (2) high performance liquid chromatography (HPLC), and (3) electron paramagnetic resonance (EPR). Surrogate measurements of radioprotective properties were obtained by performing: (1) quantitative EPR to estimate the number of stable free radicals, (2) determination of mass attenuation coefficients from the cross section used in Monte Carlo simulation, and (3) clonogenic survival assay to determine melanin's ability to protect mammalian cells (Schweitzer et al. 2009).

Melanins were designed from 5-S-cysteinyl-DOPA, L-cysteine/L-DOPA, or L-DOPA with diverse structures as shown by elemental analysis and HPLC. Sulfur-containing melanins had higher predicted attenuation coefficients than non-sulfur-containing melanins. All synthetic melanins displayed strong electron paramagnetic resonance (2.14×10^{18}, 7.09×10^{18}, and 9.05×10^{17} spins/g, respectively), with sulfur-containing melanins demonstrating more complex spectra and higher numbers of stable free radicals. There was no change in the quality or quantity of the stable free radicals after high-dose (30,000 cGy), high-energy (^{137}Cs, 661.6 keV)

irradiation, indicating a high degree of radical stability as well as a robust resistance to the ionizing effects of gamma irradiation. The rationally designed melanins protected mammalian cells against ionizing radiation of different energies. We proposed that the mechanism of melanin protection involved dissipation of Compton recoil electrons energy via interaction with numerous aromatic oligomers containing multiple π-electron system, such that the electron gradually loses energy while passing through the pigment, until its energy is sufficiently low that the electron can be trapped by stable free radicals present in the pigment. Controlled dissipation of high-energy recoil electrons by melanin prevents secondary ionizations and the generation of damaging free radical species (Schweitzer et al. 2009).

Examples of Melanin Function in Fungi as a Radioprotector

Earth Environments

Melanized fungi inhabit some remarkably extreme environments on the planet including Arctic and Antarctic regions and high altitude terrains, with the latter habitats being characterized by the naturally higher radiation levels than lower altitudes (Robinson 2001). The "Evolution Canyon" in Israel is a popular site for studying adaptation of organisms to their environment. Its south-facing "African" slope receives 200–800% higher solar radiation than the north slope and is populated by many species of melanized fungi (see Chap. 10.2 Ecological Stress: Melanization as a response in Fungi to Radiation).

Among the environments with high radiation resulting from human activities, -two examples stand out. First, melanized fungal species colonize the walls of the damaged reactor at Chernobyl where they are exposed to a high constant radiation field (Mironenko et al. 2000). Second, melanized fungal species are found in the so-called cooling pool water of nuclear reactors. This water circulates through the nuclear reactor core for cooling purposes where the radiation doses are extremely high. These pools often contain large numbers of fungi, cocci, Gram-positive rods, and some Gram-negative rods. Analysis of the reactor water microbiota led to the suggestion that high fluxes of radiation select for highly radioresistant types of microorganisms, which manifest increases in catalase and nuclease activities (Mal'tsev et al. 1996).

Stratosphere and Space

Other high radiation environments where fungi have adapted are the stratosphere and orbiting spacecraft. More than 30 years ago meteorological rockets fitted with specially designed analyzers brought back to Earth microorganisms collected at the altitudes of 48–77 km. The microorganisms were microscopic fungi having black conidia or spores (*Circinella muscae*, *Aspergillus niger*, *Papulaspora anomala*) and one species forming green conidia (*Penicillium notatum*). Colonies of *Mycobacterium luteum* and *Micrococcus albus* were also found at such altitudes. Those organisms were apparently delivered to the stratosphere by sand storms. Five out of six microbial species collected synthesized pigments (Imshenetsky et al. 1978).

Analysis of the atmosphere in the Russian orbital station Mir revealed the ubiquitous presence of many microorganisms (Alekhova et al. 2005). The likely sources of contamination of the space station are flight materials during manufacturing and assembly, the delivery of supplies to the space station, the supplies themselves, and secondary contamination from the

crew and any other biological material on board, e.g., animals, plants, and microorganisms used in scientific experiments (Alekhova et al. 2005). Fungal contamination poses certain threats to the well-being of the crew not only because some of those fungi are potential human pathogens but also because fungi possess powerful enzymatic systems and secrete various metabolites capable of degrading structural materials inside the spacecraft – from polymers to various alloys.

The survey of the environmental contamination on board of the International Space Station (ISS) revealed many fungal species on the surfaces and in the air with *Aspergillus* sp., *Penicillium* sp., and *Saccharomyces* sp. being the most dominant genera among fungi. A diverse population of *Aspergillus* spp. was recovered (13 species), whereas diversity was less pronounced in the case of *Penicillium* (5 species) and *Cladosporium* (4 species) (Novikova et al. 2006). The levels of ionizing radiation that these fungi encounter in the space stations (approximately 4 cGy/year; Baranov et al. 2006) are below fungicidal doses (Saleh et al. 1988; Dembitzer et al. 1972; Dadachova et al. 2004, 2008; Mirchink et al. 1972) and allow fungi to thrive provided the humidity levels are sufficient. Interestingly, many of the microorganisms inhabiting the space station – both bacteria and fungi – were found to be pigmented or melanized, which hints at the usefulness of pigments presence in those cells under the extreme conditions.

Another important microbiology-related aspect of space flight is the possibility of spacecraft inhabiting microorganisms changing their properties to the extent that they become dangerous for the Earth's inhabitants when the spacecraft returns to Earth. Most likely such microorganisms would be located on the outside surfaces of the craft where they would be exposed to the extremes of "open space." To investigate such possibility the researchers conducted "Biorisk" experiment (Novikova et al. 2007). The electron-microscopy investigation of *Aspergillus versicolor* and *Penicillum expansum* exposed to open space conditions for 7 months revealed many morphological changes which apparently allowed those fungi to survive. For example, the polysaccharide capsule and melanin layer in *P. expansum* were significantly increased in comparison with control samples, as well the numbers of mitochondria and vacuoles in space-exposed fungi were much higher than in controls.

Possible Mechanism of Ionizing Energy Utilization by Melanized Fungi

Despite the high prevalence of melanotic microorganisms in radioactive environments, it is unlikely that melanin is synthesized solely for the purposes of protection from ionizing radiation. For example, in high elevation regions inhabited by melanotic fungi the background radiation levels are approximately 500–1,000 higher than at sea level, which amounts to a dose of 0.50–1.0 Gy/year. Since the overwhelming majority of fungi, melanized or not, can withstand doses up to 1.7×10^4 Gy (Saleh et al. 1988; Dembitzer et al. 1972; Dadachova et al. 2004, 2008; Mirchink et al. 1972), there is no apparent requirement for melanin pigments for protection against radiation in those environments. On the other hand, biological pigments play a major role in photosynthesis by converting the energy of light into chemical energy. Other pigments such as chlorophylls and carotenoids absorb light of certain wavelengths and help convert photonic energy into chemical energy during photosynthesis. Given that melanins can absorb visible and UV light of all wavelengths (Nicolaus 1968), we hypothesized that exposure to

ionizing radiation would change the electronic properties of melanin and affect the growth of melanized microorganisms.

To test this hypothesis, we subjected fungal melanin to ionizing radiation and observed changes in its electron paramagnetic resonance (EPR) signal, consistent with changes in electronic structure. Irradiated melanin manifested a fourfold increase in its capacity to reduce NADH relative to non-irradiated melanin, suggesting that radiation affected the oxidation-reduction properties of melanin and providing a hint of how radiation could eventually be harnessed for biological energy. HPLC analysis of melanin from fungi grown on different substrates revealed chemical complexity, dependence of melanin composition on the growth substrate, and possible influence of melanin composition on its interaction with ionizing radiation. The interaction with ionizing radiation was studied for three fungal species – *C. neoformans* which can be grown in both melanized and non-melanized forms depending on the presence of exogenous substrate, and two intrinsically melanized species *Wangiella dermatitidis* and *Cladosporium sphaerospermum* with the latter being one of the predominant species inhabiting the destroyed reactor in Chernobyl. XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) and MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) assays showed increased metabolic activity of irradiated melanized *C. neoformans* cells relative to irradiated non-melanized cells, consistent with the observation that exposure to ionizing radiation enhanced the electron-transfer properties of melanin. Melanized *W. dermatitidis* and *C. neoformans* cells exposed to ionizing radiation approximately 500 times higher than background grew significantly faster as indicated by higher CFUs, increased biomass and threefold greater incorporation of ^{14}C-acetate than non-irradiated melanized cells or irradiated albino mutants. In addition, radiation enhanced the growth of melanized *C. sphaerospermum* cells under limited nutrients conditions. The observations that melanized fungal cells manifested increased growth relative to non-melanized cells after exposure to ionizing radiation raised the intriguing possibility that melanin functioned in energy capture, transduction, and utilization (Dadachova et al. 2007).

Examples of Ionizing Energy Utilization by Melanized Fungi

Zhdanova et al. reported that some of the fungi growing in the area around the site of the 1986 Chernobyl nuclear accident had the ability of growing into and decomposing so-called "hot particles" – pieces of graphite from destroyed reactor # 4 which are contaminated with various long-lived radionuclides (Zhdanova et al. 1991, 1994). They termed this attraction of fungi to radiation "radiotropism." In their more recent work they excluded possible confounding effects of carbon on directional growth of fungi by exposing them to the external collimated beams of radiation from ^{32}P and ^{109}Cd radionuclides, which are beta- and gamma-emitters, respectively (Zhdanova et al. 2004). The authors measured the "return angle" which they defined as an angle between the point of impingement of radioactivity in the culture vessel and the direction of growth of the distal portion of the emergent hyphum from each spore. A low return angle ($<90^{o}$) indicates mean hyphal growth towards the source of radioactivity and a high angle (90–180°) growth away from the source. Fungi used in the experiment were either isolated from the contaminated Chernobyl zones, or isolated before the explosion or from the remote sites. Altogether 27 responses of interactions between

fungal isolates and radiation source were investigated. Of these, 18 (66.7%) showed positive stimulation of growth towards the radiation source (low mean return angle), and eight showed no response. Statistically significant directed growth to the ^{109}Cd source of radiation was seen for *Penicillium roseopurpureum* 147 (from contaminated Red Forest soil), *P. hirsutum* 3 (hot particles), *Cladosporium cladosporioides* isolates 60 and 10 (from the fourth Block reactor room). *C. sphaerospermum* 3176, although isolated from control uncontaminated soil, also showed a positive response. A trend towards directional growth, though not statistically significant, was observed for *C. cladosporioides* 396 and *Paecilomyces lilacinus* 101 (both isolated from uncontaminated soils) and for *Penicillium lanosum* (from the fourth Block) and *Paecilomyces lilacinus* 1941 (contaminated Red Forest soil). The authors concluded that both beta and gamma radiation promoted directional growth of fungi from contaminated and clean areas towards the sources of ionizing radiation. This result raised the tantalizing possibility that radiotropism was analogous to the phototropism shown by plants towards light sources with the suggestion that like plants, fungi were growing towards the source of electromagnetic radiation because it was favorable for the organism.

In their later work published in 2006–2007 the same group investigated the influence of external radiation from ^{121}Sn (low energy gamma-emitter) and ^{137}Cs (high-energy mixed beta- and gamma-emitter) not only on hyphal growth of fungi from radioactively contaminated Chernobyl regions versus controls but also on their spore germination (Tugay et al. 2006, 2007). They observed that radiation promoted spore germination in species from contaminated regions, which they called "radiostimulation." Contrary to their previous results (Zhdanova et al. 2004) they observed the "radiostimulation" only for the species from contaminated regions but not for isolates from the clean areas. They named this phenomenon "radioadaptive response." They also observed the same results for responses of fungi from contaminated areas to light (Karpenko et al. 2006).

Melanins and Radiation in Perspective

Melanin pigments are found in all biological kingdoms, suggesting that these compounds are ancient molecules that emerged early, and possibly repeatedly, in the course of evolution. Melanins are complex polymers with a variety of properties that can be made enzymatically from relatively simple precursors. A remarkable aspect of melanins is their ability to absorb all types of electromagnetic radiation which endows them with the capacity for both radioprotection and energy transduction. The findings of melanized organisms in high radiation environments such as the damaged reactor at Chernobyl, the space station, Antarctic mountains, and reactor cooling water combined with phenomenon of "radiotropism" raises the tantalizing possibility that melanins have functions analogous to other energy harvesting pigments such as chlorophylls.

Acknowledgments

E. Dadachova is supported by the National Institute of Allergy and Infectious Disease (NIAID) grant AI60507; A. Casadevall – by NIAID grants AI033142 and AI033774.

Cross-References

- 9.3 Biochemistry
- 10.1 Deinococcus Radiodurans: Revising the Molecular Basis for Radiation Effects on Cells
- 10.2 Ecological Stress: Melanization as a Response in Fungi to Radiation
- 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria

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Part 11

New Frontiers: Microorganisms in Oligotrophic Environments

11.1 Ecology and Cultivation of Marine Oligotrophic Bacteria

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_11.1,

Though generally not perceived as an "extreme" environment, the global open ocean is a desert with respect to the organic and inorganic building blocks required to promote the growth of any potential inhabitants. In illuminated surface waters where light energy available for photosynthesis abounds, thermal stratification of the water column results in the constant starvation of (primarily microscopic) photoautotrophs for inorganic nutrients such as nitrogen and phosphorus. In the dark ocean interior, inorganic nutrients are generally in excess; however, organic carbon compounds are in short supply, and the stocks that are present are generally considered to be inaccessible as substrates to sustain active cell growth and metabolism. In spite of all of this, massive populations of microscopic cells abound in the global open ocean. Assessments based on gene- and genome-based measures have revealed a tremendous amount of diversity within the microbial plankton, but also that a vast majority of this diversity is not currently represented in existing culture collections of marine microorganisms. However, a combination of approaches that permit the interrogation of in situ microbial communities, such as environmental metagenomics, and methodological developments aimed at cultivating microorganisms present in numerically high abundance in the environment, such as high-throughput cultivation, are facilitating an unprecedented understanding of oligotrophic marine microorganisms and the roles they play in global ocean ecology.

The Oligotrophic Ocean as an Extreme Environment

Oligotrophic ecosystems can be loosely defined as systems that exhibit low ambient nutrient levels that are suboptimal for biological production. Additionally, oligotrophic systems are often characterized by a low nutrient flux (Schut et al. 1997). The global ocean subtropical gyres easily meet both criteria: they are far removed from exogenous nutrient inputs at coastal margins and, notwithstanding occasional seasonal or periodic relief, possess persistently low nutrient concentrations. In these systems, thermal stratification keeps the euphotic zone chronically deprived of nutrients that are lost to depth through sedimentation as particulate matter. As such, primary production in the global ocean gyres is often limited by either nitrogen, phosphorus, and/or iron availability. This limitation on the production of organic matter subsequently limits the magnitude of secondary production by the heterotrophic bacteria that dominate these systems (Kirchman 1990).

Nutrients that limit primary production can periodically enter the euphotic zone through regular events such as seasonal convective overturning of the water column (Hansell and Carlson 2001) or seasonal increases in mixed-layer depths (Christian 2005), or by stochastic events such as mesoscale eddy-induced upwelling (McAndrew et al. 2008) or atmospheric deposition (Aumont et al. 2008). These brief pulses of nutrients can rapidly and drastically change the structure of the otherwise perennially nutrient-starved phytoplankton community from oligotrophic picocyanobacteria to eukaryotic phytoplankton able to quickly utilize the elevated nutrients (Benitez-Nelson et al. 2007; Ewart et al. 2008). However, in the oligotrophic open ocean, the picocyanobacterial community quickly re-establishes dominance when the nutrient spike is depleted, presumably due to a variety of adaptations that enable them to dominate under oligotrophic conditions (see Chap. 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria).

A rich history of study has documented the fundamental importance of phytoplankton adapted to the oligotrophic ocean and has resulted in an expansive base of knowledge regarding photoautotrophic microorganisms tuned to persistently low nutrient conditions (e.g., Vaulot et al. 2008; Simon et al. 2009). However, it is the heterotrophic bacteria that dominate the

oligotrophic ocean in terms of biomass (Fuhrman et al. 1989), and, because they are believed to be limited in their growth by carbon (Kirchman 1990), understanding the nature and dynamics of dissolved organic carbon (DOC) in seawater becomes crucial in order to understand the ecology of pelagic marine heterotrophic bacteria and subsequently the functioning of the marine ecosystem as a whole. Phytoplankton are the major source of DOC in the pelagic marine environment, so it is not surprising that DOC concentrations mirror the horizontal and vertical distributions of phytoplankton (*Fig. 11.1.1*). In parallel with nutrient concentrations, subtropical gyres are especially oligotrophic with respect to DOC, particularly at depths below the euphotic zone (*Fig. 11.1.2*). However, it is not simply an issue of quantity; the quality of DOC affects how labile it is for bacterial utilization. Microbial oceanographers have long used the Redfield ratio to model marine biogeochemical cycles (Redfield et al. 1963). The Redfield ratio generalizes the stoichiometric molecular ratio of carbon (C), nitrogen (N), and phosphorus (P) in plankton to 106:16:1, respectively. As such, it should be expected that elements from the DOM pool be depleted in accordance with the Redfield ratio. However, DOM appears to be almost universally depleted in N and P relative to the Redfield ratio (Benner 2002). Sediment trap data also reveal increased C:N and C:P ratios in particles with increased depth (Christian et al. 1997). These data suggest that remineralization of C is sufficiently longer than N and P and that a large fraction of the DOC pool may be refractory to microbial degradation (Benner 2002). The global DOC pool is estimated to be approximately 700 Pg C, which is comparable to the mass of inorganic carbon in the atmosphere. Thus, small perturbations in the metabolism of DOC by heterotrophic bacteria could strongly impact the balance between oceanic and atmospheric carbon dioxide.

Clearly, the ecology of oligotrophic marine bacteria is of very high interest, particularly with respect to the physiological capacity and metabolic rates of the heterotrophic fraction. Unfortunately, current thought is that the vast majority of marine microorganisms have thus far eluded laboratory cultivation, precluding rigorous laboratory-based physiological examination. However, a wide variety of methods are now available that allow the interrogation of populations or communities of microorganisms without the need to first domesticate the microbes in question in the laboratory (i.e., "culture-independent" methods).

Culture-Independent Ecological Methods

Much of what we now know regarding the diversity and identity of marine bacterioplankton has been achieved by the application of molecular biological methods directly to microbial biomass harvested from seawater (Giovannoni and Rappé 2000; Giovannoni and Stingl 2005). Despite being one of the oldest, small subunit ribosomal RNA (SSU rRNA) gene cloning and sequencing remains one of the most commonly used methods for sequence-based diversity surveys of marine bacterioplankton. In a nutshell, microbial cells in seawater are concentrated (most commonly by filtration) and subsequently extracted in order to obtain community genomic DNA. This material is then used to amplify SSU rRNA genes via the polymerase chain reaction (PCR), and the resulting amplicon is cloned and sequenced. In 2000, Giovannoni and Rappé reviewed the diversity of marine bacterioplankton based on SSU rRNA gene sequences, which numbered approximately 600 at that time (Giovannoni and Rappé 2000). Now, the marine bacterioplankton SSU rRNA gene sequence database numbers in the tens of thousands.

Despite the rich information gained by cloning and sequencing, this method can be laborious when processing many samples, and costly if appreciable numbers of clones are

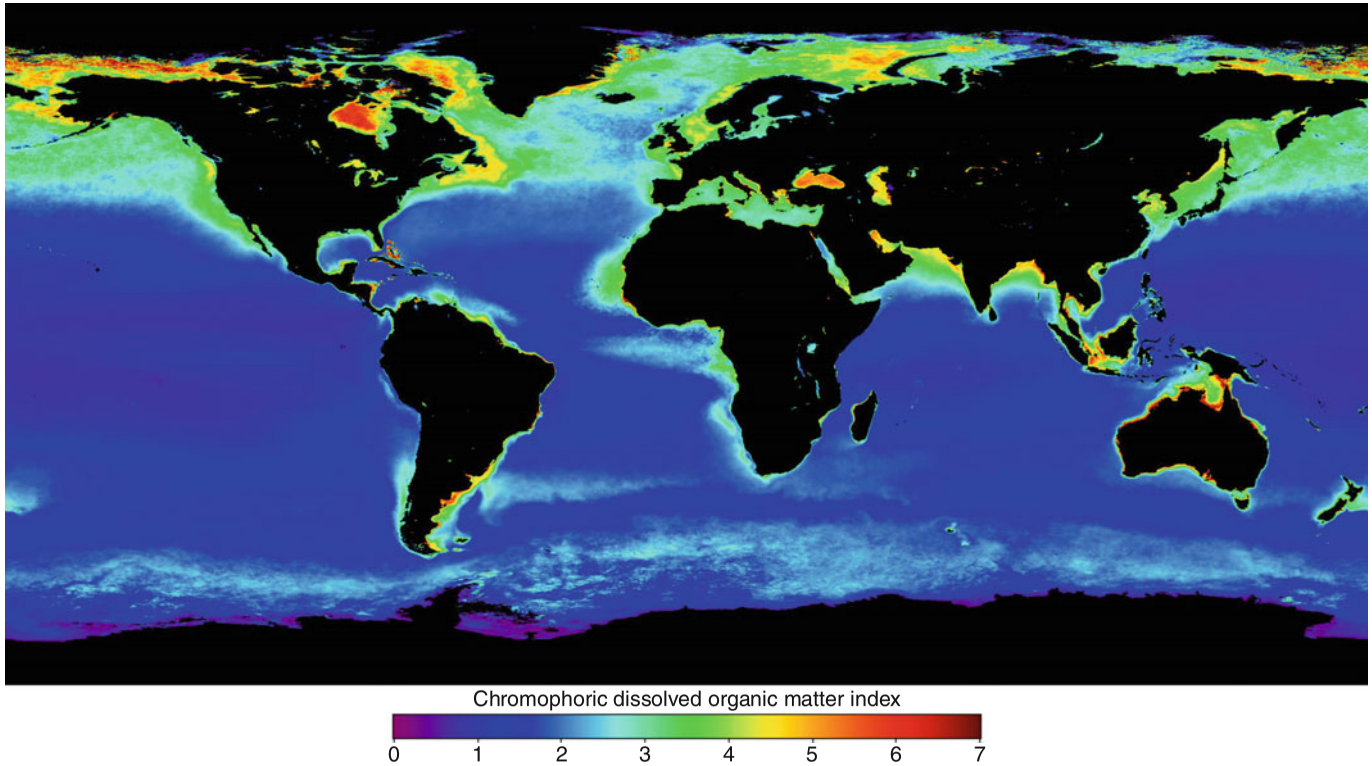

Fig. 11.1.1

Composite image of SeaWiFS Chromophoric Dissolved Organic Matter (CDOM) indices collected from 4 September 1997 to 31 August 2009, downloaded from the NASA Ocean Color website (http://oceancolor.gsfc.nasa.gov/) using the Level 3 data access browser. The CDOM index is the algorithm of Morel and Gentili (2009) applied to actual reflectance ratios derived from ocean color imagery. The index has no units and assesses the relative anomalies in CDOM with respect to its standard chlorophyll-dependant values.

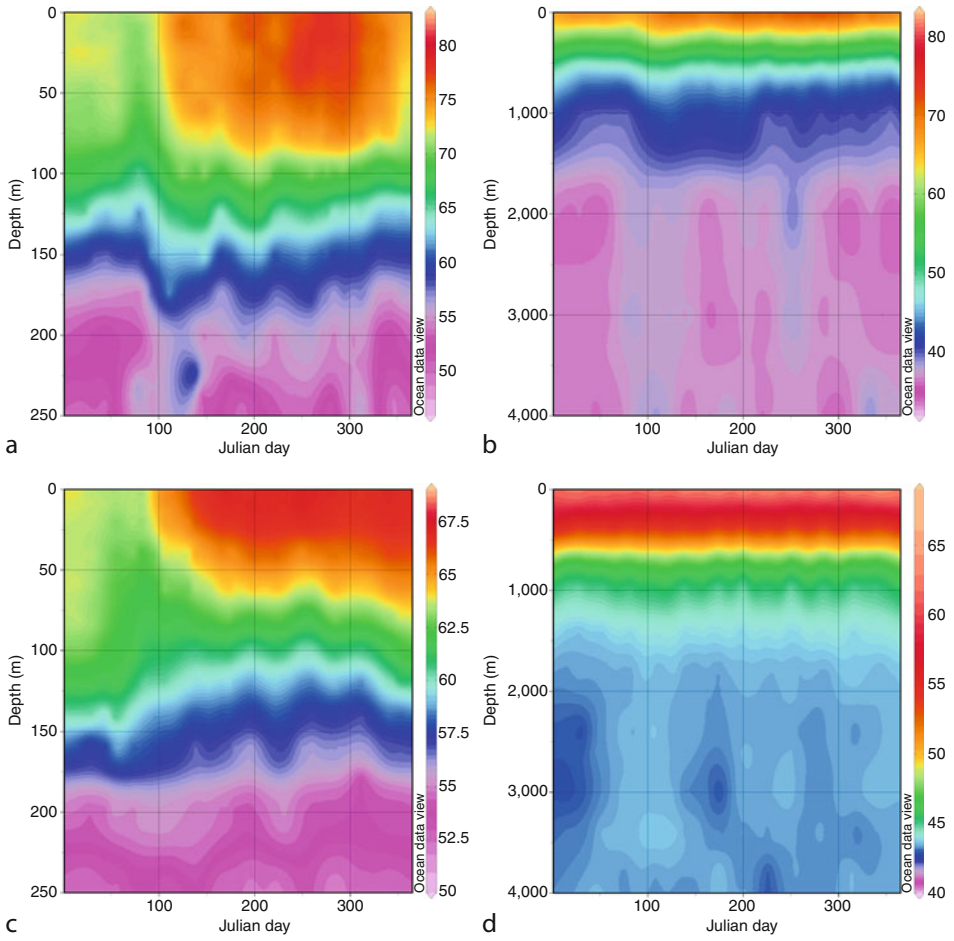

Fig. 11.1.2

Composite images of dissolved organic carbon concentrations from 2004 to 2008 at (a, b) Station ALOHA (A Long-Term Oligotrophic Habitat Assessment; 22° 45′N, 158° 00′W) in the North Pacific subtropical gyre and (c, d) BATS (Bermuda Atlantic Time-series Study; 31° 40′N, 64° 10′W) in the North Atlantic subtropical gyre. Station ALOHA bottle data was extracted from the Hawaii Ocean Time-series Data Organization & Graphical System (HOT-DOGS) website (http://hahana.soest.hawaii.edu/hot/hot-dogs/interface.html) and BATS bottle data was extracted from the Bermuda Atlantic Time-series Study website (http://bats.bios.edu/index.html). Values shown are in micromolar (μM).

sequenced per clone library. In many instances, the goals of the project may allow for more rapid and cost-effective methods to be employed, such as community fingerprinting methods. Collectively, these methods refer to a broad suite of techniques that rely on proxy information of the SSU rRNA gene rather than sequence data (Bent and Forney 2008). One of the most commonly used DNA fingerprinting techniques in the field of microbial oceanography is terminal restriction fragment length polymorphism (T-RFLP), which uses endonuclease

recognition sequences in SSU rRNA genes as the proxy information. Community genomic DNA is first used as a template for the PCR amplification of SSU rRNA genes, with at least one of the primers being end-labeled with a fluorochrome. The labeled amplicons are then restricted using one or more restriction endonucleases prior to separation on a DNA sequencing machine, which detects the migration rates and relative fluorescence of end-labeled fragments. This data can be used to infer the number of operational taxonomic units (OTUs) and their relative abundances in samples. As an example of the utility of this method, T-RFLP has been used extensively by Carlson, Giovannoni, and colleagues in their investigations of bacterioplankton community dynamics in the oligotrophic northwestern Sargasso Sea (Morris et al. 2005; Carlson et al. 2009; Treusch et al. 2009).

Metagenomics is the study of communities of microorganisms by the application of genomic techniques directly to environmental DNA. This approach acquires DNA sequence information from genomes without the need for isolating microorganisms first, and can therefore yield insights into the metabolic potential and genome properties of uncultivated microbes. The *Sourcerer II* Global Ocean Sampling expedition has afforded microbial oceanographers with a staggering amount of metagenome sequence data (Rusch et al. 2007). In one example of how this dataset has found use, a recent interrogation revealed that oligotrophic open ocean bacterioplankton communities exhibited much more microdiversity than any other marine habitat (Biers et al. 2009). Metatranscriptomics, a technique related to metagenomics, can shed light on putative metabolic processes in place at the time of sample acquisition (Poretsky et al. 2009). Metatranscriptomics works similarly to metagenomics, except the starting sample material is RNA rather than DNA. The RNA is first reverse transcribed to complementary DNA (cDNA) before being sequenced as in metagenomics. For example, a metatranscriptomic study of microbial communities inhabiting the North Pacific subtropical gyre revealed an unexpected abundance of small RNAs that may be involved in environmentally relevant processes including carbon metabolism and nutrient acquisition (Shi et al. 2009).

Various methods are also commonly used to investigate individual populations of cells. A popular microscopy-based technique is fluorescence in situ hybridization (FISH), which allows for the direct counting of targeted populations within a community (Amann et al. 1990). FISH differs from other nucleic acid-based techniques in that nucleic acids are not extracted from the harvested cells. Rather, the cells are first fixed in paraformaldehyde, and filtered onto polycarbonate membrane filters. Fluorescently labeled oligonucleotide probes targeting specific SSU rRNA sequences are then added to the fixed sample and allowed to hybridize under stringent conditions to minimize non-specific binding. The sample is then counterstained with the general nucleic acid stain DAPI, and analyzed by epifluorescence microscopy.

Quantitative PCR (qPCR), another commonly used technique to investigate single populations, measures amplified PCR products in real-time (Smith and Osborn 2008). This method uses community genomic DNA as template in a PCR reaction with primers that target specific gene sequences such as the SSU rRNA gene or other gene of interest. The amplified products are fluorescently labeled during the reaction using either one of two general approaches. The first approach employs a PCR primer that is designed to fluoresce only when it is incorporated into an amplified gene product. The second approach incorporates a general double-stranded DNA dye, such as SYBR Green I, into the amplified products during the PCR reaction. In either approach, the rate of gene amplification is measured through the detection of fluorescently labeled products. The number of target genes in the community sample can be quantified by comparing its amplification rate against that of a standard curve produced by qPCRs of known amounts of the targeted gene.

Metagenomics has thus far been unable to piece together entire genomes from complex communities, such as the oligotrophic bacterioplankton. This places a limit on what could be gleaned through metagenomics, since genes work within the context of genomes and not in isolation. Single cell genomic techniques are therefore becoming increasingly popular, particularly for bacterial groups that elude cultivation (Hutchison and Venter 2006; Rodrigue et al. 2009). These techniques usually entail the sorting of individual cells, which are then lysed to free their chromosomes. The chromosomes are denatured and undergo multiple displacement amplification using random primers. The products are then cloned and sequenced, which are assembled to form contigs *in silico*. This technique has been rigorously tested with the oligotrophic marine picocyanobacterium *Prochlorococcus* as a model system and appears to hold much promise for other taxonomic groups as well (Rodrigue et al. 2009).

Another powerful single cell technology is nanoscale secondary ion mass spectrometry (NanoSIMS), which can allow for the simultaneous analysis of microbial identity and function (Li et al. 2008). This method is based on the visualization of oligonucleotide probe-conferred hybridization signal in single microbial cells and isotopic measurement using a high-resolution ion microprobe. This relatively new technology is receiving much attention for its promise in studies of in situ ecophysiology of uncultured bacterioplankton within complex communities, such as the oligotrophic open ocean (Orphan and House 2009). This technique has already been used to demonstrate the fixation and fate of carbon and nitrogen in the oligotrophic marine cyanobacterium *Trichodesmium* (Finzi-Hart et al. 2009) and can be extended to other biogeochemically relevant microbial systems.

Cultivation of Typical Marine Oligotrophs

The cultivation of microorganisms relevant to in situ ecological processes continues to be a major challenge for microbiologists. One recent estimate suggested that, of 100 bacterial phyla, only 30 possess a cultivated representative (Achtman and Wagner 2008). Oligotrophic marine bacterioplankton communities are no exception: in general, they contain a dearth of laboratory strains that represent the numerically abundant groups of bacteria present in seawater. An important consequence of our inability to generate pure cultures of "typical" planktonic marine bacteria is a general lack of the understanding regarding even the most basic aspects of their physiology (Giovannoni and Stingl 2005; Giovannoni and Stingl 2007). Unfortunately, DNA sequence data alone do not provide conclusive information regarding the metabolism of individual microorganisms, and questions regarding the evolution, population genetics, and ecology of abundant microbial taxa are most readily addressed with isolated representatives in hand (*Fig. 11.1.3*). Because the isolation and domestication of microorganisms from marine bacterioplankton communities remains a major struggle, there is significant impetus to develop and apply novel cultivation approaches to this task, such as micromanipulation (Ziegler et al. 1990), encapsulation of cells in gel droplets (Zengler et al. 2002), growth of cells in diffusion chambers (Kaeberlein et al. 2002), dilution to extinction of cells in liquid media (Button et al. 1993; Connon and Giovannoni 2002), novel single cell technology (Liu et al. 2009), flow sorting of active cells (Czechowska et al. 2008), and co-culturing (Hwang and Cho 2009).

For some time, it has been recognized that pure culture isolation strategies based on traditional enrichment techniques tend to select for opportunistic, fast-growing organisms. In nutrient-rich artificial media, the copiotrophic community members often out-compete

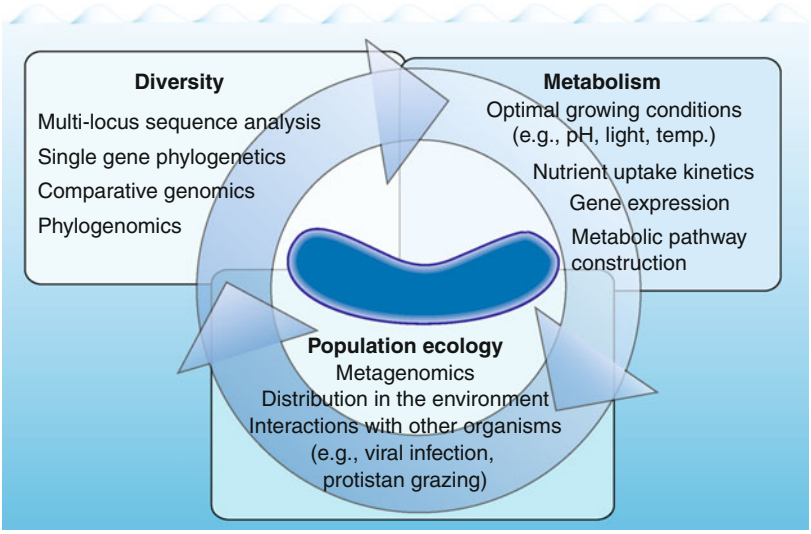

Fig. 11.1.3

Schematic representation of the integration between the major lines of research incorporating model marine oligotrophic isolates. Ecologically relevant investigations can be structured around three interconnected themes: (I) marine microbial diversity, (II) microbial metabolism and the mechanisms of nutrient and energy flow, and (III) microbial population ecology. The resulting knowledge can be incorporated into complementary field research and physical-biogeochemical modeling efforts to formulate a holistic understanding of the marine oligotrophic ecosystem.

and overgrow the more naturally abundant oligotrophic microorganisms. Consequently, these conventional approaches usually result in the cultivation of strains that do not reflect the diversity contained within the in situ microbial community. Isolates from such bacterial genera as *Pseudomonas, Alteromonas, Pseudoalteromonas*, and *Vibrio* frequently dominate the culturable fraction of seawater when using high nutrient media, yet these are generally large, fast-growing microorganisms that are not routinely abundant in situ. As a result of the bias of traditional isolation methods, most of our knowledge on planktonic marine bacteria generated from pure culture studies has historically been restricted to species that form a numerical minority in natural populations.

For the isolation of numerically dominant heterotrophic bacteria from oligotrophic marine environments, one of the more successful strategies developed to date has been dilution to extinction methodology (Button et al. 1993). Essentially, the approach involves diluting natural communities of microorganisms to extinction in liquid media that more closely reflects the nutrient and organic carbon concentrations of the natural environment, and subsequently examining the cultures for microbial growth by flow cytometry. In early experiments, up to 60% of cells from marine waters around Alaska and the Netherlands were reported to be culturable by this method when filtered, autoclaved seawater was used as the medium (Button et al. 1993; Schut et al. 1993). This work resulted in the description of two new oligotrophs, *Sphingomonas alaskensis* and "*Cycloclasticus oligotrophus*." *S. alaskensis* retains a very small cell volume (0.03–0.07 μm^3) regardless of the organic carbon concentration in the cultivation medium, which has proven to be a typical characteristic of oligotrophic bacterioplankton isolates (Eguchi et al. 2001; see Chap. 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria).

More recently, the extinction culture methods of Button and colleagues have been modified to allow for screening a much larger number of potential cultures for growth, as well as to incorporate molecular biology and DNA sequence-based techniques to rapidly identify potential isolates (Connon and Giovannoni 2002; Rappé et al. 2002). This strategy, referred to as high-throughput culturing (HTC), has resulted in a few notable achievements, including the isolation of representatives of the highly abundant marine bacterioplankton clade known generally as SAR11 (*Fig. 11.1.4*) (Rappé et al. 2002). While the moniker "SAR11" originally referred to a single rRNA gene clone sequence obtained from the Sargasso Sea (Giovannoni et al. 1990), it is now used as a label for the entire monophyletic clade of aquatic microorganisms within the *Alphaproteobacteria* related to this initial clone lineage. In aggregate, this group is thought to be one of the most abundant groups of microorganisms on Earth (Morris et al. 2002), making it an obvious target for cultivation-based studies. HTC methodology has since been used to successfully isolate a diverse range of representatives from the SAR11 clade, as well as members of other dominant bacterioplankton groups known to be numerically abundant in marine bacterioplankton communities in situ (e.g., Connon and Giovannoni 2002; Cho and Giovannoni 2004; Stingl et al. 2007; Song et al. 2009).

In general, HTC modifications to extinction culturing have involved reducing the size of cultures (i.e., to the size of microtiter plate wells), implementing rapid screening methods based on epifluorescence microscopy or flow cytometry that can detect very dilute cell concentrations, using pristine sterile seawater as a basal medium and limiting media amendments so that the cultivation conditions reflect in situ conditions, and adopting clean procedures throughout the protocol (e.g., acid washing, avoiding detergents, and using Teflon when possible). Briefly, raw seawater is collected and a portion is reserved for the inoculum. The remaining raw seawater is sterilized via a combination of filtration, microwave, and/or autoclave, and is subsequently amended with an appropriate nutrient enrichment for use as media. The abundance of microbial cells in the sample reserved for the inoculum is determined so that it can be serially diluted with sterile amended media to the final desired cell concentration (typically between 1 and 10 cells per well) and aliquoted into the wells of an appropriate vessel (such as a microtiter plate). All media preparation and manipulation of cells is usually performed in a sterile environment such as a laminar flow hood in order to limit contamination. Microtiter plates containing media but no cells are also generally included as controls. All of the microtiter plates are subsequently sealed in some fashion in order to limit evaporation (e.g., with parafilm, or sealed in a humid chamber), and incubated under light and temperature conditions that mimic the environment from where the sample originated. Incubation times are a user-controlled variable that are best determined by trial and error. However, it is noted that the longer the cultures can be incubated, the better the chance one has to isolate very slow growing oligotrophic strains.

After incubation, wells are screened for growth. A small volume from each well is removed and cell counts are determined by either microscopy or flow cytometry. For high-throughput screening, custom 48 well manifolds have been designed to be used with epifluorescence microscopy in order to facilitate the screening of large numbers of cultures (Connon and Giovannoni 2002), though flow cytometry is the more rapid and facile choice (e.g., Tripp et al. 2008). Screening for growth can be performed using general nucleic acid stains such as DAPI or SYBR Green, though DAPI has the added benefit in that it can be combined with fluorescently labeled oligonucleotide probes in an in situ assay targeting specific phylogenetic groups of bacteria (e.g., Rappé et al. 2002). However, the most common means to identify isolates is to extract genomic DNA from a small volume of culture and characterize the SSU rRNA gene

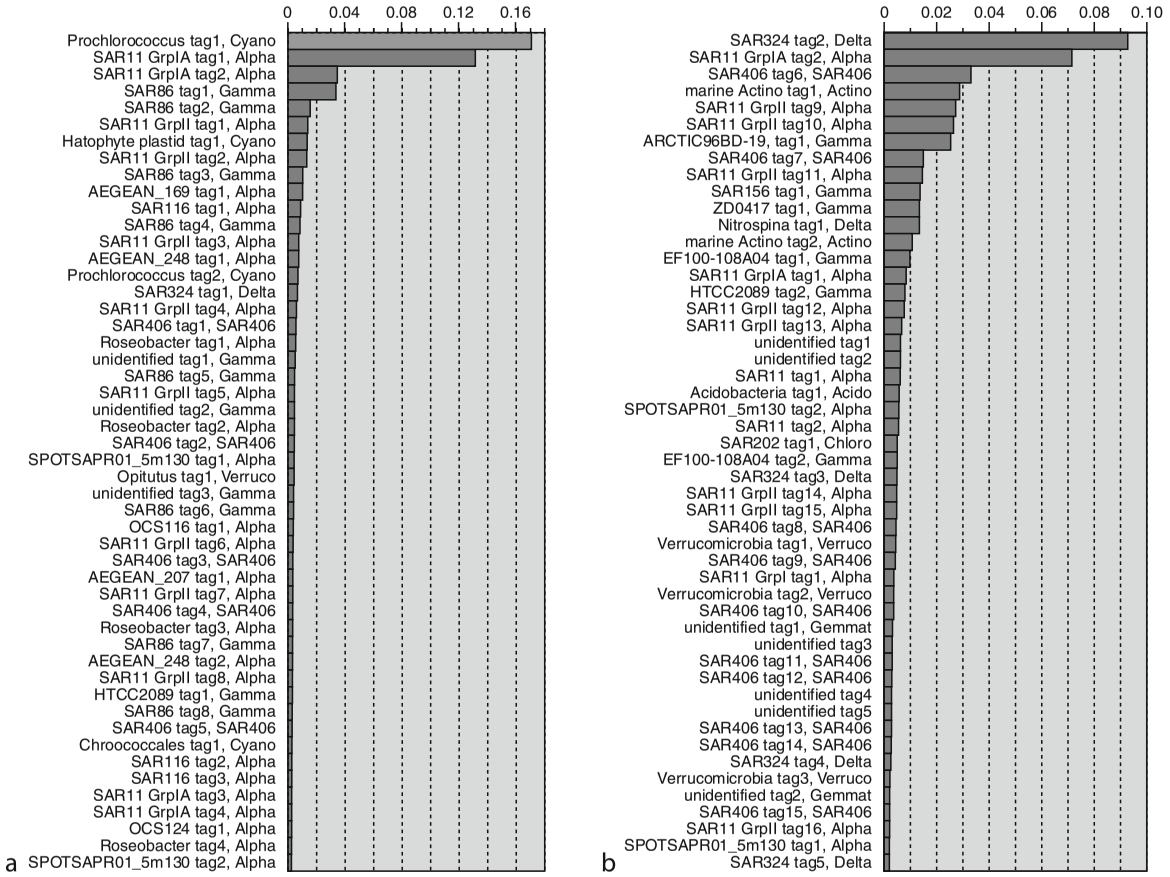

Fig. 11.1.4 (Continued)

sequence. Once identified, the remaining culture volume may be split between cryopreservation and sub-culturing into fresh sterile media in order to obtain sufficient biomass for further characterization and analyses.

We are confident that continued effort, patience, and creative modifications would undoubtedly result in the successful isolation and cultivation of important bacterial strains representative of the major clades of marine bacterioplankton in the global ocean.

Major Groups of Putatively Oligotrophic Marine Bacteria

Since their incorporation into modern microbial ecology research, new and emerging molecular biology-based methodologies have frequently focused on marine bacterioplankton, resulting in rich nucleic acid sequence datasets with which to assess the diversity and structure of marine bacterioplankton communities. Though frequently maligned for its shortcomings, the SSU rRNA gene remains a useful phylogenetic marker for distinguishing groups of bacteria, albeit probably not consistently at the species or subspecies level, and has been in wide use since the first application of SSU rRNA gene cloning and sequencing to marine bacterioplankton by Giovannoni and colleagues (Giovannoni et al. 1990). Early studies were fortunate to feature the sequencing and phylogenetic characterization of a few dozen clones (e.g., Giovannoni et al. 1990; Fuhrman et al. 1993). However, we have entered an era where new technologies now permit the sequencing of tens to hundreds of thousands of SSU rRNA gene sequence "tags" from a single sample, providing unprecedented depth of interrogation into bacterioplankton community structure, and a window into the rare (or at least periodically less abundant) biosphere (Sogin et al. 2006).

An exhaustive summary of all of the major groups of putatively oligotrophic marine bacteria revealed using modern molecular biology techniques is complicated by the fact that most of the abundant marine bacterial phylogenetic "types" in seawater have no (or, at most, very few) representative cultivated strains available for laboratory-based experimentation and interrogation (Giovannoni and Rappé 2000; Giovannoni and Stingl 2005; Giovannoni and Stingl 2007). Thus, most of the bacterial groups recovered in genomic- or gene-based cultivation-independent surveys of marine bacterioplankton communities are *hypothesized* to be oligotrophs based on their occurrence and abundance in oligotrophic ocean provinces. In fact, it is not actually known whether many of these groups are even heterotrophs: while it is

Fig. 11.1.4

Rank order histograms of SSU rRNA V6 tag sequences from (a) surface layer (5 m), and (b) oxygen minimum zone (770 m) bacterioplankton communities from Station ALOHA in the North Pacific subtropical gyre, sampled in April 2006. The relative abundance of the 50 most abundant tag sequences are shown, from a total of 27,033 (5 m) and 11,142 (770 m) tags. The identity of individual tags was determined by matching tag sequences with full-length SSU rRNA gene sequences within the ARB sequence analysis package and a sequence alignment based on the SSUref version 91 database, using the "Match Probes" function within ARB. Phylum and subphylum designations: Cyano – *Cyanobacteria*; Alpha – *Alphaproteobacteria*; Gamma – *Gammaproteobacteria*; Delta – *Deltaproteobacteria*; SAR406 – SAR406/Marine Group A candidate phylum; Verruco – *Verrucomicrobia*; Actino – *Actinobacteria*; Acido – *Acidobacteria*; Chloro – *Chloroflexi*; Gemmat – *Gemmatimonadetes*.

generally accepted that that must be the case for most, it is entirely possible that new modes of autotrophy will be revealed among the myriad of bacterioplankton phylogenetic "types" in the sea. This is a particularly intriguing possibility for planktonic microorganisms residing below the photic zone of the global ocean, where many of the major groups of bacteria are exotic (from a phylogenetic prospective) and undoubtedly possess some exciting physiological and genetic surprises awaiting discovery. An example of this is provided by a recent study from Walsh and colleagues, who employed metagenomics to determine that a bacterial group common in oxygen minimum zones of the global ocean appears to be a versatile chemolithoauthtroph (Walsh et al. 2009). Rather than attempt an up-to-date, comprehensive overview of marine bacterioplankton diversity, we will instead use two specific examples from the ultra-oligotrophic North Pacific Subtropical Gyre (NPSG) in order to make some general observations regarding marine bacterioplankton community structure and groups of common microbes, and subsequently highlight some of what is known regarding one important group of marine oligotrophic bacteria.

In *Fig. 11.1.4*, the structure of planktonic bacterial communities inhabiting the surface layer (5 m) and oxygen minimum zone (770 m) of the NPSG – a highly oligotrophic system – are shown. These are the result of a 454-based deep pyrosequencing approach applied to hypervariable region V6 of the SSU rRNA gene (Sogin et al. 2006), and are fairly typical examples of the bacterioplankton communities from these two depth horizons at this locale. In fact, only the identity and relative abundance of the 50 most abundant tag sequences are shown in *Fig. 11.1.4* because 3,734 different tags were recovered from 5 m (out of 27,033 total tags), and 2,684 different tags were recovered from 770 m (of 11,142 total). However, these 50 tags account for 61% of the total tags recovered from 5 m, and 55% of the total tags recovered from the oxygen minimum zone. A consistent feature of rank abundance curves from marine bacterioplankton communities from the oligotrophic NPSG, such as the two shown in *Fig. 11.1.4*, is that very few individual tag sequences are greater than 1% in abundance (11 tags from 5 m and 14 from 770 m), indicating a high amount of genetic diversity within the SSU rRNA gene tag pool. This observation is consistent with other genetic data such as metagenomic surveys of marine bacterioplankton from various sites around the global ocean, which have also revealed high sequence diversity within marine bacterioplankton metagenomes (e.g., Venter et al. 2004; Rusch et al. 2007).

In spite of the high frequency of low-abundance tags, the vast majority actually belong to a small number of well known, previously described lineages of marine bacterioplankton. In the surface ocean, these include the oxygenic photoautotrophic cyanobacterium *Prochlorococcus*; the SAR11, SAR116, and marine *Roseobacter* lineages of *Alphaproteobacteria*; the SAR86 lineage of *Gammaproteobacteria*; and the SAR406 lineage of the candidate bacterial phylum Marine Group A (*Fig. 11.1.4a*). In the oxygen minimum zone, these include SAR11 and SAR406, as well as the SAR324 lineage of *Deltaproteobacteria*, the ARCTIC96BD-19 lineage of *Gammaproteobacteria*, and the marine *Actinobacteria* lineage of the bacterial phylum *Actinobacteria* (*Fig. 11.1.4b*). Within the top 50 most abundant V6 tags of this particular NPSG surface ocean example, 18 different SAR11 tags were recovered which accounted for nearly 23% of the total tags recovered from this community, and 8 different SAR86 V6 tags accounted for 8% of the total. At the oxygen minimum zone, these include 19 SAR11 tags accounting for nearly 11% of the total, 10 SAR406 tags accounting for 7% of the total tags, and 5 SAR324 tags accounting for a little over 10% of the total, from within the 50 most abundant V6 tags of this oxygen minimum zone example. Collectively, we interpret this and other data to indicate that the oceans are numerically dominated by a relatively small number of bacterial

groups, but that these groups harbor a high amount of genetic diversity (e.g., see Wilhelm et al. 2007). It has yet to be determined how much of this diversity contributes to phenotypic differences within members of the same group, and how much is neutral.

Previously, we mentioned that members of the SAR11 clade of *Alphaproteobacteria* were ubiquitous in the global ocean, and possibly one of the most abundant heterotrophs on Earth (Morris et al. 2002). Ribosomal RNA gene sequence analyses have shown that this group consists of four major basal subclades: subclade I is predominantly restricted to the surface layer of the oceans, subclade II is found deeper in the water column, subclade III is restricted to estuarine/coastal waters, and subclade IV is found in fresh water (Suzuki et al. 2001; Morris et al. 2005). Based solely on SSU rRNA and ITS gene sequence comparisons, these basal subclades have been further divided into slightly finer phylogenetic groups that possess some shared characteristics, indicating some ecotypic cohesiveness on even finer scales of the SAR11 SSU rRNA gene phylogeny. For example, SAR11 subclade I consists of two sister clades (known as subclades IA and IB) that exhibit different seasonal and depth-specific distributions in the Sargasso Sea (Morris et al. 2005; Carlson et al. 2009), while SAR11 subclade II, known as the subsurface or "deep" subclade of SAR11, contains a well defined subclade that is found solely in the ocean surface layer. The first cultivated members of the SAR11 clade isolated from the coast of Oregon in the Pacific Ocean belonged to SAR11 subclade IA, and all 11 isolates contained identical SSU rRNA gene sequences (Rappé et al. 2002). Stingl and colleagues subsequently reported the isolation of five strains of SAR11 from surface waters of the Sargasso Sea, and an additional 12 strains from the Oregon coast (Stingl et al. 2007). While all of the newer strains were also restricted to SAR11 subclade IA, they did exhibit some SSU rRNA gene sequence heterogeneity.

Based on abundance and distribution alone, it is logical to hypothesize that members of the SAR11 clade play an important role in global ocean biogeochemical cycling. Support for this perspective comes from a variety of publications indicating the contribution of SAR11 cells to secondary production and the assimilation of dissolved organic matter (e.g., Malmstrom et al. 2004; Malmstrom et al. 2005; Alonso and Pernthaler 2006; Vila-Costa et al. 2007). While creative studies are combining physiological experimentation with various genetic and molecular biology tools in order to elucidate important details regarding the physiological characteristics of this group (e.g., Tripp et al. 2008; Schwalbach et al. 2009; Tripp et al. 2009), it is important to emphasize that our current window into interrogating cultivated isolates of the SAR11 clade is limited to one subgroup, and that major lineages within this important group remain uncultured.

Insights Gleaned Through Cultivated Marine Oligotrophs

Understanding the limitations on the growth and metabolism of oligotrophic marine microorganisms is central to our knowledge of ocean ecosystem functioning. In oligotrophic ocean environments, the rate of bacterial growth is set by one of two processes: the ability to transport the nutrient of greatest need into the cell, and the rate of use of previously stored reserves (Button 1985). Button's early research on the nutrient uptake kinetics of various marine isolates and the development of improved culture conditions enabled more control of the growth rate of cultures, facilitating more accurate models of growth kinetics (e.g., Button and Garver 1966; Button 1991). These advances ultimately led to the realization that, in general, small oligotrophic marine bacteria possess large transporter/limiting-enzyme ratios for

operation at very low concentrations of limiting nutrients (Button et al. 1993; see Chap. 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria). Consistent with the properties predicted for the bulk of marine oligotrophic heterotrophs, an oligotrophic marine isolate was eventually obtained that fitted these predictions: it possessed few rRNA operons, few cytoplasmic proteins, a small genome size, a small cell size, and was able to grow without any intentionally added substrate (Button et al. 1998).

As we mentioned previously, in recent years modified extinction culture methodology has facilitated the isolation of pure cultures representing several of the dominant bacterioplankton groups of the oligotrophic ocean (e.g., Connon and Giovannoni 2002; Rappé et al. 2002; Cho and Giovannoni 2004; Stingl et al. 2007), and these isolates are proving highly valuable in efforts to characterize typical bacterioplankton. For example, strains of SAR11 were the first cultured isolates shown to possess a gene encoding for proteorhodopsin, and that the expressed functioning protein occupied approximately 20% of the inner membrane surface area of these cells (Giovannoni et al. 2005a). Other examples include elucidating some of the metabolic capabilities and genome properties of the obligate methylotrophic bacterioplankton known as OM43 (Giovannoni et al. 2008), and the requirement of cultivated SAR11 strains for reduced sulfur compounds (Tripp et al. 2008).

A hypothesis that is gaining ground from isolates obtained via HTC methodology is that of "genome streamlining," whereby selection acts to reduce genome size because of the metabolic burden of replicating DNA with no adaptive value (Giovannoni et al. 2005b). Several prominent members of the bacterioplankton community appear to possess streamlined genomes, including the SAR11 clade (Giovannoni et al. 2005b), the picocyanobacteria *Prochlorococcus* (Rocap et al. 2003), and the betaproteobacterial lineage OM43 (Giovannoni et al. 2008), and this hypothesis continues to grow in strength as additional whole genome sequences from oligotrophic marine bacteria become available. Under this hypothesis, it is presumed that repetitive DNA arises when mechanisms that add DNA to genomes – for example, recombination and the propagation of self-replicating DNA (e.g., introns, inteins, and transposons) – overwhelm the simple economics of metabolic costs. However, evolutionary theory predicts that the probability that selection will act to eliminate DNA merely because of the metabolic cost of its synthesis will be greatest in very large populations of cells that do not experience drastic periodic declines (Giovannoni et al. 2005b). This theory is most efficient in microbial populations with large population sizes, and therefore the elimination of unnecessary DNA from genomes will be most pronounced in organisms such as bacterioplankton that have such large populations. An alternative hypothesis is that the marine habitat is more uniform than other habitats and so organisms can reduce their versatility. However, this is contradicted by the fact that many marine bacterioplankton retain fairly average genome sizes, such as the *Alphaproteobacteria* species *Silicibacter pomeroyi* (Moran et al. 2004) and the *Gammaproteobacteria* species *Congregibacter litoralis* strain KT71 (Fuchs et al. 2007). Thus, oligotrophic marine bacteria with small genomes such as SAR11 may be examples of an evolutionary strategy where metabolic versatility is abandoned in favor of specialization on carbon compounds that are present at low concentrations in the ambient nutrient field (Giovannoni et al. 2005b; Giovannoni et al. 2008).

Within SAR11, natural selection appears to have conserved many core features of genomes across broad oceanic scales, with significant variation associated with just four hypervariable genome regions (Wilhelm et al. 2007). Current data suggests that there are relatively few, perhaps less than a dozen, SAR11 ecotypes that are important to the ecology of the oceans, despite the accumulation of extensive neutral sequence variation in large populations of this ancient group of microorganisms (Wilhelm et al. 2007).

Concluding Remarks

By virtue of their sheer numbers and rates of metabolism, oligotrophic marine microorganisms are key components of Earth's major biogeochemical cycles, and must be explicitly considered when examining the effect of changing environmental conditions on the biology of our planet. In the near future, we envision studies that characterize the environmentally relevant physiology and growth characteristics of strains of numerically abundant oligotrophic bacterioplankton, their genomes, and regulatory mechanisms, and environmental studies such as metagenomics, metatranscriptomics, and metaproteomics will be performed in concert in order to achieve an unparalleled understanding of the ecology and evolution of members of the global oligotrophic marine bacterioplankton community.

Cross-References

11.2 Physiology of Marine Oligotrophic Ultramicrobacteria

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11.2 Physiology of Marine Oligotrophic Ultramicrobacteria

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_11.2,

Introduction

All marine heterotrophic bacteria have to contend with the same challenge in the open ocean: extracting sufficient substrates for growth from a bulk nutrient-depleted environment. In response to this challenge, oligotrophs and copiotrophs have evolved distinct trophic strategies. Oligotrophs can subsist on low concentrations of substrates, and have a physiology attuned to achieving a relatively constant rate of growth under nutrient limiting conditions of carbon (organic), nitrogen, or phosphorus. The concentration of dissolved organic carbon (DOC) in the ocean is typically below 1 mgL^{-1} of which only a small fraction is metabolically available (Carlson and Ducklow 1996). One consequence of global warming is increased stratification of the water column, which decreases the transport of nutrients from the deep water to the surface, thereby decreasing bioavailable DOC for bacteria (Sarmiento et al. 2004). As a consequence, extreme-oligotrophic provinces have been expanding, and are likely to continue to do so (Irwin and Oliver 2009).

In contrast to oligotrophs, copiotrophs are dependent on nutrient-enriched conditions, and exhibit a "feast-and-famine" response that entails rapid growth when nutrient upshifts are encountered, but respond to nutrient depletion (including starvation) by undergoing reductive cell division to form resting-stage cells (Srinivasan and Kjelleberg 1998; Cavicchioli et al. 2003). Thus, in an environment (the open sea) that has been described as a "marine desert" (Schut et al. 1997a), oligotrophs can subsist on dilute organic matter present in bulk seawater, whereas copiotrophs are restricted to the exploitation of nutrient-enriched oases that can be limited in both size and duration.

The majority of oligotrophic bacteria are planktonic and have small cell volumes ($\leq 0.1\ \mu m^3$), and have been termed "ultramicrobacteria" (Schut et al. 1997a, b; Cavicchioli and Ostrowski 2003). Unlike copiotrophs that can become small by undergoing reductive cell division, oligotrophic ultramicrobacteria retain a small cell volume when they are actively growing. Using cell volume as a criterion for cell size is particularly applicable to studies of natural bacterial populations because it provides a measurement that is independent of cellular dimensions and morphology (Schut et al. 1997a; Cavicchioli and Ostrowski 2003). Ultramicrobacteria are a major source of biomass and metabolic activity in oceanic ecosystems, and express higher metabolic activity per unit of volume of seawater than larger bacterial cells (Schut et al. 1997a). The minimal cell size affords several advantages to these bacteria: resistance to grazing by heterotrophic protozoa (González et al. 1990; Boenigk et al. 2004), improved growth under nutrient-limited conditions via higher surface-to-volume ratios (Button 1991), and the ability to generate a higher number of cells from a defined substrate pool (Fegatella et al. 1998).

The bacterioplanktonic species *Sphingopyxis alaskensis* and "*Candidatus* Pelagibacter ubique" have been used as exemplars for oligotrophic ultramicrobacterial physiology. Both belong to the Alphaproteobacteria. *S. alaskensis* is a member of the Sphingomonadales, a lineage known for its physiological and ecological versatility (White et al. 1996; Cavicchioli et al. 1999), and "*Cand.* P. ubique" belongs to the SAR11 clade, which are ubiquitous in marine ecosystems and dominate bacterioplankton communities at the ocean surface (Rappé et al. 2002; Giovannoni et al. 2005a). SAR11 bacteria are adapted to survive and proliferate in extremely low-nutrient environments, including the Sargasso Sea, where bacterioplankton is dominated by SAR11 and cyanobacteria (Giovannoni et al. 1990). The heterotrophic metabolism of SAR11 bacteria is augmented by the light-dependent proton pump proteorhodopsin, which can generate metabolic energy in addition to respiration (Giovannoni et al. 2005a, b).

"*Cand.* P. ubique" has one of the smallest genomes (1.31 Mbp for strain HTCC1062) for any organism that is capable of independent replication outside of a host (*Table 11.2.1*). This

intense genomic streamlining has led to the loss of numerous metabolic pathways and regulatory networks, leading to an inability to exploit nutrient upshifts, and a greater dependence on exogenous substrates (Giovannoni et al. 2005a; Tripp et al. 2009). The small size of the genome has the advantage of lowering the cell's dependence on nitrogen and phosphorus, and relieving the metabolic burden of DNA replication (Giovannoni et al. 2005a). "*Cand.* P. ubique" is nonmotile, and may be considered a "passive" oligotroph, whereas *S. alaskensis* is motile, with a genomic capacity for chemotaxis (Giovannoni et al. 2005a; Williams et al. 2009). The genome of *S. alaskensis* (3.35 Mbp for strain RB2256) is much larger than that of "*Cand.* P. ubique," and the loss of metabolic capacity is modest by comparison (*Table 11.2.1*). Nevertheless, the genome of *S. alaskensis* lacks certain nonessential functions, which has led to an inflexible and highly canalized metabolism compared to copiotrophs, while retaining regulatory capacity to respond adequately to nutrient shifts (Williams et al. 2009). Although genomic streamlining is reflected in the conservative metabolic strategies of both "*Cand.* P. ubique" and *S. alaskensis*, the elimination of metabolic pathways is far more apparent in "*Cand.* P. ubique" than in *S. alaskensis*.

A recent comparison of the genomes of oligotrophs (including *S. alaskensis* and "*Cand.* P. ubique") and copiotrophs emphasized the diversity of molecular mechanisms associated with the two trophic strategies (Lauro et al. 2009; *Fig. 11.2.1*). For example, compared to copiotrophs, oligotrophs tend to have fewer genes involved in motility, signal transduction, ATP-dependent binding cassette (ABC) transport, and extracellular enzymes (Lauro et al. 2009). Oligotrophs were also shown to have smaller genomes and a lower copy number of 16S rRNA genes compared with copiotrophs (Lauro et al. 2009). Similar findings were reported for free-living bacteria in ocean surface waters where the average number of genes per genome was ~1,000 genes and the number of 16S rRNA genes per genome was 1.8 copies (Biers et al. 2009). However, the distribution of such traits across marine bacterial genomes indicates that rather than representing a dichotomy, the strategies employed by model oligotrophs and copiotrophs are situated at either end of a broad ecophysiological spectrum (Lauro et al. 2009; *Fig. 11.2.1*).

This chapter discusses the physiological strategies that characterize the oligotrophic ultramicrobacteria *S. alaskensis* and "*Cand.* P. ubique" by particularly making reference to differences between them and marine copiotrophic bacteria. Throughout the chapter, reference will be made to three copiotrophic marine bacteria that were also subjected to the genomic analysis of Lauro et al. (2009) (*Fig. 11.2.1*, *Table 11.2.1*). *Photobacterium angustum* (formerly *Vibrio angustum*) S14 is defined by physiological and genomic analysis as an "extreme copiotroph" (Humphrey et al. 1983; Kjelleberg et al. 1993a, b). *Pseudoalteromonas haloplanktis* TAC125, known for its ability to exploit ephemeral nutrient patches (Médigue et al. 2005; Stocker et al. 2008), is defined by genomic analysis as a "moderate copiotroph." The genome of *Silicibacter pomeroyi* DSS-3, a species associated with phytoplankton blooms (González et al. 1999; Moran et al. 2004), is also consistent with being a "moderate copiotroph," exhibiting traits intermediate between bacteria representing the trophic extremes.

Definitions

Marine Oligotroph: A heterotrophic microorganism capable of proliferating to high cell numbers in marine environments that have low concentrations (nanomolar levels) of carbon substrates.
Ultramicrobacterium: A free-living cell that has a relatively constant cell volume of not more than 0.1 μm^3.

Table 11.2.1

Comparison of genomic characteristics for five marine bacterial strains, highlighting disparate metabolic strategies

Features	*"Cand.* Pelagibacter ubique" HTCC1062	*Sphingopyxis alaskensis* RB2256	*Silicibacter pomeroyi* DSS-3	*Pseudoalteromonas haloplanktis* TAC125	*Photobacterium angustum* S14
Trophic strategy	Oligotroph	Oligotroph	Moderate copiotroph	Moderate copiotroph	Extreme copiotroph
Genome size (Mbp)	1.31	3.35	4.60	3.85	5.10
GC content (%)	29.7	65.5	64.1	39.8	39.6
rRNA operon copy	1	1	3	9	12
Glycolysis genes	Entner–Doudoroff	Entner–Doudoroff	Entner–Doudoroff	Embden–Meyerhof	Embden–Meyerhof
Complete TCA cycle (oxidative) genes	Present	Present	Present	Present	Present
Glyoxylate bypass	Present	Present	Present	Present	Present
PHA synthesis genes	Absent	Present	Present	Absent	Present
ABC transport systems (sets of genes for complete system)	12	7	58	5	33
Incl. amino acids	3	1	16	–	5
Polyamines	1	1	6	–	2
Sugars	2	2	8	–	2
Peptides	–	–	10	1	5
Phosphate	1	1	1	(Incomplete)	1
Phosphonate	–	–	1	–	1

TRAP transporters (sets of genes for complete system)	2	–	26	–	1
PTS transport genes	Absent	Absent	Present	Absent	Present
Chitinase genes	Absent	Absent	Absent	Absent	Present
Transcription regulator families	LysR, Fur	AraC, LysR, LacI, GntR, TetR, Lrp, MarR, MerR, Fur	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp, LuxR, MarR, MerR, Fur	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp, LuxR, MarR	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp, LuxR, MarR, MerR
RpoS	Absent	Absent	Absent	Present	Present
RpoN	Absent	Present	Present	Present	Present
Nitrate regulation genes	NtrX/NtrY only	Present	Present	Present	Present
Nitrate assimilation genes	Absent	Present	Present	Absent	Present
Assimilatory sulfate reduction genes	Absent	Present	Present	Present	Present
PhoR/PhoB	Present	Present	Present	Present	Present
Phosphonate metabolism genes	Absent	Absent	Present	Absent	Present
Motility genes	Absent	Present	Present	Present	Present
Chemotaxis genes	Absent	Present	Absent	Present	Present
MCP genes	–	3	–	14	17
Photolyase genes	3	1	1	2	3

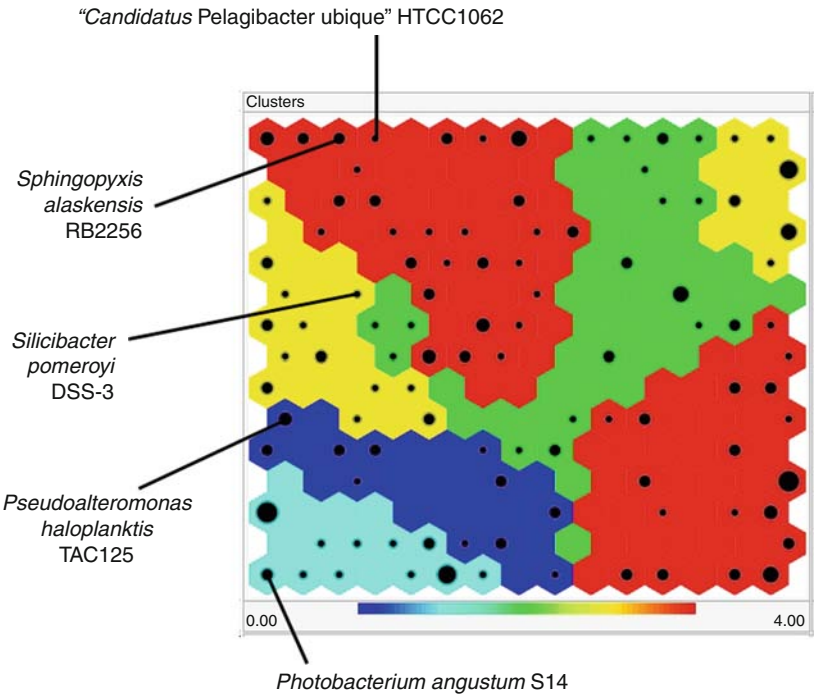

Fig. 11.2.1

Self-organizing map (SOM) of trophic strategies was generated based on the method in Lauro et al. (2009). Color coding is as follows. *Red*: Extreme oligotrophs, such as "*Candidatus* Pelagibacter ubique" HTCC1062 and *Sphingopyxis alaskensis* RB2256. Also recovered in this category were *Prochlorococcus marinus*, *Synechococcus* spp., *Caulobacter crescentus*, and green sulfur bacteria (Chlorobiaceae). *Green*: Moderate oligotrophs; this category included *Planctomyces* and the cyanobacteria *Nostoc* and *Anabaena*. *Yellow*: Moderate copiotrophs, but closer to the oligotrophic range of the trophic continuum; these included *Silicibacter pomeroyi* DSS-3. Also recovered in this category were *Ralstonia eutropha*, *Pseudomonas fluorescens*, and several Flavobacteria. *Blue*: Moderate copiotrophs, such as *Pseudoalteromonas haloplanktis* TAC125. *Cyan*: Extreme copiotrophs, such as *Photobacterium angustum* S14, and *Vibrio* spp. The *Roseobacter* clade (family Rhodobacteraceae) exhibited especially high trophic diversity, and spanned three trophic categories (*Red*, *Green*, *Yellow*)

Isolation of Oligotrophic Ultramicrobacteria

Epifluorescent and flow cytometry counts suggest that only between 0.01% and 0.1% of all microbial cells from marine environments are able to form colonies on standard agar plates (reviewed in Schut et al. 1997a). However, techniques have been developed to cultivate previously uncultured marine microorganisms using growth conditions that closely mimic those of natural environments. *S. alaskensis* strain RB2256 (Schut et al. 1993) and strain AF01 (Eguchi et al. 2001) were isolated using a dilution-to-extinction method, enabling the numerically abundant members of the population to be isolated and grown on plates. The ability to

grow the *S. alaskensis* strains on plates, and to high cell density in a range of media in batch and chemostat cultures has enabled a broad range of physiological studies to be performed (e.g., Schut et al. 1995, 1997a, b; Eguchi et al. 1996, 2001; Fegatella et al. 1998; Fegatella and Cavicchioli 2000; Ostrowski et al. 2001, 2004; Cavicchioli et al. 2003; Williams et al. 2009; Ting et al. 2009; Matallana-Surget et al. 2009a, b). SAR11 bacteria, including "*Cand.* P. ubique" strain HTCC1062, have also been cultivated using dilution-to-extinction methods, followed by growth in unamended seawater, and high-throughput screening to detect growth by direct counting (Rappé et al. 2002; Giovannoni and Stingl 2007). SAR11 bacteria remain unculturable on enriched media, and may be considered to be "obligate oligotrophs" in comparison to *S. alaskensis* strains, which may be considered "facultative oligotrophs." Seawater medium amended with reduced sulfur compounds (methionine, 3-dimethylsulfoniopropionate [DMSP]) boosted "*Cand.* P. ubique" growth rate and cell density in laboratory cultures (Tripp et al. 2008). However, maximum cell densities were achieved on the order of 10^7 cells mL^{-1}, compared to 10^6 cells mL^{-1} for native populations (Morris et al. 2002; Tripp et al. 2009). The low-density of cultures restricts the biomass yield and hence the types of studies that can be performed in the laboratory (Joint 2008). Nevertheless, finding avenues to culture members of the important SAR 11 clade has been a major breakthrough in the field.

Access to Substrates

Although the open ocean is a bulk low-nutrient environment, this oligotrophic expanse is punctuated by nutrient patches that can contain organic compounds at concentrations two to three orders of magnitude higher than the surrounding seawater. These patches are expressed as nutrient pulses, such as exudates or lysates from phytoplankton blooms, or as nutrient plumes, in the form of sinking organic particles. Utilization of these transient resources by copiotrophs requires sufficient chemotactic ability for detection and exploitation of nutrients prior to dissipation, particularly in the case of nutrient plumes where dissipation is accompanied by rapid downward motion. The copiotroph *Ps. haloplanktis* exhibits a strong chemotactic response, allowing it to exploit ephemeral nutrient sources, including plumes (Stocker et al. 2008). Consistent with a refined chemotactic ability, the *Ps. haloplanktis* genome contains numerous methyl-accepting chemotaxis proteins (MCPs; *Table 11.2.1*). By contrast, as a passive oligotroph, "*Cand.* P. ubique" specializes in utilizing low concentrations of uniformly distributed, soluble substrates that are available in ambient seawater. Accordingly, "*Cand.* P. ubique" lacks genes associated with chemotaxis and motility, and relatively few regulatory genes, precluding exploitation of sudden shifts in substrate availability (Giovannoni et al. 2005a).

S. alaskensis appears to employ a strategy that is in between these two extremes. The genome includes genes for chemotaxis and motility, plus a range of regulatory proteins (*Table 11.2.1*). Unlike "*Cand.* P. ubique," *S. alask*ensis has the metabolic capacity to respond to and exploit shifts in nutrient availability (Williams et al. 2009). However, the limited number of MCPs suggests that *S. alaskensis* is not specialized for the exploitation of ephemeral nutrient sources in the manner of specialist copiotrophs such as *Ps. haloplanktis*. An entirely different strategy is exemplified by *Si. pomeroyi*: it is motile, but lacks genes required for chemotaxis (Moran et al. 2004). However, this is consistent with a trophic lifestyle that depends on associations with phytoplankton, and sampling of a diverse array of carbon substrates

mediated by abundant ABC transport systems (see Substrate Uptake), obviating the need for mechanisms to detect individual substrates.

Copiotrophs also exhibit a bias toward scavenging of particulate organic matter, with a higher representation of genes for secreted proteins, including enzymes such as chitinase (Lauro et al. 2009). Attached bacteria generally exhibit higher extracellular enzymatic (ectoenzymatic) activities to obtain substrates from aggregated organic matter (Hoppe et al. 1993; Agis et al. 1998). The polysaccharide chitin, produced by invertebrates as well as fungi, is extremely abundant in the marine environment, and is a major component of marine snow. Chitin therefore constitutes a major reservoir of carbon and nitrogen, albeit in an insoluble form. Chitin degradation (chitinolysis) is considered to be a core function of Vibrionaceae (Hunt et al. 2008), and *Ph. angustum* S14 contains multiple secreted chitinase genes (*Table 11.2.1*). *Ps. haloplanktis* (although lacking chitinase) contains up to 16 genes involved in the synthesis of agglutinin associated with cellular adhesion to chitin (Médigue et al. 2005). Attachment to particulate organic matter (such as marine snow) would provide access to released soluble substrates at concentrations much higher than ambient seawater. By contrast, oligotrophic bacteria generally remain free-living in ambient seawater, and eschew attachment to and/or degradation of polymers in favor of deriving labile substrates from the DOC pool (Lauro et al. 2009).

Substrate Uptake

This section will focus on ABC transport systems and TRAP (tripartite ATP-independent periplasmic) transporters, in view of the importance of high-affinity uptake of substrates in oligotrophic environments. ABC transport systems are primary transporters that couple ATP hydrolysis to the translocation of solutes across the cytoplasmic membrane. A complete ABC transport system for solute uptake has a minimum of three components: periplasmic (extracytoplasmic) binding protein for solute capture, integral membrane component (permease) for translocation of the solute into the cytoplasm, and an ATPase (ATP-binding) component to drive the transport process (Tomii and Kanehisa 1998). TRAP transporters employ periplasmic solute capture in combination with secondary transport (proton-symport), and are composed of a periplasmic solute-binding component and two integral membrane (or one fused) components (Kelly and Thomas 2001). Given that ABC and TRAP transporters are adapted to extract substrates present at low concentrations, they are good systems for substrate uptake under oceanic conditions. Consistent with this, periplasmic binding components of ABC and TRAP transporters were found to dominate the metaproteome of surface water communities from the extremely nutrient-depleted Sargasso Sea (Sowell et al. 2009).

Nevertheless, genomic analysis indicates that oligotrophs are characterized by genomes relatively deplete of components for ABC transport systems (Lauro et al. 2009). This is consistent with a strategy of relying on a relatively smaller number of sufficiently high-affinity ABC transporters to scavenge essential substrates from seawater, with an emphasis on broad specificity transporters, especially for amino acid uptake (Akagi and Taga 1980; Schut et al. 1995; Lauro et al. 2009; Williams et al. 2009). High-affinity, multi-substrate transport allows oligotrophic bacteria to scavenge a wide spectrum of substrates under oligotrophic conditions by deploying a relatively small number of energy-intensive ABC transport systems. "*Cand.* P. ubique" also

uses TRAP transporters for solute capture and uptake, which unlike ABC transporters do not require ATP hydrolysis.

The small repertoires of ABC transport systems in "*Cand.* P. ubique" and *S. alaskensis* contrast with the numerous ABC transporters of *Ph. angustum* and *Si. pomeroyi*. The high numbers of ABC transport systems inferred for copiotrophs such as *Ph. angustum* and *Si. pomeroyi* attest to a strategy of primary transport diversification and specialization (Lauro et al. 2009). These bacteria are thereby capable of sampling a diverse array of substrates with high affinity in order to furnish a highly versatile metabolism. The exceptionally high number of ABC transporters in *Si. pomeroyi* accords with the *Roseobacter* strategy of obtaining substrates by associations with marine phytoplankton in algal blooms and phycospheres of zooplankton (Alonso and Pernthaler 2006; Moran et al. 2007). This parallels the strategy of free-living rhizobia in nutrient-limited soil, in which the high number of ABC transport systems facilitates the high-affinity acquisition of a very broad range of substrates from the rhizosphere (Mauchline et al. 2006). However, the presence of a high number of complete ABC transporters is not shared by all copiotrophs. *Ps. haloplanktis* has very few complete ABC transporters, which might reflect increased bioenergetic investment in chemotaxis over high-affinity substrate uptake. Additionally, there are genes for other ABC transport systems (e.g., for phosphate) that are composed of ATPase and membrane permease components, but lack a periplasmic binding protein. The effective chemotactic ability demonstrated by *Ps. haloplanktis* (Stocker et al. 2008) would allow nutrient patches to be rapidly detected and exploited prior to extensive nutrient dissipation and dilution, which would alleviate the demand for extracytoplasmic solute capture. Copiotrophs tend to have genes for ABC transport systems for peptide uptake (*Table 11.2.1*); these are lacking in "*Cand.* P. ubique" and *S. alaskensis*, indicating a shift away from the expenditure required for proteolysis in order to utilize these as carbon and nitrogen sources. Instead, free amino acids are preferred substrates in these ultramicrobacteria.

S. alaskensis has a high affinity, broad specificity uptake system and the ability to simultaneously take up mixed substrates (Schut et al. 1995). Nevertheless, *S. alaskensis* has even fewer ABC transport systems for the uptake of carbon substrates than "*Cand.* P. ubique," including only a single ABC amino acid transport system (*Table 11.2.1*). The general amino acid ABC uptake system in *S. alaskensis* has an affinity for several (but not all) amino acids, which means that the supply of the remaining amino acids is dependent upon either endogenous synthesis, or on less efficient uptake systems. The latter may be a preferred option under nutrient-enriched conditions, and would be facilitated by an ability to move toward nutrient patches, which is a strategy unavailable to passive oligotrophs such as "*Cand.* P. ubique." An inability to exploit elevated nutrient levels in order to achieve higher cell densities is characteristic of SAR11 marine bacteria (Tripp et al. 2008).

Evidence that ABC-mediated uptake is intimately coordinated to catabolism in *S. alaskensis* comes from enzyme assays, which determined that the high affinity for alanine exhibited by the general amino acid ABC transport system was matched by elevated catabolic activities of the enzyme alanine dehydrogenase (Schut et al. 1995, 1997b; Williams et al. 2009). By contrast, glutamate was not taken up by ABC transport, and levels of catabolic glutamate dehydrogenase were exceedingly weak (Schut et al. 1995, 1997b; Williams et al. 2009). Thus, the preference of *S. alaskensis* for alanine over glutamate as growth substrates under oligotrophic conditions is reflected both at the level of uptake and subsequent metabolic processing.

The genome of "*Cand.* P. ubique" has genes that encode 12 complete ABC transport systems for nutrient uptake: amino acids (three), sugars (two), proline/glycine betaine (two), and one each for polyamines, octopene, taurine, phosphate, and complexed iron (Fe^{3+}). *S. alaskensis* has only seven ABC transport systems for uptake: sugar (two), and one each for amino acids, polyamines, phosphate, molybdate, and complexed Fe^{3+}. The two TRAP transporters in "*Cand.* P. ubique" have putative specificity for dicarboxylates. In contrast, *S. alaskensis* has no genes for TRAP transporters. Thus, ABC transport systems for organic substrates, amino acids, polyamines, and sugars are present in both oligotrophs. Notably, all five marine strains (*Table 11.2.1*) have ABC transport systems for the uptake of Fe^{3+}. Iron has been proposed to be capable of limiting biomass production in marine environments (Street and Paytan 2005).

General Metabolism

Central Carbon Metabolism

The genome of the five marine strains examined (*Table 11.2.1*) show a complete oxidative tricarboxylic acid cycle (TCA cycle) to provide reducing power and biosynthetic precursors. Evidence for a glyoxylate shunt (genes for isocitrate lyase, malate synthase) is present in all these strains. This indicates that these marine bacteria are capable of metabolizing acetate, either exogenous or generated by breakdown of polyhydroxyalkanoate storage product (in those strains in which this pathway is available). All strains show a pentose phosphate pathway, and a pathway to generate pyruvate from the catabolism of sugars ("glycolysis"). However, for "*Cand.* P. ubique," although genes for the Entner–Doudoroff pathway have been identified, the complete pathway for glycolysis has yet to be determined (Giovannoni et al. 2005a; *Table 11.2.1*).

Carbon Metabolism: Amino Acids

Dissolved free amino acids (DFAAs) are ubiquitous in the open ocean, with alanine, glutamate, glycine, and serine as major constituents (Lee and Bada 1975, 1977; Andersson et al. 1985; Eguchi and Ishida 1990). Amino acids are preferred growth substrates for many Alphaproteobacteria (Cottrell and Kirchman 2003; Malmstrom et al. 2004), and are available as potentially ubiquitous sources of carbon, nitrogen, and sulfur (certain amino acids) for oligotrophic ultramicrobacteria. Nevertheless, in both "*Cand.* P. ubique" and *S. alaskensis* the utilization of amino acids for carbon and nitrogen appears to be constrained by the absence of genes for, or low level enzymatic activity of, specific metabolic enzymes. This is illustrated by the following examples describing the physiological response of cells to specific amino acids.

Biosynthetic pathways for all 20 common amino acids are apparent in the genome sequence of *S. alaskensis* (Williams et al. 2009) and were originally reported for the genome of "*Cand.* P. ubique" (Giovannoni et al. 2005a). However, growth experiments subsequently demonstrated that "*Cand.* P. ubique" is functionally auxotrophic for serine and glycine (Tripp et al. 2009). The "*Cand.* P. ubique" genome offered only one apparent avenue for glycine biosynthesis: from threonine using the reversible cleavage enzyme threonine aldolase (*ltaE*). Despite this, threonine was not able to substitute for glycine for the growth of "*Cand.* P. ubique," which indicates that this mechanism is unable to satisfy the cell's demand for glycine (Tripp et al. 2009).

Serine could possibly substitute for glycine through the activity of glycine hydroxymethyltransferase (*glyA*), but the "*Cand.* P. ubique" genome lacks any identifiable genes for transaminases (*agxt/spt*) that would allow alanine or serine to serve as amino donors for the interconversion of glycine and glyoxylate. This is consistent with experimental growth data, which showed that glyoxylate was unable to substitute for glycine (Tripp et al. 2009). However, an alternative possibility that remains to be demonstrated experimentally is that an oxidative mechanism independent of transamination could convert glycine to glyoxylate, and allow glycine to be used as a carbon and nitrogen source. One possible candidate is the gene SAR11_0847, which is annotated as a "glycine/D-amino acid oxidase (deaminating)," a member of the D-amino acid oxidase (DAO) family of flavoproteins. If the DAO protein can catalyze the oxidative deamination of glycine, the resulting glyoxylate could be fed into the TCA cycle, and (because DAO oxidation is effectively irreversible) provide an explanation for why glyoxylate cannot substitute for glycine in growth experiments.

Further to this, glycine is posited to play a key role in "*Cand.* P. ubique" metabolism (Tripp et al. 2009). This would be achieved by acting at the level of malate synthase and serving as a cue for regulating the flow of carbon through the TCA cycle to biosynthesis via the glyoxylate bypass. Thus, given that glycine cannot be synthesized endogenously, intracellular glycine levels may serve as a proxy for ambient levels of bioavailable substrates, including DFAAs (Tripp et al. 2009). However, for glycine to function as a cue for ambient substrate levels requires a suitable transport mechanism for glycine uptake by the cell (see Substrate Uptake).

Functional assessment of the metabolic capacity of *S. alaskensis* inferred from its genome sequence determined that despite the presence of a gene (*stdA*) for serine deaminase (which breaks down serine to pyruvate and ammonia), *S. alaskensis* was found to not be able to grow on serine (Williams et al. 2009). As the genes for the glycine cleavage complex are present in the genome, serine may be channeled exclusively to C-1 metabolism via glycine, and not enter the TCA cycle. The absence of *stdA* and the presence of genes for the glycine cleavage complex in "*Cand.* P. ubique" indicate that serine may serve a similar metabolic role. In contrast, alanine is oxidatively deaminated by alanine dehydrogenase (*aldA*) to pyruvate and ammonia, and is a favored growth substrate in *S. alaskensis*. Alanine is also a major product of ammonia assimilation in *S. alaskensis* (see Nitrogen Metabolism). Despite the importance of alanine to *S. alaskensis* metabolism, there is no apparent mechanism for the direct transamination of alanine to glutamate (Williams et al. 2009). Alanine is thereby precluded from entering nitrogen metabolism via glutamate or glutamine. Thus, the metabolic fates of alanine and serine (both major DFAAs in seawater) are strongly constrained. This trade-off appears to be part of a strategy to ensure that all aspects of metabolism are furnished by limited substrates, but at the cost of metabolic flexibility. In contrast, the copiotrophic strategy involves waiting in a dormant state for periods of nutrient surfeit, at which time diverse substrates are available, and carbon and nitrogen flow can be fine-tuned according to the metabolic needs of the cell.

Carbon Metabolism: Carbohydrates

Glucose and other energy-rich carbohydrates are catabolized to pyruvate ("glycolysis") for the generation of ATP, reducing equivalents, and biosynthetic precursors. Glucose is not an important natural growth substrate for *S. alaskensis*: uptake of this substrate is inducible, and upon entering the cell glucose is immediately converted to storage product, even when

glucose is the sole source of carbon (Schut et al. 1995, 1997a, b). Nevertheless, aside from lacking 6-phosphogluconolactonase, glucose is catabolized by a classical phosphorylative Entner–Doudoroff pathway in *S. alaskensis* (Williams et al. 2009). The major glycolytic route in "*Cand.* P. ubique" is unclear, although the genome encodes genes for some enzymes of the Entner–Doudoroff pathway (Giovannoni et al. 2005a). Due to the presence of carbohydrate transporters, glycolysis is inferred to occur in "*Cand.* P. ubique" although, similar to *S. alaskensis*, uptake is not mediated by the phosphotransferase system (PTS, *Table 11.2.1*). Having distinct pathways for glycolysis and gluconeogenesis removes the necessity to regulate carbon flux between glucose and pyruvate within the same pathway, and is characteristic of an oligotrophic metabolism. Moreover, the absence of strict catabolite control by PTS is consistent with an oligotrophic environment where no single carbon substrate (such as glucose) would be expected to dominate. Consistent with this, *S. alaskensis* has been shown to simultaneously utilize mixed substrates (amino acids and glucose) (Schut et al. 1995, 1997a).

By contrast, the nutrient patches required for growth of marine copiotrophs may be dominated by elevated concentrations of competing carbohydrates. This necessitates global regulatory networks to oversee a strict hierarchy of metabolic processing of available carbohydrate substrates (Deutscher 2008; Görke and Stülke 2008). Nevertheless, unlike marine vibrios, *Ps. haloplanktis* lacks PTS and cAMP-CAP complex to regulate the assimilation of competing carbon substrates (Médigue et al. 2005). Carbohydrates (including glucose) are apparently not important natural growth substrates for *Ps. haloplanktis* (Médigue et al. 2005; Papa et al. 2006).

Carbon Metabolism: Carbon Storage

Channeling carbon into storage material is generally promoted when ambient substrates are in imbalance, such as when carbon is in excess, but another substrate (e.g., nitrogen or phosphorus) is limiting. Under such conditions, excess carbon can be converted to storage products, such as polyhydroxyalkanoate (PHA). "*Cand.* P. ubique" lacks an identifiable PHA pathway, consistent with its inferred "hand-to-mouth" metabolism, a characteristic that may be typical of passive oligotrophs. In contrast, the metabolic strategy in *S. alaskensis* accommodates the detection and exploitation of elevated carbon levels, and subsequent storage as PHA when other substrates are limiting. Some copiotrophs (e.g., *Ph. angustum*, *Si. pomeroyi*) are also likely to respond to substrate imbalance by promoting carbon storage for later use in biomass production. The absence of PHA genes in *Ps. haloplanktis* is explained by this copiotroph being specialized for growth under highly nutrient-enriched conditions (Médigue et al. 2005), which better allows carbon substrates to be converted immediately into biomass and driving a rapid rate of growth in response to nutrient pulses. Thus, the ability to synthesize PHA does not correlate with an oligotrophy versus copiotrophy strategy per se, but is associated with different specific metabolic requirements of the individual bacterial species.

Nitrogen Metabolism

S. alaskensis uses nitrate, ammonia, amino acids, and polyamines as nitrogen sources (Williams et al. 2009). Despite being a more energy-intensive process than ammonia assimilation, nitrate assimilation allows marine bacteria to exploit this abundant inorganic nitrogen source.

Pathways for the reduction of nitrate and sulfate are absent from "*Cand.* P. ubique." The lack of ability to reduce nitrate and sulfate is likely to be associated with a metabolic strategy that entails the acquisition of nitrogen and sulfur at a lower energetic cost to the cell, and is consistent with the trade-off advantages of possessing a smaller genome (Giovannoni et al. 2005a; Tripp et al. 2008). The loss of a nitrate utilization ability can generally be associated with genome simplification favoring the retention of the uptake and utilization of reduced nitrogen in the form of amino acids, and particularly ammonia; an example of this is the cyanobacterium *Prochlorococcus marinus*, which is a dominant member of the marine phytoplankton that is present throughout oligotrophic intertropical areas of the oceans (García-Fernández et al. 2004).

A metaproteomic survey of the Sargasso Sea indicated that the SAR11 population was metabolically poised to assimilate ammonium as a nitrogen source, with the detection of proteins involved in the uptake and assimilation of ammonium (Sowell et al. 2009). Consistent with nitrogen-depleted conditions prevailing in the Sargasso Sea, glutamate synthase (GS) was one of the most frequently detected proteins. The higher affinity of GS for ammonium compared to other ammonia assimilatory enzymes (glutamate dehydrogenase, alanine dehydrogenase) would facilitate the scavenging of low concentrations of ammonia, at the cost of ATP hydrolysis. For "*Cand.* P. ubique," transporters exist for the uptake of all potential sources of reduced nitrogen for biosynthesis (ammonia, amino acids, and polyamines) (Giovannoni et al. 2005a). Extracytoplasmic solute-binding components of ABC transport systems for amino acids and polyamines were also detected as some of the most frequent protein identifications within the SAR11 metaproteome (Sowell et al. 2009). These data indicate that, despite generally paring back energetically demanding processes, investment in ATP-dependent enzymes (e.g., GS) and substrate uptake systems to extract and assimilate essential nitrogen from ambient seawater provides a competitive strategy for SAR11 bacteria in oligotrophic waters.

Mechanisms to regulate the response to competing nitrogen sources are also absent from "*Cand.* P. ubique." One possible exception is the NtrY/NtrX two-component regulatory system, encoded in the genome. NtrB/NtrC and NtrY/NtrX systems have been inferred to be involved in regulatory responses to alternative nitrogen sources (Pawlowski et al. 1991; Assumpção et al. 2007). However, similar to a proposed role in the streamlined genome of an intracellular parasitic bacterium (Kumagai et al. 2006), NtrY/NtrX may regulate other responses in "*Cand.* P. ubique" unrelated to nitrogen metabolism. "*Cand.* P. ubique" also lacks genes for σ^{54} (RpoN) regulated PTS control of nitrogen metabolism (Giovannoni et al. 2005a; Commichau et al. 2006; *Table 11.2.1*). It therefore appears questionable whether nitrogen metabolism is subject to any regulation in "*Cand.* P. ubique."

The ability of *S. alaskensis* to utilize nitrate as a nitrogen source is consistent with a complete repertoire of genes for regulatory proteins associated with the efficient utilization of competing nitrogen sources (*Table 11.2.1*). In addition to GS and glutamate dehydrogenase, *S. alaskensis* assimilates ammonia using alanine dehydrogenase. This allows alternative carbon skeleton precursors (pyruvate and 2-oxoglutarate) to be used for ammonia assimilation. This may provide an advantage under oligotrophic conditions when carbon flow through the full TCA cycle is restricted by carbon limitation (Williams et al. 2009). It remains to be determined if "*Cand.* P. ubique" has adopted a similar strategy. This would be interesting to determine in view of the restricted availability of 2-oxoglutarate in the cytoplasm that would be expected to result from induction of the glyoxylate shunt in response to the detection and uptake of DFAAs (see Carbon Metabolism: Amino Acids).

Phosphorus Metabolism

Phosphorus can be a limiting substrate in ocean surface waters (Rivkin and Anderson 1997), and genes that encode proteins associated with the uptake and utilization of inorganic phosphorus and organic phosphorus in the form of phosphonates (C–P-bonded phosphorus) were found to be highly abundant in a metagenomic survey of oligotrophic surface waters in different oceans (Feingersch et al. 2009; Hewson et al. 2009; Martinez et al. 2010). Similarly, periplasmic solute-binding proteins for phosphate and phosphonate transporters were prevalent in a metaproteome derived from surface waters of the Sargasso Sea (Sowell et al. 2009). Although *S. alaskensis* and "*Cand.* P. ubique" possess relatively few ABC transport systems, both include a complete ABC transport system for phosphate binding and uptake (*Table 11.2.1*). The genes for the phosphate uptake system (*pstBACS*) are located adjacent to a phosphate uptake regulator gene (*phoU*). The genomes of all five strains have homologues of the PhoR/PhoB two-component systems, suggesting regulated responses to phosphate limitation. Genes for ABC-mediated phosphate transport is also evident in the three copiotroph genomes (although *Ps. haloplanktis* lacks an identifiable phosphate-binding protein; see Substrate Uptake). Notably, *Ph. angustum* and *Si. pomeroyi* possess ABC transport systems for both phosphate and phosphonate, whereas the latter is absent from the *S. alaskensis* and "*Cand.* P. ubique" genomes. Thus, the two oligotrophs focus on deriving their phosphorus from phosphate rather than organophosphorus compounds such as phosphonate that require further catabolization. Accordingly, genes of the *phn* cluster that are required for the utilization of phosphonate (Metcalf and Wanner 1993) are present in *Ph. angustum* and *Si. pomeroyi*, but absent from the genomes of the three other strains examined (*Table 11.2.1*). Overall, the genomic data highlight the importance of ATP-driven primary transport in competing for available phosphorus in ambient seawater, with *S. alaskensis* and "*Cand.* P. ubique" using inorganic phosphate as a preferred phosphorus source.

Sulfur Metabolism

While marine bacteria typically face a bulk medium that is generally unbalanced in terms of carbon, nitrogen, and phosphorus, sulfur is not usually a limiting nutrient (Moran et al. 2004; Médigue et al. 2005). Most aerobic marine bacteria can import and reduce sulfate. However, "*Cand.* P. ubique" has been shown experimentally to be unable to provide sulfur for biosynthesis by performing assimilatory sulfate reduction (Tripp et al. 2008). "*Cand.* P. ubique" and other SAR11 bacteria that lack this ability are dependent on exogenous reduced sulfur compounds, such as methionine and DMSP (Tripp et al. 2008). A pathway for the conversion of cysteine to methionine also appears to be absent in "*Cand.* P. ubique" (Tripp et al. 2008).

Translation and Growth Rate

Low rRNA copy numbers are characteristic of oligotrophs, whereas high rRNA copy numbers are advantageous for rapidly growing cells (Fegatella et al. 1998; Aiyar et al. 2002). Laboratory cultures of *S. alaskensis* respond to nutrient upshift by immediately increasing their growth rate, and maximum growth rate is achieved when nutrient-starved cultures are exposed to excess levels of glucose (Eguchi et al. 1996; Fegatella et al. 1998). Nevertheless, the maximum growth rate of *S. alaskensis* ($<0.2\ h^{-1}$) is low compared to copiotrophs (Fegatella et al. 1998)

and remains largely unchanged within a large range of organic carbon concentrations (Eguchi et al. 1996). On this basis, it was hypothesized that the translational machinery was constitutively regulated in *S. alaskensis*, which would limit growth rate, but allow the cell to be poised to take advantage of nutrient upshifts (Eguchi et al. 1996; Fegatella et al. 1998). However, subsequent research indicated that the number of ribosomes per cell was tightly regulated, and ribosome content was therefore not the limiting factor responsible for a low maximum growth rate; in fact, translational capacity appears to exceed the cellular requirements for growth (Fegatella et al. 1998).

Glucose taken up by *S. alakensis* cells is not immediately catabolized, but stored, even during glucose-limiting growth (Schut et al. 1995, 1997a). The absence of 6-phosphogluconolactonase may impede efficient catabolism of glucose and other carbohydrates, because this step therefore depends on spontaneous hydrolysis (Williams et al. 2009). Nevertheless, although certain amino acids (especially alanine) are taken up by high-affinity transporters, their metabolic fates are highly constrained (see Carbon Metabolism: Amino Acids). Thus, *S. alaskensis* has evolved a physiological strategy that ensures that all facets of metabolism are served during growth on amino acids, at the cost of metabolic flexibility. This represents a very coarse mechanism for regulating carbon flow, but it ensures that biosynthetic precursors can be supplied from a subset of the DFAA pool with minimal metabolic fine-tuning.

For "*Cand.* P. ubique," the elimination of energy-intensive anabolic (especially reductive) and chemotactic pathways not only allows further compaction of the genome, but makes global metabolism less dependent on appropriate redox poise and ATP levels inside the cytoplasm. The trade-off is that metabolic building blocks for growth are increasingly dependent on exogenous organic compounds. Growth is therefore dependent on the availability of these substrates; the corollary is that absence of these substrates (such as glycine, or reduced sulfur compounds) would limit proliferation (Tripp et al. 2008). "*Cand.* P. ubique" would be drawn into intense competition for eligible substrates (such as ammonia, amino acids, and DMSP), especially if these substrates are limiting. Moreover, as a passive oligotroph, detection and contact with substrates cannot be facilitated by chemotaxis. Accordingly, SAR11 populations in the North Sea do not show the pronounced increase in biomass demonstrated by other bacterial species (including roseobacters) in response to increased DMSP levels generated by phytoplankton blooms (Simo et al. 2002). However, while increased nutrient availability does not lead to large increases in cell density, "*Cand.* P. ubique" populations remain, in effect, very abundant and widespread.

The factors that are responsible for keeping cell densities of SAR11 populations low, both under natural growth conditions and in laboratory cultures, are not yet well understood. Growth rates of laboratory cultures of "*Cand.* P. ubique" remain low ($<0.75\ d^{-1}$) and largely unchanged irrespective of nutrient addition (Giovannoni et al. 2005a; Tripp et al. 2008). It is possible (but untested) that limited translational capacity including constitutive ribosome synthesis might contribute to low growth rate in "*Cand.* P. ubique." In effect, the extreme metabolic streamlining in "*Cand.* P. ubique" may limit the cell's ability to respond to environmental variation in substrate availability, and minimize the metabolic capacity to efficiently convert substrates into biomass.

Adaptive Responses and Stress Resistance

Bacteria can be exposed to a range of biotic (e.g., viral infection, predation) and abiotic (e.g., UV, temperature, nutrient limitation) factors that control their ability to proliferate in the

marine environment. After exposure to sublethal levels of a particular environmental stress, or during starvation, many bacteria become resistant to unrelated stresses, a phenomenon termed "cross protection." Cross protection has been described for marine *Vibrio* spp. (Kjelleberg et al. 1993b) and has been generally linked to gene expression pathways mediated by the alternate sigma factor, RpoS (σ^S). This molecular response enables marine copiotrophic bacteria to rapidly respond to a variety of possible stressful conditions (e.g., starvation-stress response). In contrast, starvation in *S. alaskensis* does not induce cross protection to the stress-inducing agents hydrogen peroxide, ethanol, heat (Eguchi et al. 1996), and UV-B (Joux et al. 1999). Instead, *S. alaskensis* has inherently high levels of resistance during logarithmic growth phase, and even higher levels of resistance when grown at low growth rates under nutrient-limited conditions in chemostats (Ostrowski et al. 2001), the latter being growth conditions similar to what may be encountered in the ocean. The *rpoS* gene is not typically present in Alphaproteobacteria (Rava et al. 1999; Cavicchioli et al. 2003; Zech et al. 2009; *Table 11.2.1*). A protein was detected in *S. alaskensis* protein extracts by Western-blot analysis using polyclonal antibodies raised against *E. coli* RpoS (Cavicchioli et al. 2003). However, in contrast to growth-phase-regulated synthesis of the protein in *E. coli*, the protein in *S. alaskensis* remained constant throughout the growth phase. The protein is likely to have been a cross-reacting species (perhaps another sigma factor), as the genome sequence of *S. alaskensis* encodes two sigma factors (*rpoD* and *rpoH*) but does not encode *rpoS*. Similarly, although "*Cand.* P. ubique" possesses genes for two sigma factors (Giovannoni et al. 2005a), an *rpoS* gene is not present.

Oligotrophic oceans are characterized by clear water where ultraviolet radiation (UVR, 280–400 nm) penetrates deeper than in coastal waters (Tédetti and Sempéré 2006). Exacerbating the effect of UVR is the stratospheric depletion of ozone, which is increasing UVB (280–315 nm) fluxes in high and mid-latitude regions, and the impact that climate change is likely to have on the thermal stratification of ocean waters resulting in the increased exposure of organisms to solar radiation in surface waters (Zepp et al. 2007; Häder et al. 2007). Solar UVB is a major mutagen that damages DNA through the formation of dimeric photoproducts between adjacent thymine and cytosine bases. It has been recently demonstrated that formation of cytosine-containing photoproducts increases with the GC content of the bacterial genome (Matallana-Surget et al. 2008).

Because cytosine-containing photoproducts are highly mutagenic, it is reasonable to expect that species with genomes exhibiting a high GC content are more susceptible to UVB-induced mutagenesis. Bacteria with small genomes, including "*Cand.* P. ubique," also have low GC content (29.8%; Giovannoni et al. 2005a; *Table 11.2.1*), and oligotrophs tend to have smaller genomes than copiotrophs (Lauro et al. 2009). Moreover, the mean GC value determined from large-scale DNA shotgun sequencing of oligotrophic surface waters is low (~34%) (Foerstner et al. 2005). In combination with the presence of three DNA photolyase genes in the genome (*Table 11.2.1*), these findings suggest that "*Cand.* P. ubique" (with a small, low GC content genome) may be less affected by UV-induced mutations.

S. alaskensis, has only one photolyase gene and a GC content of 65%, illustrating that a high GC content and low photolyase gene copy number does not, in itself, preclude a marine bacterium from being an oligotroph. To define the molecular basis for adaptation of *S. alaskensis* to solar radiation (both UV and visible), a study was performed using quantitative proteomics (Matallana-Surget et al. 2009a). Key factors that were identified that could be linked to a solar radiation adaptive response included: DNA-binding proteins implicated in reducing DNA damage, detoxification of toxic compounds such as glyoxal and reactive oxygen

species, iron-sequestration to minimize oxidative stress, chaperones to control protein re/folding, and specific changes to transcriptional and translational processes (Matallana-Surget et al. 2009a).

The inherently high resistance of *S. alaskensis* to UVB compared to a range of marine bacteria has been demonstrated (Joux et al. 1999). However, the copiotroph, *Ph. angustum* has been shown to exhibit superior UVB resistance with an unexpected capacity to repair dimeric photoproducts during UVB exposure, and in this case the presence of very efficient DNA photolyase-mediated repair (involving up to three photolyase genes) was hypothesized to be linked to the high UVB resistance (Matallana-Surget et al. 2009b; *Table 11.2.1*). The studies to date illustrate that diverse mechanisms have evolved in oligotrophs and copiotrophs to facilitate resistance to, and repair of UV-induced DNA damage.

Conclusion

Oligotrophic ultramicrobacteria such as "*Cand.* P. ubique" and *S. alaskensis* represent the extreme end of the trophic spectrum, exhibiting physiologies that are well adapted to proliferation under oligotrophic conditions. While "*Cand.* P. ubique" is an obligate oligotroph and *S. alaskensis* a facultative oligotroph, their genomic signatures clearly cluster them together, distinguishing them from marine copiotrophic bacteria (Lauro et al. 2009). However, as individual species, "*Cand.* P. ubique" and *S. alaskensis* possess a number of defining genomic and ecophysiological traits which demonstrate that evolution for broadly convergent lifestyles based on distinct phylogenetic blueprints.

The SAR11 clade is ubiquitous in marine environments, at all depths of the water column, and accounts for approximately a quarter of all microbial plankton cells at the ocean surface (Morris et al. 2002; Giovannoni and Stingl 2007). *S. alaskensis* has a more limited distribution compared to SAR11, with *S. alaskensis* strains isolated from three locations (Gulf of Alaska, North Pacific waters, and the North Sea) and found not to be abundant in the Global Ocean Survey metagenomic data (Thomas et al. 2007). The recipe for the success of the SAR11 clade lies in its ability to survive and proliferate under extreme nutrient limitation, whereas the physiology of *S. alaskensis* represents a trade-off between genomic streamlining and the ability to efficiently exploit fluxes in the quality and quantity of bioavailable substrates.

"*Cand.* P. ubique" has evolved competitiveness through pronounced genome streamlining resulting in decreased metabolic capacity and loss of motility without compromising fitness. Although it has not been established in situ, *S. alaskensis* can exploit elevated levels of nutrients resulting in strains becoming amenable to a broad range of laboratory studies (Cavicchioli et al. 2003). Ascertaining why this is achievable with *S. alaskensis* and not with "*Cand.* P. ubique" and other SAR11 bacteria is likely to shed new light on the molecular mechanisms underpinning their specific physiologies and the distinction between obligate and facultative oligotrophs. Clearly there is a great scope and a strong need for learning more about the unique physiologies of marine oligotrophic ultramicrobacteria.

Cross-References

11.1 Ecology and Cultivation of Marine Oligotrophic Bacteria

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Part 12

New Frontiers: Applications and Global Impacts

12.1 Actinobacteria of the Extremobiosphere

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_12.1,

The Actinobacteria

The class *Actinobacteria* is one of 27 currently comprising the Domain *Bacteria*; this chapter is the only one in the Handbook devoted to a single taxonomic group of bacteria, members of which are found in the complete spectrum of extreme environments. Actinobacteria are Gram-positive organisms characterized by having a high mol% G+C ratio, filamentous or nonfilamentous morphologies, and some members that produce spores. Regarded until recently as predominantly components of the soil microbiota, they are known now to have a ubiquitous distribution in the biosphere, including the extremobiosphere.

The class is organized into four subclasses and eight orders (*Actinomycetales, Acidimicrobiales, Bifidobacteriales, Coriobacteriales, Rubrobacterales, Nitriliruptorales, Solirubrobacterales, Thermoleophilales*) the latter three of which have been described very recently (Sorokin et al. 2009; Reddy and Garcia-Pichel 2009). Reference throughout this chapter to *Figs. 12.1.1* and *12.1.2* will assist those less familiar with the actinobacteria in negotiating the taxonomy of this large class of bacteria. Refinements in 16S rRNA gene phylogenies have led to the reclassification of the genus *Thermoleophilum* (Perry 2006) as an actinobacterium and description of the new order *Thermoleophilales* (Reddy and Garcia-Pichel 2009); and removed the order *Sphaerobacteriales* to the class *Chlorofexi* (Hugenholtz and Stackebrandt 2004) although the invalid taxon continues to be cited (e.g., Ventura et al. 2007). The size and diversity of the *Actinobacteria* defines it as one of the principal classes of bacteria – 54 families, 286 genera, >3,000 species (Euzeby 2009), while the comparative figures of a decade ago (30 families, 95 genera) (Stackebrandt et al. 1997) reveal the rapid rate of discovery within this taxon. In "The All-Species Living Tree" database of all 16S DNA sequenced bacteria and

Class	Sub-class	Order	Sub-order	Family
Actinobacteria	*Actinobacteridae*	***Actinomycetales***	*Corynebacterineae*	
			Frankineae	
			Micrococcineae	***Microbacteriaccae***
				Micrococcaceae
			Streptomycineae	*Streptomycetaceae*
			Actinopolysporineae	***Actinopolysporaceae***
		Bifidobacteriales		
		Nitriliruptorales		*Nitriliruptoraceae*
	Acidimicrobidae	***Acidimicrobiales***	*Acidimicrobineae*	***Acidimicrobiaceae***
	Coriobacteridae	*Coriobacteriales*		
	Rubrobacteridae	***Rubrobacteriales***	*"Rubrobacterineae"*	***Rubrobacteraceae***
		Thermoleophilales		*Thermoleophilaceae*
				Conexibacteraceae
				Patulibacteraceae
		Solirubrobacterales		*Solirubrobacteraceae*

Fig. 12.1.1

Hierarchical classification of the Class *Actinobacteria*. Taxa in bold indicate those containing a particularly high incidence of extremophilic/-trophic organisms. Selected Sub-orders and Families included here are ones notable for their extremophilic/-trophic members (Based on data provided by JP Euzéby (www.bacterio.cict.fr/classiphyla.html) and from Zhi et al. 2009)

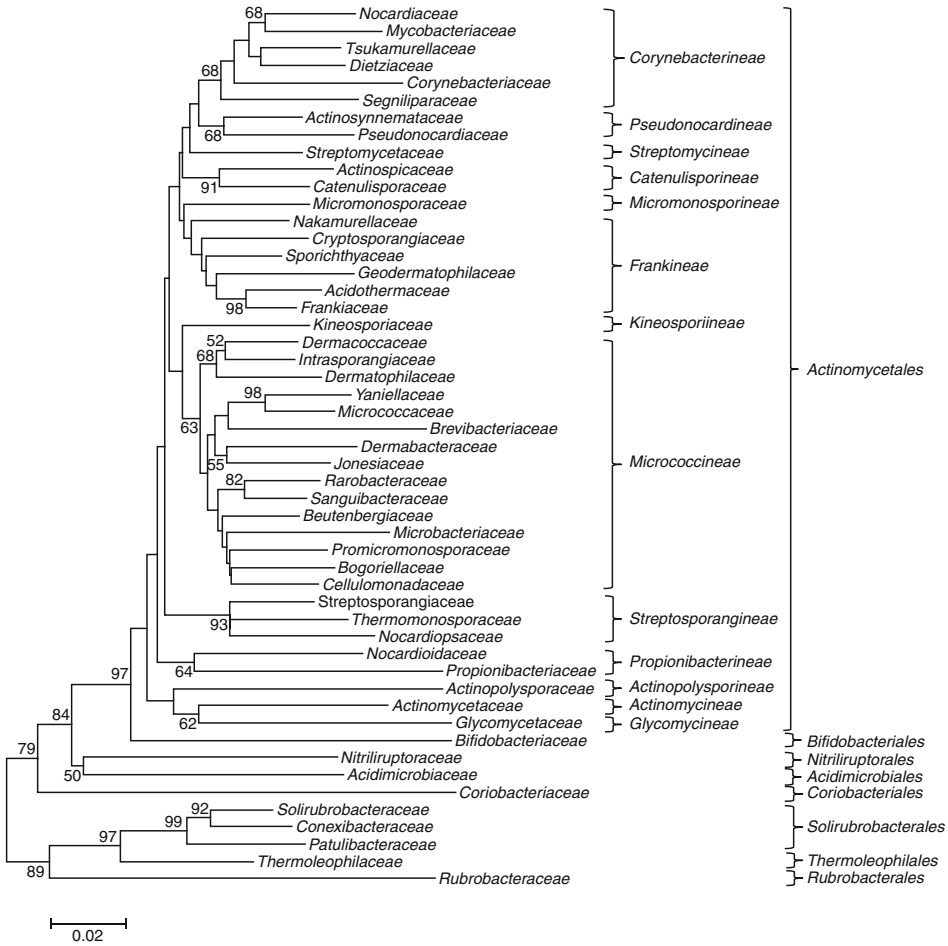

Fig. 12.1.2

Intraclass relatedness within *Actinobacteria*. Relatedness is based upon 16S rRNA gene sequence comparison; bootstrap values of 50% or more are indicated ar branch points. The scale bar represents 2 nucleotide substitutions per 100 nucleotides (Figure kindly provided by Dr Wen-Jun Li and Zhi Xiao-Yang)

archaea established in 2007 (Yarza et al. 2008) over 80% of validated genera contained only 1–5 species but, interestingly, the largest genus in the database (*Streptomyces*) comprised 488 species. Of course, such numbers in part reflect comparative ease of study, perceived importance and historical contexts, nevertheless the case of *Streptomyces* highlights evolutionary divergence and ecological success to be found within the *Actinobacteria*.

The rationale for considering actinobacteria in the context of this volume is several-fold: overall size and diversity of the taxon, their widespread global and environmental dispersal, the pace of discovery of new taxonomic radiations, and their ecological and economic value. Geochemical methods and molecular clock methods calibrated against the microbial fossil

record have enabled estimates to be made of microbial divergence times. Thus analyses by Brenchley's group (Sheridan et al. 2003) place the Last Common Ancestor at about 4.29 Ga (10^9 years) ago, while those of Hedges (Hedges et al. 2006) indicate a divergence of the *Archaea* and *Bacteria* occurring at about 3.97 Ga. Battistuzzi and Hedges (2009) subsequently have proposed that a land clade of bacteria, which they have termed Terrabacteria that includes the *Actinobacteria*, arose in the mid-Archaean era (ca. 3.18 Ga), members of which are characterized by multiple adaptations to terrestrial conditions such as resistance to desiccation, ultraviolet radiation, and high salinity. Based on gene transposition data Kunisawa (2007) concluded that *Rubrobacter xylanophilus* is the earliest diverging species among fully sequenced *Actinobacteria*, thus suggesting that the *Rubrobacterales* is the most ancient lineage in this class of bacteria. However, Lake and his colleagues (Servin et al. 2008) have argued that on the evidence of gene insertions and deletions found in GyrA and ParC, the root of life is not located within the *Actinobacteria*. *Arthrobacter-Bifidobacterium-Streptomyces* divergence is estimated to have occurred at 2.76 Ga (Sheridan et al. 2003). The influence of extreme terrestrial environments on actinobacterial speciation and adaptation is explored later in this chapter.

In this chapter the following topics are examined as they relate to actinobacteria: the determinants of extreme ecosystems and actinobacterial distribution; actinobacteria of selected extremo-ecosystems; natural product discovery from the latter organisms; and concluding issues and challenges. First, however, some circumspection is necessary in discussions of actinobacterial extremophily.

Extremophily versus Extremotrophy

By definition extremophiles are microorganisms that are adapted to grow optimally at or near to the extreme ranges of temperature, pH, pressure and/or salinity. In contrast a much larger collection of microorganisms is able to tolerate extreme conditions and, in addition, grow but not necessarily optimally, under conditions of high radiation exposure, high desiccation/low moisture and/or dilute nutrient availability; these organisms are properly referred to as extremotrophs (Mueller et al. 2005) (see Box 12.1).

The distinction between extremophilic and extremotrophic organisms is imperative when discussing actinobacteria and rather than being a mere semantic issue it is subject to a number of important caveats: (1) inappropriate methods may have been used to isolate putative extremophiles (e.g., agar plate-based isolation of acido-/alkaliphiles); (2) the nature of the extremophily may not have been tested rigorously (e.g., specific growth rate measurements in liquid culture); (3) putative extremophily may be compromised by subsequent serial cultivation under laboratory conditions (e.g., loss of obligate salt requirement); and (4) little or no attempt to determine whether organisms are adaptable to only small differences in environmental variables (e.g., tests for stenothermic, stenobaric adaptation). Consequently, the literature on actinobacteria from the extremobiosphere needs to be assessed with caution. For example, in descriptions of the new genus, *Subtercola*, its species were stated to be psychrophiles (Mannisto et al. 2000) and similarly the newly described *Salinibacterium xinjiangense* is also defined as being psychrophilic (Zhang et al. 2008) but, on the basis of the usually accepted criteria of extremophily (Antranikian et al. 2005; Pikuta et al. 2007), these organisms are psychrotolerant (growth optima 15–17°C and 18–19°C, respectively, compared to <10°C; see Cryo-Environments below). These are far from being isolated examples of the lack of stringency in defining actinobacterial extremophily; therefore, throughout this chapter

Box 12.1 Defining Bacterial Extremophily and Extremotrophy

The following categorization of extremophilic and extremotrophic bacteria used in this chapter is based on definitions proposed by Antranikian et al. (2005), Pikuta et al. (2007) and Mesbah and Wiegel (2008). The values quoted for optimum, minimum, and maximum growth characteristics are not absolute and should always be accompanied by those of other culture conditions (note, e.g., the variation in pH and pressure optima as a function of temperature).

A guide also is offered for defining obligate oligotrophy where growth is restricted to growth rate limiting substrate (s) concentrations in the range of $\mu g\ L^{-1}$ (Button 2004), and μ_{max} values occur at nanomolar concentrations of s (Cavicchioli et al. 2003). Similarly, an upper value for bacterial growth under conditions of hyper irradiation is included (Daly 2009).

Category	Growth		
	Minimum	Optimum	Maximum
Thermophily			
Moderate		≥50°C	>60°C
Extreme	>35°C	>65°C	<85°C
Hyper	>60°C	>80°C	≥85°C
Psychrophily	<0°C	<10°C	ca. 20°C
Psychrotrophy		7–15°C	
Acidophily			
Moderate	pH 4	pH 6	
Obligate	pH 0	pH ≤ 4	
Alkaliphily			
Moderate	pH < 7.5	pH > 8.5	pH ≥ 10
Obligate	pH ≥ 7.5	pH > 8.5	pH ≥ 10
Halophily			
Moderate	NaCl 1 M	NaCl ≥ 1.5 M	
Extreme	NaCl ≥ 1.5 M	NaCl ≥ 2.5 M	
Halotrophy		NaCl ≥ 0.25–1.5 M	NaCl < 2.5 M
Piezophily		≥40 MPa	ca. 110 MPa
Piezotrophy		≤40 MPa	
Oligotrophy			
Obligate		$s \leq \mu g\ L^{-1}$	nM
Irradiation tolerance			60 Gy h^{-1}

organisms are referred to as extremotrophs (or extremotolerants) unless the caveats above have been respected. This is not to dismiss the ecological fact that extremotrophic actinobacteria may be active in prevailing extreme environments (e.g., psychroactive actinobacteria in ancient permafrost, Vishnivetskaya et al. 2006).

Tables 12.1.1 and *12.1.2* illustrate the diversity and numbers of actinobacterial genera that are reported as containing members definable as being *bona fide* extremophilic, or, as extremotrophic. The listings are not exhaustive and further research on particular organisms may result in their reassignment to one category or the other; however, inclusion is based on

Table 12.1.1

Genera of *Actinobacteria* containing Extremophilic species

Genus	Order/family	References
Acidimicrobium (acidophilic)	Acidimicrobiales/ Acidimicrobiaceae	Clark and Norris (1996), Cleaver et al. (2007), Okibe and Johnson (2004)
Actinospica (acidophilic)	Actinomycetales/ Actinospicaceae	Busti et al. (2006)
Actinopolyspora (halophilic)	Actinomycetales/ Actinopolysporaceae	Aislabie et al. (2006), Grochnauer et al. (1975), Zhi et al. (2007)
Arthrobacter (psychrophilic)	Actinomycetales/ Micrococcaceae	Reddy et al. (2002), White et al. (2000)
Acidothermus (thermo-, acido-philic)	Actinomycetales/ Acidothermaceae	Bergquist et al. (1999)
Brachybacterium (psychrophilic)	Actinomycetales/ Dermabacteraceae	Junge et al. (1998)
Catenulispora (acidophilic)	Actinomycetales/ Catenulisporaceae	Busti et al. (2006)
Cryobacterium (psychrophilic)	Actinomycetales/ Micrococcaceae	Zhang et al. (2007)
Dietzia (halophilic)	Actinomycetales/ Dietziaceae	Gontang et al. (2007), Kleinsteuber et al. (2006), Al-Awadhi et al. (2007)
Ferrimicrobium (acidophilic)	Acidimicrobiales/ Acidimicrobiaceae	Johnson et al. (2009)
Ferrithrix (acidophilic)	Acidimicrobiales/ Acidimicrobiaceae	Johnson et al. (2009)
Frigoribacterium (psychrophilic)	Actinomycetales/ Microbacteriaceae	Kämpfer et al. (2000)
Glaciibacter (psychrophilic)	Actinomycetales/ Microbacteriaceae	Katayama et al. (2009)
Haloactinospora (halophilic)	Actinomycetales/ Nocardiopsaceae	Tang et al. (2008)
Haloglycomyces (halophilic)	Actinomycetales/ Glycomycetaceae	Guan et al. (2009)
Kocuria (halophilic, psychrophilic)	Actinomycetales/ Micrococcaceae	Gontang et al. (2007), Reddy et al. (2003)
Marmoricola (halophilic)	Actinomycetales/ Nocardioidaceae	Gontang et al. (2007)
Microbacterium (halophilic)	Actinomycetales/ Microbacteriaceae	Gontang et al. (2007)
Microcella (alkaliphilic)	Actinomycetales/ Microbacteriaceae	Tiago et al. (2005)
Modestobacter (psychrophilic)	Actinomycetales/ Geodermatophilaceae	Mevs et al. (2000)
Mycobacterium (halophilic)	Actinomycetales/ Mycobacteriaceae	Gontang et al. (2007)

Table 12.1.1 (Continued)

Genus	Order/family	References
Nesterenkonia (alkaliphilic)	Actinomycetales/ Micrococcaceae	Luo et al. (2009)
Nitriliruptor (haloalkaliphilic)	Nitriliruptorales/ Nitriliruptoraceae	Sorokin et al. (2009)
Pseudonocardia (halophilic)	Actinomycetales/ Pseudonocardiaceae	Gontang et al. (2007)
Rubrobacter (thermo-, acidophilic)	Rubrobacteriales/ Rubrobacteraceae	Carreto et al. (1996), Chen et al. (2004), Ferreira et al. (1998)
Strain Pa33 (acidophilic)	Rubrobacteridae	Bryan and Johnson (2008)
Streptacidiphilus (acidophilic)	Actinomycetales/ Streptomycetaceae	Cho et al. (2006)
Streptomonospora (halophilic)	Actinomycetales/ Nocardiopsaceae	Zhi et al. (2007), Cui et al. (2001)
Thermoleophilum (thermophilic)	Thermoleophilales/ Thermoleophilaceae	Yakimov et al. (2003)
Yaniella (alkaliphilic, halophilic)	Actinomycetales/ Micrococcaceae	Li et al. (2005), Ghauri et al. (2007)

studies of cultivated organisms whereas those based on surveys of phylotypes have not been considered.

Despite the paucity of data, a number of salient points begin to emerge from this analysis. First, regarding extremophilic actinobacteria, the majority of taxa currently reported are found in the suborder *Micrococcineae* of the *Actinomycetales*; this taxon includes *Arthrobacter*, one of the proposed earliest divergences within the *Actinobacteria*. Second a very similar trend can be seen in extremotrophic actinobacteria with members of the *Micrococcineae* again apparently being dominant in extreme environments. Third, members of the *Actinomycetales* are the most frequently recorded actinobacteria in extreme environments within which members of a large number of suborders are represented. Such facts probably belie the facility of isolating these bacteria and the comparatively recent discovery, and hence low level of research on the deep lineage actinobacteria. Fourth, the picture of actinobacterial occupation of extreme environments is consistent with the hypothesis of the evolution of a land clade of bacteria (Battistuzzi and Hedges 2009). Founded on phylogenetic (SSU, LSU), organismal (cytology, physiology), and environmental (colonization of arid and related environments) evidence, these authors have defined a major group of bacteria comprising *Actinobacteria, Chloroflexi, Cyanobacteria, Deinococcus-Thermus*, and *Firmicutes* that evolved ca. 3.18 Ga ago, the Terrabacteria. Especially, significant in this context is the conclusion that actinobacteria are the dominant bacterial populations of arid soils. Thereafter, as the authors declare, "many descendant species living today are adapted to other environments." Fifth, on the evidence of the present survey, actinobacteria have been particularly successful in colonizing the arid cryosphere within which closely related groups have evolved ecological strategies for survival under the austere conditions of low temperature, desiccation, and radiation exposure.

Table 12.1.2

Genera of *Actinobacteria* containing Extremotrophic species

Genus	References
acI clades (high acidity, oligotrophic, UV-radiation)	Warnecke et al. (2005)
Aeromicrobium marinum (high salinity)	Bruns et al. (2003)
Agrococcus (low temperature)	Liu et al. (2009)
Amycolatopsis (low moisture, high salinity)	Okoro et al. (2009a)
Arthrobacter (low temperature, oligotrophic)	Aislabie et al. (2006), Chanal et al. (2006), Steven et al. (2007), Steven et al. (2008), Vishnivetskaya et al. (2006), Junge et al. (1998), Gilichinsky et al. (2007), Hansen et al. (2007), Liu et al. (2009), White et al. (2000), Gratia et al. (2009)
Blastococcus (low moisture)	Chanal et al. (2006), Genoscope (2009)
Brachybacterium (low temperature, UV-radiation)	Junge et al. (1998), Miteva et al. (2009)
Brevibacterium (low temperature)	Miteva et al. (2009)
Cellulomonas (low temperature)	Liu et al. (2009), Hansen et al. (2007)
Clavibacter (low temperature)	Liu et al. (2009)
Conexibacter (low temperature)	Wagner et al. (2009a)
Cryobacterium (low temperature)	Johnson et al. (2007)
Demequina (low temperature)	Finster et al. (2009)
Dermacoccus (high pressure)	Pathom-aree et al. (2006a), Pathom-aree et al. (2006b)
Dietzia (high salinity, high pressure)	Kleinsteuber et al. (2006), Al-Awadhi et al. (2007), Pathom-aree et al. (2006c), Helmke and Weyland (1984)
Friedmanniella (low temperature)	Aislabie et al. (2006), Schumann et al. (1997)
Frigoribacterium (low temperature)	Liu et al. (2009)
Geodermatophilus (low moisture, γ-radiation, UV-radiation)	Genoscope (2009), Gorbushina et al. (2007), Rainey et al. (2005)
Gordonia (low moisture)	Brandao et al. (2001)
Kocuria (low temperature, high salinity, high pressure)	Steven et al. (2007), Pathom-aree et al. (2006c), Tang et al. (2009)
Lechevalieria (low moisture, high salinity)	Okoro et al. (2009a)
Microbacterium (low temperature)	Liu et al. (2009), Miteva et al. (2009)
Microbacteriaceae (oligotrophic)	Vishnivetskaya et al. (2006), Hahn et al. (2003)
Micrococcaceae (oligotrophic, low temperature)	Vishnivetskaya et al. (2006)
Micrococcus (low temperature, UV-radiation)	Steven et al. (2007), Miteva et al. (2009), Dib et al. (2008), Shivaji et al. (1988), D'Amico et al. (2006)
Micromonospora (high pressure)	Pathom-aree et al. (2006c)
Modestobacter (low moisture, oligotrophic, UV radiation)	Chanal et al. (2006), Genoscope (2009), Mevs et al. (2000)

Table 12.1.2 (Continued)

Genus	References
Nocardia (low temperature, UV-radiation)	Dib et al. (2008), Al-Mueini et al. (2007), Babalola et al. (2009)
Nocardiodes (low temperature)	Gilichinsky et al. (2007)
Patulibacter (low temperature)	Reddy and Garcia-Pichel (2009)
Propionobacterium (low temperature)	Liebner et al. (2008)
Pseudonocardia (low temperature)	Al-Mueini et al. (2007), Babalola et al. (2009)
Rhodococcus (low temperature, UV-radiation, high pressure)	Liu et al. (2009), Steven et al. (2007), Helmke and Weyland (1984), Alvarez et al. (2004), Colquhoun et al. (1998)
Rhodoglobus (low temperature)	Sheridan et al. (2003)
Rubrobacter (low temperature, low moisture, γ-radiation, high salinity)	Aislabie et al. (2006), Chanal et al. (2006), Asgarani et al. (2000), Chen et al. (2004), Empadinhas et al. 2007, Ferreira et al. (1998)
Sanguibacter (low temperature)	Liu et al. (2009), Miteva et al. (2009)
Salinibacterium (low temperature)	Zhang et al. (2008)
Salinispora (halophilic)	Maldonado et al. (2005a)
Serinicoccus (halophilic)	Yi et al. (2004)
Sporichthya (low temperature)	Babalola et al. (2009)
Streptomyces (low temperature, high temperature, high salinity, low a_w, high pressure)	Okoro et al. (2009a), Pathom-aree et al. (2006c), Kuznetsov et al. (1992), Ivanova et al. (2009)
Subtercola (low temperature)	Mannisto et al. (2000)
Tsukamurella (high pressure)	Pathom-aree et al. (2006c)
Verrucosispora (high pressure)	Riedlinger et al. (2004)
Williamsia (high pressure)	Pathom-aree et al. (2006c)
Yonghaparkia (high alkalinity)	Yoon et al. (2006)
"Candidatus *Limnoluna*" (oligotrophic)	Hahn (2009)
"Candidatus *Microthrix*" (low temperature)	Knoop and Kunst (1998), Kumari et al. (2009)
"Candidatus *Planktoluna*" (oligotrophic)	Jezbera et al. (2009)

Extreme Ecosystem Determinants and the Distribution of Actinobacteria

Planet Earth presents a large number and diversity of environments that can be described as extreme either in terms of geochemical (pH, salinity, moisture), physical (temperature, pressure, radiation), or, both determinants. The anthropocentric view of extremophilic and extremotrophic organisms is one defined by a single environmental extreme but, as pointed out by Mesbah and Wiegel (2008), many environments pose multiple extremes as exemplified by deep-seas, geothermal springs, and high altitude deserts. Polyextremophily and

polyextremotrophy (adaptation to more than two extremes) have received comparatively little attention by microbiologists and this is especially evident with regard to actinobacteria. However, certain actinobacteria have evolved tolerances to multiple extreme conditions; some examples are given in the next section.

The recent attempt by Pikuta et al. (2007) to consider the distribution of archaea and bacteria in 2-dimensional matrices of geochemical-physical variables generates several challenging issues. Thus, evidence for the existence of acidopsychrophilic, acidohalophilic, and thermohalophilic organisms as yet is lacking, a finding consistent with the authors' hypothesis of a primary origin of acidophiles on a hot, cooling Earth, a non-salty primordial ocean, and psychrophily evolving only after a significant temperature drop. Moreover, alkaliphily probably developed only after accumulation of critical levels of mineral precipitation and atmospheric carbon dioxide, while halophily may have evolved at a still later stage when an arid climate dominated on the landmasses; it follows, therefore, that we might expect to find examples of actinobacterial polyextremophily and polyextremotrophy of the type described in non-actinobacteria and archaea (Mesbah and Wiegel 2008). Such a hypothesis is broadly in line with current proposals on actinobacterial evolution wherein the deepest ancestral lineages (subclasses *Acidimicrobidae* and *Rubrobacteridae*) contain acidophilic and thermoacidophilic members with some showing multiple extremophily and extremotrophy (*Rubrobacter*). Later diverging actinobacteria appear to have become especially successful microbiota of the arid cryosphere (e.g., members of the suborders *Frankineae* and *Micrococcineae*). While these interpretations must be regarded as very preliminary and considerably more data are needed in order to substantiate or modify such initial conclusions, the opportunities for microbiologists to discover new extremophilic/-trophic actinobacteria is timely, challenging, and exciting.

The reported distribution of actinobacteria in extreme environments is shown in *Table 12.1.3*. Again it should be stressed that the information is not exhaustive, and whereas a diversity of actinobacterial taxa are found in many of these environments, others appear to be colonized by solitary or only a few species of these bacteria. Deep-seas in this context are defined as depths greater than 200 m (i.e., bathyal and below, presenting hydrostatic pressures >2 MPa). Freshwater may seem to be an incongruous descriptor for an extreme environment; the notable extreme features here are very low nutrient concentrations, and waters that are influenced by extreme physical and geochemical conditions. Much of the research relating to actinobacteria in extreme environments has been published only in the past decade with particular interest focussed on cryo-, marine, and desert ecosystems. Moreover, the majority of this research has been directed at biodiversity surveys and the search for novel bioactive natural products, from which several conclusions can be drawn.

First, a major incongruence exists between the inventories of actinobacteria in extreme environments based on culture-dependent and culture-independent experimental approaches. Such discrepancy has been highlighted recently in a meta-analysis of deep-sea actinobacterial diversity (Jensen and Lauro 2008), uncovered in PCR-based and metagenomic surveys (Sogin et al. 2006; Rusch et al. 2007). Most surprisingly, bathyal and abyssal ocean sites (ca. 500–4,500 m) appeared to be dominated by 16S rRNA gene sequences attributable to the subclass *Acidimicrobidae* (up to 87% of all sequences). Comparison with Ribosomal Database Project and other culture collection-derived sequences of marine actinobacteria revealed that less than 1% represented members of the *Acidimicrobidae*.

Table 12.1.3

Global distribution of extremophilic and extremotrophic actinobacteria

Marine	**Inland waters**	**Desert**
1. Sediment: Sublittoral <200 m Bathyal 200–2,000 m Abyssal 2,000–6,000 m Hadal >6,000 m 2. Pelagic: Sublittoral Abyssal	Oligo- and ultra-oligotrophic lakes High altitude lakes Soda lakes Salt lakes Groundwaters: Permanently cold Alkaline	1. Australasia realm: Australia 2. Palaearctic realm: Egypt, Oman, Tunisia 3. Afrotropical realm: Namibia 4. Nearctic realm: USA 5. Neotropical realm: Argentina, Chile
Cryo-environments	**Geothermal sites**	**Soils**
1. Arctic: permafrost, sea-ice, saline springs 2. Antarctic: permafrost, dry mineral soils, lakes 3. Glaciers and Ice cover: Alaska, Antarctica, China, Everest, Greenland, Tibet	Hot springs Geothermal soils Volcanic mud	Acid Alkaline Soil crusts Exposed rock
Anthropocentric related		
Heavy metals, metalloids: As, Cd, Cu, Zn Salt mines Thermal pollution Solvent and diesel pollution Uranium heap leaching Mill liquors Activated sludge		

Second, while care must be taken in interpreting these data (e.g., biases resulting from culture-independent sequences based largely on pelagic sampling and culture-dependent sequences largely from benthic samples; and from detection limits of respective methodologies), it is clear that renewed efforts to develop innovative recovery and cultivation techniques are now de rigueur. Greater attention to ecosystem characteristics, for example, can drive successful new approaches as illustrated in the marine actinobacterial context by in situ isolation and cultivation (Kaeberlein et al. 2002; Gavrish et al. 2008), and dispersion and differential centrifugation of sediments (Maldonado et al. 2005b). In addition, having patience to accept long incubation times (e.g., up to 12 weeks, Gontang et al. 2007) and the rigors of dilution-to-extinction culture (e.g., Stingl et al. 2008) clearly benefit the recovery of "rare" extremotrophic actinobacteria.

Third, the fact that the majority of studies on actinobacteria in extreme environments have relied on PCR and metagenomic approaches leads to difficulties in determining the members of which taxa are dominant in ecological terms. Indeed, very few studies have been directed at their functional ecology or even to determine the metabolic state (actively growing, dormant) of such populations. I return to this latter point in the Concluding Observations.

Actinobacteria in Selected Extreme Ecosystems

In this section I examine the actinobacteria adapted to a selection of ecosystems and habitats in extreme environments; an important proviso is that in many cases adaptation is presumptive (i.e., organisms simply *detected* in the prescribed environment), and in others the mechanism (s) of adaptation are uncertain or unknown.

While these ecosystems are very varied in character (see *Table 12.1.3*), most relevant research has focussed on deep-sea, desert, and cryo-environments, environments that also constitute most of the Earth's biosphere. Greater than 70% of the planet's surface is covered by oceans, the average depth of which is 3,800 m (>38 MPa pressure) and are permanently cold (<5°C). The seafloor per se presents numerous habitats including sediments of various geochemical types and depths, cold seeps, saturated brines, and carbonate mounds. Approximately, 20% of the Earth's surface is frozen soil (permafrost), terrestrial, and polar ice (glacial, sea-ice), or under snow cover; while deserts occupy more than 22% of the terrestrial environment, of which about 7% are hyper-arid (see Desert Ecosystems below). It merits reiterating at this point that all three of these environments challenge life with a complexity of extreme conditions against which adaptation has had to contend.

Deep-Sea Ecosystems

The pioneering work of Zobell (Zobell and Morita 1959) showed conclusively that bacteria could be cultured from marine sediments at extreme depths while subsequently the presence of actinobacteria in such environments was reported in Weyland's classic paper published 40 years ago (Weyland 1969). However, it is only comparatively recently as developments in ocean engineering, and particularly in submersible technology, have become available to a wider community of microbiologists that deep-sea actinobacteria have attracted serious interest. Consequently, our understanding of this topic has progressed in a number of significant ways: recognition of a very wide diversity rather than a paucity of taxa, establishment of bona fide marine actinobacteria as opposed to run-off terrestrial organisms, global distribution throughout the world's oceans, presence at depths from shallow seas to deepest ocean trenches, and occurrence of pelagic and benthic populations (Bull et al. 2005; Bull and Stach 2007; Gontang et al. 2007; Jensen and Lauro 2008).

The principal environmental parameters that determine the distribution of actinobacteria in the deep-seas are pressure, temperature, nutrient concentrations, salinity, and particulate matter. The older view that actinobacteria in the deep-seas occurred largely as benthic populations is no longer tenable as rRNA gene clone library studies in particular (Rappé et al. 1999; Yoshida et al. 2008) have revealed abundant pelagic populations. The fewer reports of pelagic actinobacteria probably reflect, in part, minimal holistic appreciation of the ecosystem being studied and thence the use of inadequate isolation methods. A clear illustration of the latter can be seen in the work of Bouvier and del Giorgio (2006) who found dramatic increases in the proportion of actinobacteria (up to 35% total ocean bacterioplankton) when isolations were made in the absence of indigenous viruses.

Many deep-sea actinobacteria grow at low temperatures but to date no strictly psychrophilic members are known. A similar situation exists regarding pressure: obligate piezophilic actinobacteria have not yet been reported but members of a large taxonomic diversity have been isolated by our group from abyssal and hadal depths, including the extremity of

the Mariana Trench (Colquhuon et al. 1998; Pathom-aree et al. 2006a). Some of these strains (isolated at atmospheric pressure) grow well at pressures as high as 40 MPa (Heald et al. 2001) and in some cases (e.g., *Dermacoccus abyssi*; Pathom-aree et al. 2006b) growth at such elevated pressures may be greater than at atmospheric pressure. In this context the critical experiments would be to attempt isolations at the in situ pressures – to our knowledge these remain to be done. Actinobacteria also occur in deep below sea-floor sediments, sometimes as dominant populations (see Bull et al. 2005). The first bone fide Gram-positive piezophiles (*Carnobacterium*) were isolated recently (Lauro et al. 2007) and helix changes in 16S rRNA genes are proposed as adaptations to improve ribosome function in the deep-sea.

Obligate oligotrophic marine actinobacteria have not been detected but it is increasingly evident that the use of high nutrient concentrations is likely to impede the recovery of many such bacteria; the studies of Köpke et al. (2005) and Gontang et al. (2007) are especially relevant here. The German group used most probable number cultivation under conditions of varying but very low concentrations of carbon substrates to recover a much greater than usual proportion of the total bacteria in coastal sediments, among which were actinobacteria. In an analysis of a large collection of inter-tidal and deep-sea sediments the Scripps group recovered the majority of actinobacteria (>70%) using low nutrient seawater media, some of which were isolated exclusively on such media. Both of these groups were cognizant of low growth rates that might be anticipated by marine oligotrophs and imposed long incubation times (12 weeks) on their experiments. Gontang et al. (loc cit) also found that a high proportion of their actinobacterial collection required, or had enhanced growth in the presence of seawater. The seawater-requiring strains represented new taxa or were most closely related to known marine-derived actinomycetes. Apart from strains related to the first described seawater-obligate actinobacterium (*Salinispora*, Maldonado et al. 2005a), the seawater-requiring actinomycetes represented several genera (see *Table 12.1.1*). Some halotrophic marine actinomycetes are reported to require seawater for sporulation and secondary metabolite production (Sunga et al. 2008). It should be noted in passing that several instances exist of seawater-requirements being lost by actinomycetes on prolonged laboratory cultivation.

As recently as a decade ago the taxonomic diversity of actinobacteria in the marine environment was thought to be very low. This view has been dispelled categorically by culture-dependent and culture-independent inventories and the recognition of indigenous marine actinobacteria and other ecologically less certain marine-derived actinobacteria. The results of all recent studies point to a great diversity of these bacteria in the oceans: for example, (1) over 1,000 reported OTUs in deep Atlantic Ocean sediments using richness estimators (Stach and Bull 2005) the majority of which were judged to be novel species or genera; (2) representatives of 6 families in the *Actinomycetales* cultured from Pacific Ocean sediments (Prieto-Davo et al. 2008); and (3) meta-analyses referred to above (Jensen and Lauro 2008) revealed large, unexpected occurrence of uncultured members of the subclass *Acidimicrobidae*. This is not the place for a detailed discussion of infraspecific diversity but in considering marine actinobacteria – as is the case throughout environmental microbiology – such variation has bearing on ecology and the search for natural products. Thus, fine scale molecular fingerprinting may be required to dereplicate (differentiate) marine collections of taxonomically "critical" genera such as *Micromonospora* (Maldonado et al. 2008), while concatenated gene sequences and numerical phenetic analyses have differentiated six ecovars of alkaliphilic *Streptomyces griseus* isolated from adjacent locations in a coastal marine habitat (Antony-Babu et al. 2008). The significance of ecovar diversity in this instance remains to be established.

On the basis of few pertinent investigations, we have speculated previously on the possible ecological roles of actinobacteria in the deep-seas (Bull et al. 2005) and suggested methodological approaches that might be deployed in opening up this subject. We can add "reverse ecology" to techniques such as stable isotope tracing and microarray applications for probing deep-sea ecology now that rapid, inexpensive whole genome sequencing is becoming available (discussed in section Concluding Observations). To date the only complete genome sequence reported for a deep-sea actinobacterium is that of the obligate marine species *Salinispora tropica* (Udwary et al. 2007) and most interrogation of this genome has focussed on natural product synthesis. Given the dominant occurrence of members of the *Acidimicrobidae* in certain deep-seas, it is timely to increase efforts for their cultivation and thence genome analyses in order to explore their adaptation to marine environments and the evolution of this deep lineaged group of actinobacteria.

Desert Ecosystems

Hyper-arid or true deserts are large contiguous landmasses with low or zero vegetation cover, and where rainfall is extremely low and unpredictable in time and space. In these biomes the ratio of mean annual rainfall to mean annual evaporation is less than 0.05 and those where the ratio falls below 0.002 are defined as extreme hyper-arid. Whereas most large deserts are located inland, others, typified by the Atacama Desert (northern Chile) are coastal and aridity develops as a consequence of cold off-shore currents. Some dry polar and high mountain areas also are defined as deserts but given their permanent low temperatures they are treated in the section on Cryo-environments.

The extreme desiccation status of hyper-arid deserts is often compounded by intense radiation exposure, oligotrophic nutrient availability, high salinity, and high concentrations of metals and inorganic oxidants. It has been suggested that such conditions are inimical even to microbial life (McKay et al. 2003; Navarro-Gonzalez et al. 2003) a notion that may help explain the relative neglect of hyper-arid deserts by microbiologists. Although reports can be found of actinobacteria in soils of most desert realms (*Table 12.1.3*), greatest attention has focussed on Nearctic (e.g., Sonoran, Mojave) and Neotropical (e.g., Atacama) (Gómez-Silva et al. 2008) deserts. The Atacama Desert is especially interesting as it is probably the oldest on Earth and has been conditioned by extreme hyper-aridity for at least 3 and possibly 15 million years (Houston and Hartley 2003).

While some authors (Navarro-Gonzalez et al., loc cit) have experienced little or no success in recovering actinobacteria or amplifiable DNA from hyper-arid desert sites, a substantial diversity of these bacteria has been revealed over the past decade principally as a result of applying DNA and other chemical fingerprinting techniques. However, culture-dependent studies are indispensable in providing material for investigating physiology and enabling whole genomes to be sequenced. Most records of actinobacteria in desert soils are members of the *Actinomycetales*, an unsurprising fact given the prevalent use of traditional actinomycete isolation media and conditions. Once again we find great incongruence between culture-dependent and culture-independent inventories of desert actinobacteria and this is seen dramatically in the work of Chanal et al. (2006). These authors analyzed prokaryotic communities in hot, arid deserts sites in Tataouime (Tunisia) and found that the culture library was dominated by members of the suborder *Micrococcineae* of the *Actinomycetales* (*Arthrobacter*, *Beutenbergia*). However, these organisms were not detected in PCR-generated clone libraries,

the latter being dominated by phylotypes most closely related to *Rubrobacter* and *Thermoleophilum*, that is, representatives of the deepest branching actinobacterial lineage, the subclass *Rubrobacteridae*. Another compelling outcome of this study was the increasing number of clones with reducing percent similarity to closest known phylotypes (5% at 98–100% similarity, rising to >40% at <78% similarity).

The Puna de Atacama is the location of some of the world's highest volcanoes where the soil water content is frequently below detection limits, the carbon content very low, and UV radiation very high. The microbiology of this extreme environment on the Volcan Socompa has been investigated recently by Schmidt's group (Costello et al. 2009) who found that actinobacterial phylotypes dominated non-fumarole soil (5,235 m altitude, −5°C) and formed a major fraction of warm-fumarole (5,824 m, 25°C) communities. Moreover, soils sampled from the adjacent Volcan Llullaillaco are rich in novel sequences related to the *Rubrobacteridae* (Steve Schmidt 2009, personal communication). These findings taken together with those discussed above reinforce the urgent need to increase efforts to develop isolation methods for "as-yet-uncultured" actinobacteria.

Studies in the extreme hyper-arid Yungay area of the Atacama Desert also showed actinobacteria phylotypes dominating (94%) the soil community, the majority of which had closest affinity with the genus *Frankia* (Connon et al. 2007). Signatures of the *Frankineae* also were found among DNA clone libraries recovered from the Tataouime Desert.

The first extensive isolations of actinobacteria from hyper-arid desert soils were reported recently by (Okoro et al. 2009a). These authors used selective isolations methods to recover actinomycetes, which, in the case of extreme hyper-arid soil (water content 0.004%, organic matter 0.03%) were the only culturable bacteria detected. A high proportion of these actinomycetes represent novel centers of taxonomic variation (e.g., new species of *Lechevalieria*, Okoro et al. 2009b).

As stated previously, the physico-chemical factors determining the diversity and abundance of bacteria in desert ecosystems are multiple and in most cases poorly understood. For example, either by direct experimentation or by inference we can point to members of the genera *Rubrobacter* and *Geodermatophilus* being extremotrophic or resistant to UV- and γ-radiation, desiccation, and high temperature (Ferreira et al. 1998; Rainey et al. 2005; Chanal et al. 2006). However, a consensus is emerging that water availability is the primary controlling factor for microbial diversity and activity in desert soils. Water activity (a_w, ratio of vapor pressure of sample to that of pure water) measurements taken on Atacama Desert (Yungay) soils were deemed to be too low to support microbial growth and even the diurnal maximum of 0.52 was below the lowest a_w reported for microbial growth (Connon et al. 2007). These authors suggest that microbial communities in this extreme environment may show periodic growth coinciding with rare precipitation events. Similar studies of hypolithic community diversity in hyper-arid deserts of China were correlated with availability of liquid water but not with temperature or precipitation alone (Pointing et al. 2007). On the basis of surveys made on Antarctic Dry Valley, Atacama Desert, and Volcan Socompa non-fumarole soils, Costello et al. (2009) conclude that actinobacteria dominate those bacterial communities that exist below the dry limit of photoautotrophy. The question arises, therefore, how do actinobacteria survive extreme desiccation stress in hyper-arid ecosystems?

Resistance to ionizing radiation (both UV and gamma) is a feature of several desert-derived actinobacteria (*Table 12.1.2*) but the DNA repair mechanisms that have evolved may be more in response to extreme desiccation (surface radiation exposure on Earth over geologic times has been of the order of 0.05–20 rads/year; Makarova et al. 2001; but see below). This correlation has

been tested by exposing arid (Sonoran Desert) and non-arid (Louisiana forest) soils to high doses of γ-radiation and analyzing the radiation-resistant organisms that survived (Rainey et al. 2005). The most resistant bacteria in the arid soil were strains of *Deinococcus* (a well-documented radiation-resistant bacterium) and, more surprisingly, the actinobacterium *Geodermatophilus* (order *Actinomycetales*), all of which withstood up to 30 Gy (3 krads) soil irradiation. Isolates belonging to these genera were not recovered from non-arid soil even after low irradiation.

Members of the superclade Terrabacteria (Battistuzzi and Hedges 2009) increase in abundance after soil drying whereas members of Gram-negative taxa decrease, and it has been suggested that peptidoglycan confers ancestral desiccation resistance in *Actinobacteria* and *Fermicutes*. Definition of the Terrabacteria was made on the basis of concatenated datasets of small and large subunit rRNA genes and protein coding genes, and supported by cytological and physiological characteristics. A striking outcome of these analyses was that arid soils were the predominant habitat of *Actinobacteria* (64%), whereas deep-seas constituted a minor habitat (5%) for these bacteria. Thus, this superclade likely evolved on land early in the Precambrian era, and thereafter some actinobacterial lineages invaded or reinvaded marine environments.

Strains of extremophilic and extremotolerant *Rubrobacter* have been shown to express classic phenotypes of ionizing radiation and desiccation resistant bacteria, for example, DNA repair mechanisms (Asgarani et al. 2000), physiological protectants such as trehalose (Nobre et al. 2008), and protection of proteins from oxidation damage (Daly et al. 2007). Protein protection may be more significant than DNA repair for these organisms and Daly and his colleagues have established that both radiation and desiccation resistance depend on the abundance of cellular manganese complexes (Daly 2009 and Chap. 10.1 Deinococcus radiodurans: Revising the Molecular Basis for Radiation Effects on Cells). The fact that within deep below seafloor sites are those that are both Mn-rich and exposed to high natural γ-radiation has led Daly to propose (see Sghaier et al. 2008) that ionizing radiation levels may have been much higher in ancient geological time and hence helped shape the evolution of *Rubrobacter* and similar bacteria. The discovery of the *treT* gene in the thermophilic, halotolerant, extreme radiation/desiccation resistant *R. xylanophilus* was exceptional and suggests a very ancient origin of this trehalose synthesis pathway in *Rubrobacter* (Nobre et al. 2008). Clearly findings of this sort should encourage further research into the mechanisms of extremotrophy in this ancient lineage of actinobacteria, while the recent in silico prediction of positively selected genes having potential roles in the resistance to γ-radiation and desiccation provides a rational basis for experimental enquiry (Sghaier et al., loc cit).

Cryo-Environments

Among the relatively under-explored ecosystems are those that are persistently cold. The results of recent research commend that "the role of permanently cold ecosystems in global ecology must be reassessed. . .(and). . .that the Earth's cryosphere. . .should be included as biosphere components of our planet" (Priscu and Christner 2004). Impetus for studying the microbiology of the cryosphere has grown in the light of climate change debates and the search for extraterrestrial life. Excellent overviews of the subject are provided by Priscu and Christner (2004), D'Amico et al. (2006), and Deming and Eicken (2007). The following discussion is intended to highlight some of the data emerging on actinobacteria in the cryosphere.

Five major ice formations are found on Earth that contain liquid water: ice sheets (and their ice-sealed lakes), glaciers, marine ice shelves, sea ice, and permafrost. These cryo-environments

frequently harbor distinctive ecological niches such as polygon formations in permafrost soils, cryoconite holes on the surface of ice sheets and glaciers, and permanently cold groundwater. Moreover, as in the case of hyper-arid deserts, actinobacteria in cryo-environments often have to respond to stressors other than temperature, viz radiation, oligotrophy, and salinity.

Descriptions of actinobacteria from cryo-environments not infrequently are deficient and adherence to accepted definitions is important for the understanding and publication of their ecology and physiology. The determination of cardinal temperatures in this regard is critical. Only organisms growing optimally at or below 10°C are defined as obligate psychrophiles, whereas many actinobacteria grow over wide temperature ranges (ca. 0°C to >28°C) but optimally only above 10°C. Helmke and Weyland (2004) have proposed the term moderate psychrophile for organisms having a $T_{min} \leq 0°C$, $T_{max} \leq 25°C$, and a T_{opt} of >10°C; on this basis cryotolerant actinomycetes such as *Modestobacter multiseptatus* (Mevs et al. 2000), *Subtercola boreus, S. frigoramans* (Mannisto et al. 2000), and *S. xinjiangense* (Zhang et al. 2008) must be classified as moderate psychrophiles. These latter species – and many others recovered from the cryosphere – are eurythermic, that is, adapted to grow over a wide range of temperature; in contrast cold adapted stenothermic actinobacteria grow within a narrower temperature range. Recent research reported by Liu et al. (2009) has attempted to dissect the actinobacterial population in meltwater on the Rongbuk Glacier into ecophysiological types on the basis of survival strategies; putative stenotherms were versatile in carbon substrate utilization, while putative eurytherms showed more restrictive nutrition. Unfortunately, the temperature range studied omitted that at which the isolations were made (4°C), but studies of this type will be important for establishing which members of cryosphere communities are residents, pioneers, or tourists (sensu Deming and Eicken, loc cit).

The need for new methods in order to isolate actinobacteria from extreme environments has been emphasized earlier in this discussion. Metagenomic inventories notwithstanding, the access to cultured organisms is crucial for defining those phenotypic traits that affect adaptation to and survival in the extremobiosphere. This prescription applies very forcibly to the study of cryosphere actinobacteria as demonstrated by the following recent investigations.

Perhaps most pointedly (ominously?) is the finding by Hansen et al. (2007) that in an ancient (2,347 years; Spitzbergen) permafrost soil only about one quarter of observed bacterial organisms were viable, and that "standard" methods of isolation recovered only a minor fraction of the community. Manipulation of the isolation media and conditions by imposing oxic or anoxic states, low water activity, or by nutrient supplementations led to a much greater recovery of bacteria over 80% of which were actinobacteria. The latter population was dominated (77%) by members of the suborder *Micrococcineae*, particularly by isolates most closely related to *Cellulomonas* and *Arthrobacter*. Compared to permafrost soil, the underlying ground ice presents an even more severe environment for bacteria; thus viabilities of ca. 13% down to 0.01% have been reported in various layers of permafrost soil but viable bacteria were not recovered from the ground ice (Steven et al. 2008).

Miteva and Brenchley (2005) observed the microbial population in a Greenland glacier ice-core (ca. 3,000 m deep) to be dominated by ultrasmall organisms. Enrichment for these bacteria was made by selective and successive filtrations of melted ice and cultivation in anaerobic, low nutrient liquid media at 5°C over long incubation times. This protocol greatly increased the culturability, numbers, and diversity of ultrasmall high G+C bacteria related to *Arthrobacter* and *Microbacterium*; the relatedness of the *Arthrobacter* isolates was distant in most cases, suggestive of distinct new phyletic line(s) within the *Micrococcaceae*. Members of the genus *Arthrobacter* are usually regarded as aerobes but several authors have shown that this

metabolically versatile and ecologically successful genus contain ones that grow anaerobically using nitrate respiration, for example. Subsequently, Miteva et al. (2009) found that >60% of bacteria isolated from clear ice cores in this environment were actinobacteria (all members of the *Micrococcineae)*, and in some cores were the only culturable bacteria.

The final case considers the use of dilution-to-extinction culture of planktonic actinobacteria from a permanently ice-covered lake in Antarctica. Isolations were made under oligotrophic, aerobic, and low temperature (4°C) conditions; whereas the vast majority of isolates were not related to known species (Stingl et al. 2008), there was substantial overlap between them and 16S rRNA gene-derived clone libraries. The actinobacterial isolates represented members of deeply branching lineages (*Acidimicrobium*, suborder *Acidimocrobineae*, and *Sporichthya*, suborder *Frankineae*).

Earth's cryosphere has developed from ice formed as a result of accumulated snowfall at the poles and trans-globally in high mountains, and actinobacteria have been recovered from or identified in each of the ice formations mentioned above, and at all geographic cryosphere sites. In one monumental study, Yergeau et al. (2007) examined the pattern of bacterial diversity over a >3,200 km Antarctic terrestrial transect covering 27° of latitude and increasingly severe environments. Actinobacterial phylotypes were detected at all of the sites and comprised ca. 25% of the clone library in Mars Oasis polygon soil (72° south, seasonal temperature range 9°C to −40°C). In a number of cases, data are available on the presence and abundance of actinobacteria in cryo-environments of known age. Thus, representatives of the *Micrococcineae* appeared to be the dominant bacteria in Spitzbergen permafrost soil of ca. 2,350 years old (Hansen et al. 2007) while members of this suborder also were preponderant in ancient (up to 2–3 million years continuously frozen) Siberian permafrost sediments (Vishnivetskaya et al. 2006). Of particular interest is the study of bacterial diversity at different depths (≅ ages) of a Greenland ice core (Miteva et al. 2009). Actinobacterial phylotypes were dominant in ice layers representing a relatively milder climatic period (i.e., −45°C, 57,000 years ago) and less so in older ice. Again, there is evidence of members of the *Micrococcineae* being part of the bacterial communities in this ancient cryosphere, while actinobacteria may prove to be markers for defining specific deposition climates. Permafrost depth analysis has been reported by Steven et al. (2008) on cores taken at Ellesmere Island (Canadian high Arctic); this permafrost is believed to have the lowest ground temperature (−15°C) in the world. Actinobacterial phylotypes dominated (55%) the active layer (subject to seasonal freeze and thaw), were rather less abundant at the permafrost table (the surface layer of the permanently frozen permafrost), and were minor components of the permafrost per se (15%) and ground ice (30%). No viable bacteria were recovered from ground ice samples.

As studies grow of actinobacteria in the cryosphere, an interesting pattern of presence, and most often dominance, emerges; namely, that members of the suborder *Micrococcineae* are a common feature of cold environments. Thus, isolates and phylotypes are found in the complete range of ice formations (Antarctic soil 77°S, Siberian permafrost 72°N, Asian and Greenland glaciers, cryoconite holes, sea ice, ice cover of and accretion ice in subglacial lakes, cryptoendoliths in rocks, etc.), ecosystems that have little in common apart from experiencing permanent cold or freezing. Moreover, of the newly described cryosphere-derived actinobacteria most are members of this suborder (*Subtercola*, Mannisto et al. 2000; *Rhodoglobus*, Sheridan et al. 2003; *Salinibacterium*, Zhang et al. 2008; *Demequina*, Finster et al. 2009; *Glaciibacter*, Katayama et al. 2009). Of the 96 genera currently comprising the *Micrococcineae*, over 25% are reported as cryosphere actinobacteria. Further discussion of the prevalence of members of the *Micrococcineae* in the extremobiosphere follows below.

A question common to discussions of actinobacteria in the extremobiosphere is: are the organisms reported bona fide indigenous residents, pioneers, or simple tourists? In the context of the cryosphere, resident actinobacteria will be cold-adapted and strong competitors with pioneer organisms among which on the basis of present evidence are likely to be other actinobacteria originating in other climes. Tourist actinobacteria are those transported to cryosphere ecosystems, most frequently but not invariably via airborne particles, and while surviving are noncompetitive with residents. For example, bacterial isolates in Greenland clear ice cores were related to organisms from Asian deserts, marine aerosols, and volcanic dust (Miteva et al. 2009) that may be indicative of the origins of the deposited microbiota. Deming and Eicken (2007) comment that sea-ice "is the best studied and most biologically active of all ice formations," and unlike others in which thermophilic tourists have been preserved, "no thermophiles and rarely even mesophiles have been cultured from sea-ice." This use of thermophilic indicator organisms as a means of circumscribing indigenous bacterial populations has been used previously to define deep-sea actinobacterial communities as opposed to those originating from terrestrial run-off (Colquhoun et al. 1998). Candidates for resident cryosphere actinobacteria are those shown in *Table 12.1.1*; even given the probable incompleteness of the data it is interesting to note that most of the genera containing psychrophilic members are of the *Micrococcineae* while some (*Arthrobacter, Brachybacterium*) have been isolated from sea-ice. The key issue is whether or not these organisms are metabolically active or growing in ice formations.

Evidence regarding microbial activity in situ in the cryosphere is limited, decidedly so for actinobacteria. As Deming and Eicken (2007) note, there have been very few direct attempts to investigate bacterial activity or growth either in natural ice formations or ex situ, and as yet a lower temperature limit that permits maintenance metabolism but not growth remains to be determined. Data for actinobacteria are very few, as pertinent research is in its infancy and even basic information comparable to that on viability (Hansen et al. 2007; Steven et al. 2008) is lacking for almost all cryo-environments. However, a number of recent studies of permafrost soils indirectly support the presence of metabolically active actinobacterial populations. Bottos et al. (2008) isolated a number of bacteria from permafrost melt-water and the majority of isolates that grew at −5°C were actinobacteria (closest matching to *Cryobacterium*); several other members of the *Micrococcineae* were isolated that grew at 0°C. Radiorespirometric experiments with permafrost core material showed a correlation between the presence of these culturable actinobacteria and the highest rates of ^{14}C-glucose mineralization. Similarly, Steven et al. (2008) recovered *Arthrobacter* strains from permafrost that could grow at subzero temperatures or 0°C and occurred in horizons where microbial activity was detected by ^{14}C-substrate mineralization. The use of BIOLOG plate screening was used by Wagner et al. (2009) to describe the activity of bacterial communities in different horizons of a Siberian permafrost soil. The diversity and viable numbers of actinobacteria increased (together with that of the *Firmicutes*) at the permafrost table, a finding that led the authors to propose the specialization of these organisms to a cold, constant anaerobic environment. Finally, Vishnivetskaya et al. (2006) found that actinobacteria, especially strains of *Arthrobacter*, dominated an ancient Siberian permafrost and that >90% of these grew at −2.5°C indicating, in the authors' opinion, that they were psychroactive.

Preliminary and tentative as these conclusions are, they begin to reveal correlation trends that argue for ecological roles of actinobacteria in the cryosphere. The application of modern methods for in situ interrogation of defined members of microbial communities (see Concluding Observations) promises to resolve such questions.

If we accept that the first glaciation events on Earth occurred in the Paleoarchaean era (ca. 2.3 Ga) and that the *Actinobacteria* appeared in the Mesoarchaean era (ca. 3.18 Ga), these bacteria would have been evolving for nearly 900 million years before the onset of persistently cold conditions. Moreover, the proposed divergence of *Arthrobacter* (and thence the suborder *Micrococcineae*) at the beginning of the Neoarchaean era (Sheridan et al. 2003) occurred approximately 450 million years prior to this glaciation event. Deming and Eicken (2007) opine that "the genetic steps from mesophily to psychrophily do not appear to be particularly daunting" so *Arthrobacter*-like organisms could have been among the earliest to evolve cold adaptation features.

Current evidence points to a cosmopolitan distribution of psychrophilic and psychrotrophic actinobacteria and the presence of closely related taxa within the complete range of ice formations suggest that clades within such taxa have evolved common cold-adaptive colonization and survival mechanisms. As D'Amico et al. (2006) have indicated, the critical problems that organisms in permanently cold environments have to overcome are high viscosity and low thermal energy, which combine to impose overall low metabolic flux. Comparative genomics of cold-adapted microorganisms show that they have evolved a portmanteau of synergistic phenoptypes that enable their successful colonization of the cryosphere (Methé et al. 2005; Grzymski et al. 2006) and affect membrane fluidity (see below), transcription and translation, DNA repair, and ice nucleation.

It is well known to microbial physiologists that temperature affects the specific growth rate and the specific maintenance rate of organisms. Low temperature causes both functions to fall and hence reduce the overall energy demands of the cell. Chemostat experiments with *Arthrobacter* species show conclusively that low specific growth rates and low temperatures induce a rod to coccus morphological switch (Luscombe and Gray 1974) with a concomitant increase in the thickness of the cell walls (Duxbury et al. 1977). The occurrence of ultrasmall cocci of arthrobacters in glacier ice cores (Miteva and Brenchley 2005) and of thick walled bacteria in cryo-environments is not without interest in this context. Similarly, microbial physiologists have long appreciated the relationship between temperature and membrane chemistry, and thence fluidity (for review see Russell 1997), a recent case in point being that of *Arthrobacter chlorophenolicus* (Unell et al. 2007). This bacterium adapts to low temperature by increasing the ratio of anteiso- to iso-fatty acids in its cell membrane thereby decreasing its viscosity and maintaining a functional fluidity. The findings of White et al. (2000) are particularly notable in view of the consensus on cold-adaptation. Of eight dominant bacteria cultured from an Antarctic fellfield soil, five of which were members of the *Micrococcineae*, there was no single strategy of thermal adaptation vis-à-vis maintenance of membrane fluidity; a combination of changes in fatty acid branching, unsaturation, and chain length was observed that were not correlated with phylogeny.

Although dormancy has been reported to be the most effective strategy for survival in cryo-environments, the work of Johnson et al. (2007) seriously challenges this hypothesis. These authors investigated DNA repair capacities of bacteria in permafrost samples from the Canadian High Arctic, Siberia, and Antarctica by screening the phylotypes recovered following DNA treatment with uracil-*N*-glycosylase (UNG) treatment. Analysis of UNG treated sequences effectively discriminates between dormant (no DNA repair) and metabolically active (DNA repair) physiological states; no bacteria having a known capacity for dormancy were detected in the oldest samples (400–600 Ky) for which amplicons were obtained, instead the latter DNA was completely dominated by *Actinobacteria* chiefly related to *Arthrobacter*. Earlier studies by Berger and colleagues (Berger et al. 1996, 1997) showed that the cold acclimation

(adaptive) protein CapA was induced in cold-shocked, pyschrotrophic *Arthrobacter globiformis*, was continually expressed throughout growth at low temperature, and thought to be involved in restoring and subsequently maintaining active translation. More recently cold-adapted bacteria (including *Arthrobacter*) isolated in Antarctica expressed cold adaptive protein CapB and the ice-binding protein IBO when grown at low temperature (Mojib et al. 2008), again suggesting essential roles for survival and protection from freeze challenge. Finally in this context of cold-adaptive mechanisms in actinobacteria, the elevated synthesis of cryoprotectants such as α,α-trehalose and glycerol by psychrolerant species grown at low temperature is to be noted (Ivanova et al. 2009).

Miscellaneous Extreme Environments

Of other extreme environments, two will be considered briefly here: inland waters, and polluted sites. Inland waters include soda and salt lakes and, perhaps incongruously on first encounter, freshwater lakes but the contextual interest in freshwater lakes revolves around their oligotrophy and high UV radiation exposure. Only recently have indigenous, "typical freshwater bacteria" (Zwart et al. 2002) been recognized and usually the dominant types are actinobacteria (≥70% abundance) characterized by ultrasmall organisms (Hahn et al. 2003). Warnecke et al. (2005) confirmed that the planktonic bacteria dominating high altitude, UV transparent lakes were indigenous actinobacteria (the acI lineage) but cautioned that this adaptation to UV stress was correlative, not necessarily causal. Currently no pure cultures have been obtained of these indigenous actinobacteria with which to assess their UV resistance. Phylotypes and cocultures of these freshwater organisms have been most frequently related to members of the *Micrococcineae* and recently Hahn has described a novel monophyletic clade within the family *Microbacteriaceae* (Hahn 2009). Seven novel taxa have been described but to date only as candidate species because pure cultures have not been achieved; helper bacteria mainly belonging to the *β-Proteobacteria* are needed to establish quite intimate contact to enable growth of the actinobacteria. The mechanistic basis of this synergy remains unknown.

Apart from freshwater, inland waters such as salt and soda lakes also are a rich source of novel actinobacteria. New salt lake-derived genera include *Streptimonospora* (Cui et al. 2001), *Haloactinospora* (Tang et al. 2008), and *Haloglycomyces* (Guan et al. 2009), while comparable discoveries from soda lakes include *Microcella* (Tiago et al. 2005), *Yonghaparkia* (Yoon et al. 2006), and *Nutriliruptor* (Sorokin et al. 2009). *Nutriliruptor alkaliphilus* is a particularly exciting organism because it is the first described member of a new, deeply branching order within the *Actinobacteria*, and is obligately alkaliphilic, moderately halophilic, and grows on a range of nitriles. Thermophilic actinobacteria have been isolated from hot springs (*Rubrobacter*, Chen et al. 2004), while culture-independent methods have shown for the first time that a major diversity of actinobacteria can be found in these environments at temperatures as high as 81°C (Song et al. 2009); phylotypes were related to members of the *Rubrobacteriales*, and the actinomycete suborder *Frankineae*.

Finally, a brief sample of cases in which actinobacteria have adapted to extreme environments caused by human intervention. The thermophilic *Rubrobacter xylanophilus* (T_{opt} 60°C) was first isolated from a thermally polluted site (Carreto et al. 1996), and subsequently other members of deep lineage actinobacteria have been confirmed as components of metal leaching or metal stressed communities. The first member of the ferrous ion oxidizing genus *Acidimicrobium* (*Acidimicrobium ferrooxidans*) was isolated by Clark and Norris (1996) and

thereafter Johnson's group have described several Fe^{2+} oxidizing actinobacteria recovered from mine and leaching sites. An *Acidimicrobium* sp. isolated from a nickel leaching operation had the ability to outcompete *A. ferrooxidans* (Cleaver et al. 2007), while members of novel Fe^{2+} oxidizing taxa belonging to the *Acidimicrobidae* were described very recently (Johnson et al. 2009). The latter organisms are extremely acidophilic and have been placed in the new genera *Ferrimicrobium* and *Ferrithrix*. Acidiphilc Fe^{2+} oxidizers also are found in the *Rubrobacteridae* (Bryan and Johnson 2008). The increasing numbers of reports of acidophilic and thermo-acidophilic actinobacteria in the *Acidimicrobidae* and *Rubrobacteridae* are consistent with their proposed ancient lineages and the broad trends of the Earth's geochemical history.

Natural Products

The growing awareness of the diversity of actinobacteria in the extremosphere has stimulated renewed efforts to explore their natural product chemistry, not least given the imperative to discover novel drugs. To date, most attention has been applied to marine and, especially, deep-sea environments as a source of novel organisms and, presumptively, novel exploitable chemistry. This expectation of discovering novel, bioactive natural products has been borne out in practice as demonstrated by the selection of examples shown in *Table 12.1.4*. Extensive discussions of marine natural products can be found in Blunt et al. (2009), in reviews of actinobacterial products (Fenical and Jensen 2006; Bull and Stach 2007; Williams 2008) and in accounts of specific bioactivities of marine actinomycetes, for example, antitumor compounds (Olano et al. 2009). This is not the place for an exhaustive review of marine actinobacterial natural products, rather I wish to make a few overall points relating to their producing organisms and chemistry.

A notable feature of the data in *Table 12.1.4* is the preponderance of organisms that are members of just two actinomycete families: *Streptomycetaceae* (45%; *Streptomyces*) and *Micromonosporaceae* (36%; *Micromonospora*, *Salinispora*, *Verrucosispora*); a very similar distribution is seen among marine antitumor compound producing actinomycetes (58% and 23%, respectively). Inasmuch as members of these two families have long been known as most prolific producers of secondary metabolites, these findings are not surprising; however, given the great diversity of actinobacteria in the deep-seas, they suggest that a too conservative approach to isolation and screening still prevails. With regard to screening, the case of *Rhodococcus* is a compelling one. Traditionally regarded as lacking interest from a screening prospective *Rhodococcus* species are abundant and diverse in the world's oceans (see, e.g., Colquhoun et al. 1998). Recently, novel antibiotic activities have been described in members of this genus (Kitagawa and Tamura 2008), while the large number of non-ribosomal peptide synthase (24) and polyketide synthase (7) gene clusters in the genome of the first completely sequenced *Rhodococcus* (McLeod et al. 2006) commend that extremotrophic rhodococci be included in screening campaigns. This general notion is supported by the fact that other extremotrophic actinobacteria previously ignored as producers of secondary metabolites are revealing unexpected promise in the field of natural products (e.g., deep-sea *Dermacoccus* isolates).

The high diversity that appears to be a feature of microbial communities in several extreme ecosystems may be another manifestation of "the paradox of plankton" phenomenon (see Bull 2004), namely, that the number of coexisting species exceeds the number of limiting resources.

Table 12.1.4

Natural products from actinomycetes in the extremobiosphere

Compound	Organism	Origin	Chemistry	Bioactivity	References
Albidopyrone	*Streptomyces* sp.	DSS	α-Pyrrone	Insulin agonist	Hohmann et al. (2009)
Ammosamides	*Streptomyces* sp.	DSS	Pyrroloquinoline alkaloids	Cell cycle modulators	Hughes et al. (2009)
Arenamides	*Salinispora arenicola*	MS	Depsipeptides	NFκB inhibitors	Asolkar et al. (2009)
Caboxamycin	*Streptomyces* sp.	DSS	Benzoxazole	Antibacterial Antitumor	Hohmann et al. (2009)
Dermacozines	*Dermacoccus abyssi*	DSS	Phenazines	Antioxidant Antitumor	Wagner et al. (2009b)
Echinomycin	*Streptomyces* sp.	MS	Peptide	Antibacterial	Socha et al. (2009)
Erythronolides	*Actinopolyspora*	SS	Erythromycin congeners	Antibacterial	Huang et al. (2009a)
Indoxamycins	Streptomycete	MS	Polyketide	Antitumor	Sato et al. (2009)
Marinosporolides	"*Marinispora*" sp.	MS	Macrolides	Anti-*Candida*	Kwon et al. (2006)
Splenocins	*Streptomyces* sp.	MS	Lactones	Anti-inflammatory	Strangman et al. (2009)
Lynamicins	"*Marinispora*" sp.	MS	Pyrroles	Antibacterial	McArthur et al. (2008)
Marineosins	*Streptomyces* sp.	MS	Spiroaminals	Antitumor	Boonlarppradab et al. (2008)
Marinopyrroles	*Streptomyces* sp.	MS	Halogenated pyrroles	Antibacterial	Hughes et al. (2008)
Pacificanones	*Salinispora pacifica*	MS	Polyketides	Antitumor	Oh et al. (2008)
Proximicins	*Verrucosispora* sp.	MS	Aminofuran	Antitumor	Fiedler et al. (2008)
Salinipyrrones	*Salinispora pacifica*	MS	Polyketides	Antitumor	Oh et al. (2008)
Arenicolides	*Salinispora arenicola*	MS	Polyketides	Antitumor	Williams et al. (2007b)
Lucentamycins	*Nocardiopsis lucentensis*	MS	Non-ribosomal peptides	Antitumor	Cho et al. (2007)
Piperazimycins	*Streptomyces* sp.	MS	Non-ribosomal peptides	Antitumor	Miller et al. (2007)
Saliniketals	*Salinispora arenicola*	MS	Polyketides	Antitumor	Williams et al. (2007a)
Daryamides	*Streptomyces* sp.	MS	Polyketides	Antitumor	Asolkar et al. (2006)

Table 12.1.4 (Continued)

Compound	Organism	Origin	Chemistry	Bioactivity	References
Marinomycins	"*Marinispora*" sp.	MS	Polyketides	Antitumor	Kwon et al. (2006)
Streptokordin	*Streptomyces* sp.	DSS	Methylpyridine	Antitumor	Jeong et al. (2006)
Chinikomycins	*Streptomyces* sp.	MS	Polyketide	Antitumor	Li et al. (2005)
Frigocyclinone	*Streptomyces griseus*	AS	Angucyclinone	Antitumor	Bruntner et al. (2005)
Gephyromycin	*Streptomyces griseus*	AS	Angucyclinone	Glutaminergic	Bringmann et al. (2005)
Lipocarbazoles	*Tsukamurella* sp.	DSS	Carbazole	Antioxidant	Fiedler et al. unpublished data
Abyssomicins (atrop-abyssomicin C)	*Verrucosispora maris*	MS	Polycyclic polyketide	Antibacterial	Riedlinger et al. (2004), Nicolaou and Harrison (2006), Keller et al. (2007)
Chandrananimycins	*Actinomadora* sp.	MS	Phenoxazin	Antitumor	Maskey et al. (2003)
Salinosporamide A	*Salinispora tropica*	MS	Polyketide/non-ribosomal peptide	Antitumor	Feling et al. (2003)
Neomarinone	Actinomycete	MS	Meroterpenoid	Antitumor	Hardt et al. (2000), Kalaitzis et al. (2003)
Thiocoraline	*Micromonospora marina*	MS	Non-ribosomal peptide	Antitumor	Romero et al. (1997)
Marinone	Actinomycete	MS	Isoprenoid	Antitumor	Pathirana et al. (1992)

MS, marine sediment; DSS, deep-sea sediment; AS, Antarctic soil

Various hypotheses have been put forward in attempts to explain this "supersaturated coexistence" among which are allelopathic interactions affecting the inhibition of competitors (antibiosis, siderphore production), action of signalling chemicals, and direct resource competition. Although these community interactions for the most part are poorly understood, the widespread production of antimicrobial compounds by extremophilic/-trophic actinobacteria may be significant. The model developed by Czaran et al. (2002) gives credence to this suggestion, showing that local interference caused by antibiosis could produce a quasi-stable equilibrium in which the coexistence of a large number of purportedly competing bacteria would be sustained.

Another but unfortunate message coming from *Table 12.1.4* is the inadequacy of taxonomic rigor in describing organisms responsible for natural product synthesis. Nearly 60% of actinomycetes included herein in the past 5 years are accorded only candidatus status or are not given species descriptions; in view of the sophistication and speed of modern methods for taxonomic circumscription these are lamentable omissions. Moreover, incorrect taxonomic reporting in this context perpetuates confusion; for example, the antitumor mechercharmycins are not actinomycete products as stated by Olano et al. (2009), rather *Thermoactinomyces* is a member of the Order *Bacillales*.

The position regarding chemical characterization of extremobiosphere-derived actinobacterial natural products is in sharp contrast to that of producer organism characterization. Almost without exception, high resolution chemical structures are being reported for these natural products thereby highlighting the novel chemistry that is associated with newly discovered bacteria. The abyssomicins produced by *Verrucosispora maris* clearly demonstrate this point; these polycyclic polyketides are new chemical entities and show first-in-a-class bioactivity (first natural product inhibitor of *p*-aminobenzoic acid synthesis) (Riedlinger et al. 2004). Thiocoraline, a thiodepsipeptide synthesized by *Micromonospora marina* is in late preclinical development as an anticancer drug; it acts by binding to double-stranded DNA through bisintercalation. Another anticancer drug produced by a member of the *Micromonosporaceae* is salinosporamide A (Feling et al. 2003). Produced by *S. tropica*, salinosporamide A is a potent proteasome inhibitor currently in Phase 1 clinical trials against solid tumors/lymphomas (2006) and multiple myeloma (2007) (Fenical et al. 2009).

One additional point merits consideration in the context of searching for actinobacterial products in the extremobiosphere, namely, that biogeographical information can be valuable when making strategic decisions about where to collect samples (Bull et al. 2005). In a recent stimulating paper Mukherjee et al. (2009) argue that if greater attention was paid to accurate taxonomy and ecological observations when collecting samples, then resulting hits could be amplified in a number of ways including: (1) targeting environmental stressors, which may induce a wide variety of natural products (e.g., the recent paper by Haferburg et al. 2009 describing nickel- and cadmium-induced antibiosis in metal tolerant actinobacteria recovered from a former uranium mining site); (2) targeting environments known to yield natural products of interest due to metabolic specialization such as in extreme environments; and (3) screening the same taxa from a variety of locations in the knowledge that natural product synthesis varies considerably with environmental parameters. These authors have developed a web-based geo-spatial referencing and visualization tool with which to aid the discovery of tropical marine natural products and it is available for individuals to apply to their own search programs. With the increased availability, range, and sophistication of databases, this "geobibliography" concept could make a valuable contribution to biodiscovery.

Concluding Observations

Traditionally, actinobacteria have been viewed as authochthonous members of soil and freshwater microbial communities. This perception has to be completely revised in the light of recent research and, it is hoped, conveyed in this discourse. It is becoming increasingly evident that members of the class *Actinobacteria* can be counted among the more successful colonizers of all the environmental elements in the extremobiosphere, a proposition that would have been difficult to defend even as recently as a decade ago. Moreover, whereas the diversity of actinobacteria in extreme environments was considered to be very low, now in many cases the reverse situation, as well as their frequent dominance, is being revealed as they become more intensively investigated.

A recurring feature of this discussion has been the incongruity of culture-dependent and culture-independent assessments of actinobacterial diversity in extreme environments, and the reports of novel taxa for which there are no cultured representatives. Several innovative and effective procedures for improving isolations from extreme environments have been advocated and others continue to be introduced that are particularly suitable for actinobacteria (e.g., Gavrish et al. 2008; Epstein et al. 2010); they should be used more vigorously. A similar plea is made to integrate formal taxonomic characterization into such studies.

Actinobacteria, most regularly reported from extremely hot and/or acidic and intense radiation/desiccation environments (hot springs, deserts and other arid climes, mining operations) tend to be representatives of the deepest actinobacterial lineages (*Acidimicrobidae*, *Rubrobacteridae*). As yet, this is not a hard and fast relationship and more cultured members and full genome sequences of these taxa are needed to better understand their adaptation to and evolution from early Earth conditions. The metagenome analysis of marine actinobacteria referred to above (section Extreme Ecosystem Determinants and the Distribution of Actinobacteria, Jensen and Lauro 2008) led to the conclusion that a deeply rooted marine actinomycete has yet to be cultured, and that the ability to adapt to both terrestrial and marine environments has evolved independently in multiple lineages of actinomycetes. In contrast, marine representatives of the *Acidimicrobidae* formed deeply rooted lineages and, as these authors point out, "once obtained in culture" it will be enlightening to see if they "display specific adaptations to life in the sea." The universal occurrence of members of the suborder *Micrococcineae* in ice formations (permafrost, sea-ice, glacial ice cores) of all geographic locations (Arctic, Antarctic, Siberia, Spitzbergen, Greenland) is striking and is consistent with the evolutionary divergence of this taxon and glaciation history.

Our knowledge of the ecology of extremophilic and extremotrophic actinobacteria is at best fragmentary. Regarding adaptation mechanisms, the results of some new researches are beginning to challenge previously accepted dogma. For example, DNA repair capacity and maintenance of translation machinery are now considered to be prominent evolutionary features of cold adapted actinobacteria (cf. the dormancy hypothesis). Conjecture of this sort must be tempered at this stage by evidence pointing not to unique genotypes but to combinations of synergistic, and variable phenotypes, the latter clearly exemplified by the biochemistry that has evolved to maintain membrane fluidity in actinobacteria at low temperatures. However, the development of a range of innovative techniques is stimulating ecological studies of actinobacteria in the extremobiosphere.

Consider first indigenicity. The question often arises: which organisms or communities are native to a particular environment? The UniFrac statistical tool developed by Lozupone and her colleagues (Lozupone et al. 2006) enables multiple environments to be compared and to

determine which are significantly different. In a test of this methodology it was found that estuarine bacteria were more similar to river bacteria than to ocean bacteria, and suggesting that freshwater organisms adapt more readily to estuarine conditions than do marine relatives. Relationships of this sort only became apparent on application of the UniFrac tool. Analyses of this type can provide a sound starting point in testing for indigenicity in extreme environments.

The second issue of relevance here is the advent of "reverse ecology," a term coined by Matthew Rockman (see Li et al. 2008) to describe the identification of adaptive traits in the absence of prior information of ecological traits per se. The recent discovery of rhodopsin-like genes in inland waters and in cultured freshwater actinobacteria illustrates this approach (Sharma et al. 2009). These actinorhodopsins (ActR) were discovered by metagenome mining of data obtained from studies of aquatic environments including hyper-saline and fresh waters. Subsequently, ActR genes were found in a cultured member of the *Microbacteriaceae* and in metagenome libraries containing the ac1 clade. This research now opens up an intriguing question regarding the functional significance of ActR in the adaptation of certain actinobacteria to radiation in exposed freshwater lakes (see Actinobacteria in Selected Extreme Ecosystems above). These exploring-the-potential methods of investigation have been complemented by the development of micro-array technology and applied successfully in analyses of the extremobiosphere. *Acidimicrobium* and *Ferrimicrobium*, for example, were located in extremely acidic waters of the Rio Tinto using an acidophile micro-array, while RNA micro-arrays provided information on the most metabolically active ecotypes in the community (Garrido et al. 2008). From comparable experiments Mason et al. (2009) reported actinobacteria (mainly unclassified or distantly related to *Acidimicrobium*) in abyssal ocean crust basalts. Micro-array analyses of functional genes in this environment revealed several biochemical processes not detected previously in basalts. Valuable as micro-arrays are, they are limited in what they can reveal about ecological function, the final issue to be addressed in this chapter.

Within this new armamentarium are environmental transcriptomics and environmental proteomics (also known as metatranscriptomics and metaproteomics) that enable functional gene expression and translation to be examined in situ. Environmental transcriptomics was introduced by Poretsky et al. (2005) with an initial application to microbial populations in a hypersaline soda lake and a tidal salt marsh. In this proof-of-concept study transcripts of actinobacterial genes were obtained from both environments but due to their minor presence in the communities little could be deduced about the roles of actinobacteria in these environments. Similar innovative research on community proteogenomics is being made by Banfield's group. In a recent paper (Wilmes et al. 2008) strain-variant protein expression (and its ecological implications) within populations of Candidatus *Accumulibacter phosphates* in an activated sludge system has been determined in the course of which even small numbers of identified actinobacterial proteins were detected.

Finally, mention must be made of stable isotope probing (SIP), originally developed by Murrell's group (Radajewski et al. 2000) and subsequently adapted and refined by others, for defining relationships between metabolic activities and phylogeny in the environment. While the original SIP procedure focused on DNA, RNA-SIP and most recently protein-SIP have been developed. Furthermore, when combined with other complementary methodologies such as fluorescence in situ hybridization (FISH), magneto-FISH, cell sorting, Raman spectroscopy or secondary ion mass-spectrometry, SIP-based technologies are set to transform our approach to studying microorganisms in the environment. One example will suffice here to demonstrate

the power of these contemporary techniques. In a groundbreaking study WE Huang and colleagues (Huang et al. 2009) combined rRNA- and mRNA-SIP with single-cell Raman-FISH to resolve metabolic function within a community of naphthalene-degrading bacteria in a polycyclic aromatic hydrocarbon contaminated water. They established an unequivocal link between an uncultured bacterium (a species of *Acidovorax*) and its primary ecological role in the degradation of naphthalene. They were able also to recover naphthalene dioxygenase genes of Gram-negative bacterial origin but – interesting from the actinobacterial perspective – not from the well-known naphthalene degrader genus *Rhodococcus*. I conclude this chapter with the comment of Huang and colleagues on this exciting new "toolbox," which will "serve to reveal the secret lives of (as yet) unculturable microorganisms which are essential to the functioning of our natural environment."

Since the time that this chapter was completed, several new actinobacterial taxa have been described that contain extremophilic/extremotrophic members. Such bacteria have been isolated from deep-sea sediment (*Verrucosispora sediminis* sp. nov., Dai et al. 2010); coastal sediments (*Corynebacterium marinum* sp. nov., Du et al. 2010; *Saccharopolyspora marina* sp. nov., Liu et al. 2010); salt lakes (*Haloactinobacterium album* gen. nov., suborder *Micrococcineae*, Tang et al. 2010a; *Haloechinothrix alba* gen. nov., sp. nov., suborder *Pseudonocardineae*, Tang et al. 2010b); alpine and high plateau soils (*Arthrobacter alpinus* sp. nov., Zhang et al. 2010a; members of 22 genera ex Qinghai-Tibet plateau, Zhang et al. 2010b); and volcanic ash ex Jeju Island, Republic of Korea (*Marmoricola scoriae* sp. nov., Lee and Lee 2010).

The importance of actinobacteria for novel natural product discovery continues to be promoted. Miao and Davies (2010) opine that actinobacteria "are arguably the richest source of small molecule diversity on the planet," and point out that it is the understanding of the roles of such compounds that will lead to the "inexhaustible supply of novel therapeutic agents." In a similar vein, Goodfellow and Fiedler (2010) discuss the importance of actinobacterial systematics as a guide to successful biodiscovery and make a strong plea for resources necessary to train taxonomists. Further progress on using multilocus sequence analysis (MLSA) to differentiate ecovars (see *Deep-Sea Ecosystems*: Antony-Babu et al. 2008) has been made by Rong et al. (2010). These authors also worked with *Streptomyces griseus* isolates that were indistinguishable on the basis of 16S-rRNA gene sequences but showed that MLSA based on housekeeping genes could provide robust dereplication at the intraspecific level and predict secondary metabolite potential, thereby facilitating biodiscovery. Screening for genes associated with secondary metabolite synthesis has been made by Gontang et al. (2010) on a large collection of marine sediment–derived actinobacteria. Again rapid identification of isolates possessing the potential to synthesize novel secondary metabolites facilitated natural product discovery by prioritizing those for fermentation and chemical analysis. This approach has also been deployed by Zotchev's group in Trondheim to discover a new thiopeptide antibiotic in a marine species of *Nocardiopsis* (Englehardt et al. 2010).

Acknowledgments

I am most grateful to several colleagues who sent me research papers prior to their publication; and to Michael Goodfellow, Marcel Jaspars, and Jem Stach for reading an earlier draft of this review and providing me with helpful comments. Wen-Jun Li and Zhi Xiao-Yang (The Key Laboratory for Microbial Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, China) very generously prepared the 16S rRNA gene tree of the *Actinobacteria* that is

an updated version of the one contained in Zhi et al. (2009). Preparation of part of this review was made when the author was a Leverhulme Trust Emeritus Fellow.

The literature search for this chapter was completed on November 30, 2009.

Cross-References

- 6.5 Ecological Distribution of Microorganisms in Terrestrial, Psychrophilic Habitats
- 7.1 Microbiology of Volcanic Environments
- 9.1 Sub-seafloor Sediments - An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)
- 10.1 *Deinococcus radiodurans*: Revising the Molecular Basis for Radiation Effects on Cells
- 11.1 Ecology and Cultivation of Marine Oligotrophic Bacteria
- 11.2 Physiology of Marine Oligotrophic Ultramicrobacteria

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Part 13

Epilogue

13.1 Epilogue

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Koki Horikoshi (ed.), *Extremophiles Handbook*, DOI 10.1007/978-4-431-53898-1_13.1,

> *If it be true that good wine needs no bush, 'tis true that a good play needs no epilogue. Yet to good wine they do use good bushes, and good plays prove the better by the help of good epilogues.*
>
> William Shakespeare: *As You Like It*. Epilogue

About 50 years ago, few microbiologists were studying microorganisms living in extreme environments. Almost all microbiologists had thought that these environments were abnormal, within which living creatures could not survive. No one conjectured that there were many microorganisms loving exotic and extreme environments while the expectation of an *Extremophiles Handbook* was remote if not the product of science fiction. Now, we cannot discuss biology, especially microbiology, without the knowledge of and reference to extremophiles. As the reader can see in this handbook, extremophiles have been found everywhere on Earth, and clearly they are not exotic! So now the question arises what we are going to do next?

The New Frontiers section of the handbook illustrates only a part of future areas that seem certain to grow very fast and develop into wider fields of research and development. Beside these, such topics as the effects of gravity and water activity in extreme environments, for example, have yet to be exhaustively studied. Yet the acceptance of an extremobiosphere and its microbiota has not always been straightforward as attempts to publish the following discoveries make clear. First there is a very interesting story in *Nature* about Low Life (Mascarelli 2009). In the 1980s and 1990s, some of the first missions of the Ocean Drilling Program made it possible for researchers to explore deeper than ever before. However, when John Parkes and his colleagues tried in 1990 to publish results in *Nature* showing that bacteria could colonize much greater subsurface depths than previously thought, they were met with "very skeptical reviews" and the paper was rejected, he says. But in 1994, they succeeded in publishing their results and reported viable microbes living in ocean sediments at depths greater than 500 m below the seabed. Another example comes from the research of Akira Inoue. Initially Inoue tried, for almost 1 year, to isolate microbes from oil field soils containing toluene and other hydrocarbons but he was unsuccessful. However, the results of his following experiments surprised us; he discovered a toluene tolerant strain of *Pseudomonas putida* IH2000 in a forest soil of Kyushu, Japan, and toluene tolerant microorganisms in many other places including seawater. He submitted his data to *Nature*, but his paper also was rejected. Although the manuscript included growth curves of the bacteria in the presence or absence of toluene, one of the reviewers did not trust his experiments. Perhaps this is no wonder because toluene is very toxic. Finally Inoue sent photographs showing very good growth in test tube cultures containing 30% toluene together with the invitation "if you have further questions, please come to Japan. We would pay all of the travel expenses." Fortunately they believed Inoue's data, and his discovery was published in *Nature* in 1989. Both of these microbiologists, of course, are contributors to the handbook, and Stephen Jay Gould's retort bears repeating in the context: "Bacteria dwell in virtually every spot that can sustain any form of life. And we have underestimated their global number because we, as members of a kingdom far more restricted in potential habitation, never appreciated the full range of places that might be searched" (Gould 1996).

Although the widespread distribution and diversity of extremophilic and extremotrophic microorganisms now is universally recognized, their study remains beset by several challenges; we will point to four such as a means of gaging current research requirements. First, cultivation, a problem highlighted by the deep subsurface, as detailed by Sass and Parkes (see Chap. 9.1 Sub-seafloor Sediments: An Extreme but Globally Significant Prokaryotic Habitat (Taxonomy, Diversity, Ecology)), but also affecting other aspects of the extremobiosphere.

Among the reasons for the under representation of deep subsurface microorganisms in culture are the inaccessibility of sampling sites, the low culturability of strongly energy-limited populations and the frequent inability to grow in or be severely harmed by a sudden exposure to high substrate concentrations, and the potential damage or even inactivation of organisms due to depressurization during sample recovery. Second, the taxonomy and curation of organisms underpin all aspects of extremophile research and which is endangered by what Cotterill and Foissner (2010) have described recently as "a pervasive denigration of natural history." These authors argue that such denigration includes a failure to appreciate and support biodiversity α-taxonomy and monitoring, a disregard for epistemological functions of specimens (cultures), and a decline in taxonomic expertise that inevitably weakens research capacity. Third, the ecology of extremophilic and extremotrophic microorganisms in many cases is poorly understood, and again compounded by the accessibility of habitats. The application of "reverse ecology" as a means of identifying adaptive traits in extremophiles in the absence of prior information of ecological traits per se; and of techniques such as those based on stable isotope probing for defining relationships between metabolic activities and the phylogeny of extremophiles in the environment undoubtedly will greatly augment the still very relevant ecophysiological studies of organisms and communities in the laboratory (further details can be found in Bull, ❯ Chap. 12.1 Actinobacteria of the Extremobiosphere). The fourth challenge, and one intimately connected with ecology, relates to the nature of adaptation mechanisms that have evolved to sustain growth under extreme conditions. In some cases old orthodoxies are needed to be revised as is the position over radiation resistance (see Daly, Chap. 10.1 this handbook), while the possibility of newly suggested generic mechanisms, such as nitric oxide synthesis (Patel et al. 2009), that enable organisms to survive a range of environmental stresses needs to be evaluated.

Finally we invite the reader to join us in *gedankenexperiment* and to indulge in some subjunctive reasoning that might prompt predictions about the presently unknowable extent of extremophily. Such thought experiments are neither fanciful nor in the realm of science fiction, as the experiences of some of the extremophile pioneers have shown (see ❯ Chap. 1.1 Prologue: Definition, Categories, Distribution, Origin and Evolution, Pioneering Studies, and Emerging Fields of Extremophiles). Returning briefly to the deep biosphere, presentations in this handbook have reported that there are many microorganisms in deep-sea sediments and lithospheric crusts. Dramatic progress in cultivation methods has made clear that these microorganisms are entirely different from the *E. coli* cultivated in the presence of organic compounds that are synthesized by using solar energy. On the other hand, there are deep-sea microbial communities that graze on deeply buried organic carbon, such as methane, while deep subterranean communities may have been sustained by geochemically derived H_2 and sulfate over millions of years without inputs from photosynthesis. The generation times of these deep biosphere microorganisms are, of course, not clear, but data collected from South African gold mines provide estimates that they may reproduce once every 1,000 years. How we can measure such long generation time (1,000 years!) in our laboratories even if this is not so long on the geological timescale? According to Paul Davis (Davies et al. 2009) our planet may have forms of "weird life" (Baross 2007) different to life as we know it. In this final discussion, therefore, we believe that the possibilities of weird life both beyond Earth (astrobiology) and on Earth (shadow biosphere) are germane to the themes of this handbook and take us "outside the box" that defines the known extremobiosphere – we make the tacit assumption that weird life would have an extremophilic nature. But first it is essential to decide upon a working definition of "life."

In 1994 a group assembled by NASA defined life as a "chemical system capable of Darwinian evolution" (Joyce et al. 1994) and based on this definition Brenner et al. (2004) in an excellent exploration of the question "is there a common chemical model for life in the universe?" proposed that life may indeed exist in a wide range of environments dependent only on a minimum number of criteria. Thermodynamic disequilibrium and temperatures consistent with chemical bonding were deemed to be absolute criteria, while less stringent criteria that also may define habitable environments were considered to include a solvent system; the availability of elements such as carbon, hydrogen, oxygen, and nitrogen; certain thermodynamic features available to metabolic pathways; and the opportunity for environmental isolation. Moreover if life was constrained to water, other specific criteria might follow such as soluble metabolites, genetic materials with repeating charges, and a well-defined temperature range.

At the time of writing the most recent discussion of the possibilities for and detection of extraterrestrial life took place at the Royal Society, London, in January 2010 whose proceedings (Dominik and Zarnecki 2010), taken together with the Benner review (loc cit), provide a comprehensive account of current thinking. The search for life beyond Earth has focused particularly on Mars because of the probable presence of liquid water at its surface at some stage in its history, thereby providing a common chemical model for Earth-like environments. However, alternative solvent systems that exist in the solar system (e.g., sulphuric acid aerosols [Venus], liquid ammonia [Jupiter], liquid hydrocarbons [Titan], and even supercritical dihydrogen) have been advanced as ones that could be compatible for life to have evolved. Horneck (2008) presents a compelling case for the habitability of Mars arguing that "it is legitimate to assume that conditions on early Mars were as favorable for life to emerge as on early Earth." The existence of a one-time aqueous environment is supported by geochemical clues (e.g., presence of hematite and salt, both of which require water for their formation), and the detection of abundant water-ice by recent Mars missions. Although the present surface of the planet is unlikely to be habitable, Horneck has suggested that potential sub surface oases on Mars that could act as refugia for a putative Martian microbiota might be inferred from terrestrial analogs such cryosphere, cryptoendolith, deep biosphere, hydrothermal, and intense radiation exposed habitats.

What are the chances of finding weird life on Earth? Several commentators have argued that we should keep an open mind regarding such a conjecture, pointing to the fact, among others, that members of the Domain *Archaea* were discovered only a few decades ago. For example, might remnants of a postulated archaic RNA-based world exist on Earth? The "RNA World" hypothesis (Gilbert 1986) has growing scientific support (e.g., Lincoln and Joyce 2009; Breaker 2010; Turk et al. 2010) but as Davies et al. (2009) point out our existing life-detecting methods that target ribosome components may be inappropriate for the purpose. Reasoning on the basis of RNA chemistry, its mechanism of replication, and its ease of refolding after denaturation, Benner et al. (2004) have suggested that the search for such remnants might be focused on low sulfur, severely space-constrained, and fluctuating very high–very low temperature dominated environments, and so to the question of an alternative "life on Earth as we know it" (Davies et al. loc cit) that constitutes a "shadow biosphere." In their stimulating *Hypothesis Article* Davies and colleagues advanced three possibilities for the relationship between a posited weird shadow life and the standard life with which we are familiar: ecologically separate, ecologically integrated, biochemically integrated. In the first case, search for weird life could focus on multidimension parameter environments *beyond* the currently established boundaries for extremophiles. Signatures of an ecologically integrated shadow

biosphere might be revealed as a distinctively different organic chemistry, e.g., use of a wider range of amino acids, homochirality, the use of a novel source of energy (e.g., UV radiation), or the use of a selection of elements different from those used by standard life (e.g., arsenic) (Wolfe-Simon et al. 2009; Stolz et al. 2010). This shadow life may be hidden in various natural and man-made environments such as hyper-arid deserts, hydrothermal vents, deep subsurface, and those exposed to intense radiation, salinity, pH, or depleted in key standard life elements. In conclusion the advice proffered by Davies et al. (loc cit) is worth citing at length: "The best strategy for discovering the existence of weird terrestrial life, assuming for sake of argument that it exists, is to search for biological anomalies. . . . . anomalies are the driving force behind scientific revolutions; they stand out against the backdrop of accepted scientific belief, driving new conceptual schemes and paving the way for yet more discoveries. There is, of course, no guarantee that such a biosphere exists or has ever existed. But a systematic search for one, even if unsuccessful, is a worthwhile strategy anyway, if only because it may well uncover hitherto unknown highly exotic forms of standard life."

► *There are more things in heaven and earth, Horatio, than are dreamt of in our philosophy.*
William Shakespeare: *Hamlet*. Act 1 Scene 5

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