Caroline Chénard · Federico M. Lauro
Editors

Microbial Ecology of Extreme Environments

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For statistical purposes, this dedication has been performed in triplicate. To our parents, without whom we would not have been possible and this book would have never existed. To our partners, Rachelle and Xavier, who still question why we study microbes. To our own microbiomes, may they keep us healthy and in good spirits.

Preface

Anyone who has recently attended a conference on microbial ecology would agree that it is hard not to appreciate how much the field has evolved in the past few decades. We are gaining unprecedented insights in the lifestyles of microbes at different spatial levels. With molecular tools, we can study microbial communities at the level of single cell interactions while with remote sensing we can detect their presence and activity at a planetary scale. Nucleic acids and proteins can be sequenced from minute amounts of starting material and in just a few hours, giving us detailed descriptions of the diversity and activity of microbial communities. And using these techniques, everywhere we look, and as long liquid water is available, we are discovering new branches of the microbial tree of life. In seemingly inhospitable places, microbes are exploiting resources, cycling organic and inorganic compounds, fighting with each other and promiscuously exchanging massive amounts of genetic information. Extreme environments select for unique adaptations at the level of enzymes, compounds, and processes which are the future arsenal of biotechnology. The exploitation of these natural resources will require not only the development of new bioinformatic tools for data mining, but also new culturing techniques and systems biology approaches for environmental engineering.

This book is meant to provide a useful reference for those who want to start a research program in extreme microbiology and, hopefully, inspire new research directions. Assembling it has taken a long time. For one reason or another, there was always an excuse such as too much teaching or administrative duties or the lack of enough material or just procrastinating because of another grant deadline approaching. And it was just by chance that we ended up working at the same institution and found the time to convince enough experts in the field that it would be fun to write this book.

So here it is. The chapters are organized so that they roughly follow a vertical profile through the biosphere.

After a brief introductory chapter, we begin in the deepest depths of the Ocean with Chap. 2 comprehensively reviewing the phylogenetic diversity of cultured and uncultured piezophiles (pressure-adapted microbes) and discussing the need for a

better representation of piezophilic phylogenetic diversity in culture collections. We also discover how the genomic features of pressure-adapted microorganisms differ from those of close phylogenetic relatives from shallower waters.

The deep sea is further explored in Chap. 3, which goes into detailing the adaptations of motility and cell envelopes of piezophiles and Chap. 4, which explores the effects of hydrostatic pressure on extracellular electron transfer and how this might be ecologically relevant to environments with low energy input.

The following two chapters describe life in hot environments. Chapter 5 reviews the diversity and activity of microbial populations in many high-temperature habitats around the world. Thermophilic bacteria and archaea produce many enzymes which have biotechnological applications but proteins from thermophilic viruses might also have similar or even better potential. Chapter 6 describes the ecology viruses and the concurrent coevolution of CRISPR/Cas systems in hot springs.

The next two chapters take the readers to the opposite extreme of temperature. In Chap. 7, the diversity of bacteria in polar deserts is discussed in the context of their remarkable adaptations, while Chap. 8 looks at the ecological function of phages in high-latitude aquatic systems. A particular emphasis is devoted to phage–host dynamics and their relevance to the control of biogeochemical fluxes though the polar ecosystem.

Chapter 9 deals with solvent tolerance, which is important for many industrial processes. The chapter discusses the evolution of efflux pumps as a way to cope with solvent stress and how these can also provide resistance to antibiotics. Therefore, understanding solvent tolerance has implications in the control of infectious disease and human health.

And finally, Chap. 10 presents a comprehensive review of microbial life in clouds from their diversity to the adaptations to environmental challenges of living air.

This book only deals with a small subset of the extreme habitats of our biosphere. Notable omissions include extremes of pH or salinity on which other, more specific volumes have been written. One important point made by all authors is that the molecular and physiological basis of life at any extreme is still largely unknown and that a lot more work is needed to fully understand it. It is easy to see why, since most of the habitats are difficult to access. Yet, if technology keeps progressing at the same pace, it will not be long before we can answer many of the questions raised in this book about evolution, biogeography, and biodiversity.

And even if the study of microbes in extreme environments will remain challenging, hence not for the faint-hearted, the potential rewards are worth the effort.

Singapore

Caroline Chénard
Federico M. Lauro

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It is a big debt to repay no matter how long you made us wait. And a big thanks to all our readers: we hope you enjoy this book as much as we have.

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Chapter 1
Introduction

Caroline Chénard and Federico M. Lauro

Abstract This introductory chapter provides a brief historical perspective on the field microbial ecology and its aims. After dispelling the misconception that "extreme" environments represent just a small portion of the biosphere, the discussion highlights the importance of understanding the function and ecological roles of microbial communities in every environment and concludes by putting the current knowledge in the context of potential new discoveries of enzymes and activities with applications in industry, medicine and biotechnology.

1.1 Microbial Ecology

Ecology is the study of organisms in their natural environment and starts with the observation of natural systems, which becomes the foundation of hypotheses and theories.

Antonie Van Leeuwenhoek was a curious observer and, one could say, the first microbial ecologist. More than three centuries ago, using a rudimentary microscope he saw what he called "animalcules" and the field of microbiology was born. For the following two centuries, the field advanced mostly with laboratory experiments and using cultured isolates. The focus was on understanding the role of microorganisms in a mechanistic way, particularly in disease. Yet the pioneering work of the likes of Louis Pasteur and Sergei Winogradsky struggled to define the relevance of microbial communities and their interactions with the natural environment. In fact, the real extent of microbial diversity was not really appreciated until the advent of molecular ecology pioneered by Carl Woese in 1977 (Woese and Fox 1977) This

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opened the door to characterize not just those microbes that could be cultured, but many others, which had never been seen before on a Petri dish.

As with all new discoveries, microbiologists were left with more questions than answers: were 16S phylotypes really the most appropriate biological unit for Bacteria and Archaea and the best proxy for a microbial "species"? Moreover, how did individual phylotypes relate to functional traits and ecological niches? The widespread use of molecular phylogenetics suddenly allowed for studies of taxonomic diversity, but the advent of meta-omics technologies in the mid-2000 provided a completely new perspective. Microbial assemblages could be understood in terms of communities of genes, functions and biological activities rather than communities of taxa. Every new environmental sample yielded millions of new putative genes, mostly with unknown functions. And the increased sequencing throughput revealed the universal presence of a rare biosphere (Sogin et al. 2006). The role of these low-abundance taxa was thought to represent a permanent seed-bank, providing support to the Baas Becking hypothesis (Becking 1934) that "everything everywhere and the environment selects". Yet even this notion was soon considered too simplistic (Baltar et al. 2015) and that a combination of many biotic and abiotic factors had to be responsible for the establishment and persistence of the rare biosphere (Lynch and Neufeld 2015).

It is clear that with molecular data accumulating at an ever-increasing pace, microbial ecology requires a solid theoretical framework: do the same principles of ecological theory described by macroecologists apply and, if so, at what scale? What defines a microbial species or an ecotype? What are the rules governing the assembly of multiple taxa in complex communities? With careful observations, extreme environments provide an opportunity to address some of these questions with carefully designed natural experiments. They also represent an endless source of innovation for enzymes and compounds for the benefit of mankind.

1.2 Extreme Environments Are "The Norm"

Extreme environments are those environments where at least one physical (temperature, radiation, pressure) or chemical (pH, salinity) property is outside the normal range for human survival. Some examples of extreme environments include extremely cold environment such as those found in the Arctic and Antarctic or high pressure environment such as the deep ocean. Other severe environments include hydrothermal vents, hypersaline lakes and pools, alkaline soda lakes, dry deserts and clouds. Many environments can also be considered "extreme" under more than one parameter. For example, deep-sea hydrothermal vents are both under high hydrostatic pressure and low pH.

Contrary to popular belief, extreme environments are the norm rather than the exception. Indeed, 80% of the biosphere is permanently at low temperature (Gounot 1999) while 70% is under high pressure (Picard and Daniel 2013). These extreme environments are also home to the majority of the Earth's biomass. They support a

large diversity of microorganisms, called extremophiles, which thrive in extreme physical and or chemical conditions. For example, the bacteria *Planococcus halocryophilus* Or1, isolated from high Arctic permafrost, grows at −15 °C (Mykytczuk et al. 2013) while the chemolithoautotrophic archaea *Pyrolobus fumarii* isolated from a deep sea vent can live at 113 °C (Cowen 2004).

Some of the challenges that extremophiles must face include: changes in membrane fluidity, effects on transcription and translation and DNA damage. Indeed, nucleic acids are especially vulnerable to high temperature, radiation, oxidative damage and desiccation. However, to adapt to their environment, extremophiles have developed unique mechanisms that enables them to remain viable and active. For example, *Deinococcus radiodurans* is able to withstand an acute dose of 5000 Gy of ionizing radiation, which is 1000 time the amount that would kill a human. The radiation-induced double-strand breaks in DNA are repaired with a mechanism that consist of re-assembling fragmented DNA with remarkable accuracy (Battista 1997). Another example is the development of adaptations to deal with the denaturation and chemical modification that DNA encounters at high temperature (>70 °C). Indeed, hyperthermophiles are believed to contain more stable DNA due to the presence of monovalent and divalent salts. These salts screen the negative charges of the phosphate groups and protect the DNA from depurination and hydrolysis (Marguet and Forterre 1998).

1.3 "Extremozymes" and Their Biotechnological Interests

Microbial communities that live in extreme environments represent an innovative source of novel enzymes, which can be used for a wide range of products and industrial processes. These extremophile–derived enzymes, also known as "extremozymes", perform the same enzymatic function as their non-extremophilic homologs but with greater versatility and adaptability to extreme conditions.

The field of molecular biology has benefited from many extremophile-derived enzymes (see Table 1.1 for example). Arguably the most famous "extremozyme" is Taq polymerase, the heat resistant enzyme commonly used for Polymerase Chain Reaction (PCR). Taq polymerase was first described in the thermophilic bacterium *Thermus aquaticus*, isolated from a hot spring in Yellowstone National Park (USA) (Chien et al. 1976).

Cold-adapted enzymes are another class of "extremozymes" with applications in molecular biology. These enzymes carry the advantage that they can be selectively inactivated in a complex mixture simply by increasing the temperature of the reaction. One example is an alkaline phosphatase (AP) derived from an Antarctic bacterium (Kobori et al. 1984; Sarmiento et al. 2015). In molecular biology, alkaline phosphatases remove 5′-terminal phosphates from linearized DNA molecules, preventing self-ligation for a higher cloning efficiency. Once the activity is no longer needed, the psychrophilic AP can be easily heat-inactivated. Cold-adapted DNA ligases also confer advantages in comparison to mesophilic homologs.

Table 1.1 Few examples of potential biotechnological application derived from microbial organisms isolated from extreme environments

Organism	Group	Environments	Product/application	References
Galdieria sulphuraria 074G	Thermophilic cyanobacteria	Hot, acidic springs (T > 40 °C and pH 1–3)	Blue pigment phycocyanin (PC) used as a fluorescent marker in histochemistry	Sloth et al. (2006)
Raphidonema sp.	Psychrophilic algae	Arctic snow and permafrost	α-Tocopherol (vitamin E) and xanthophyll cycle pigments	Leya et al. (2009)
Thermus aquaticus	Thermophilic bacteria	Hotspring, Yellowstone National Park	Heat-resistant enzyme Taq polymerase	Chien et al. (1976)
Chlorella sorokiniana UTEX 2805	Thermophilic green algae	Wastewater stabilization ponds	Wastewater treatment (Ammonium removal)	De-Bashan et al. (2008)
Ralstonia sp.	Bacteria	Various environments	Biosensors for heavy metals	Nies (2000)
Antarctic psychrophile HK47	Psychrophilic bacteria	Antarctic seawater	Alkaline phosphatase (AP)	Kobori et al. (1984)
Pseudoalteromonas haloplanktis	Psychrophilic bacteria	Antarctic sea water	DNA ligase	Georlette et al. (2000)
Glaciozyma antactica strain PI12	Yeast	Antarctic	Serine protease (detergents)	Alias et al. (2014)

The function of DNA ligase is to join DNA fragments with the formation of a phosphodiester bond but at high temperature residual nuclease activity can interfere with the ligation. The use of a cold-adapted enzyme has the advantage of maintaining high specific activity at low temperature while concurrently minimizing nuclease interference (Sarmiento et al. 2015). The DNA ligase from the psychrophilic bacterium *Pseudoalteromonas haloplanktis* offers great potential given that it is active at temperatures as low as 4 °C (Georlette et al. 2000). Cold-adapted enzymes are also of interest for improving laundry and dishwasher detergents. Some of these potential enzymes include a high-performance lipase isolated from *Pseudomonas stuzeri* PS59 which has optimal activity at 20 °C (Li et al. 2014) or a serine protease isolate from Antarctic yeast *Glaciozyma antactica* strain PI12 with optimal activity also at 20 °C (Alias et al. 2014).

The food and beverage industry is also taking advantage of the discovery of new extremozymes. To date, there are many examples of commercially available thermostable enzymes especially for the starch-processing industry. Indeed, enzymes isolated from thermophilic microorganisms are optimal between 80 and 100 °C and at pH levels from 4.0 to 7.5, which are the optimal conditions for starch liquefaction (Niehaus et al. 1999).

1.4 Conclusions

In the various chapters of this book we describe the aspects of the microbial ecology from different extreme environments including polar desert soil, hyperthermophilic environments and clouds. Some of these chapters also highlight some of the potential industrial or biotechnological applications.

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Chapter 2
Ecogenomics of Deep-Ocean Microbial Bathytypes

Logan M. Peoples and Douglas H. Bartlett

Abstract The deep ocean is one of the largest and least studied biomes on Earth. The microbes inhabiting these locales require physiological adaptations to handle the associated extreme environmental conditions, including high hydrostatic pressure, low temperatures, and low organic carbon. Few microbes have been successfully cultured that are capable of growth under in situ high-pressure conditions, especially at hadal depths, thanks to the relative inaccessibility of these sites, an inability to collect samples and maintain them under in situ conditions, and difficulties in culturing methodology. However, genome sequencing and high-throughput community analyses have provided insight into the prokaryotes which inhabit the deep sea and their lifestyles. This review discusses our current understanding of microbial adaptation to the deep-ocean through genomic comparisons of deep-ocean adapted microbial ecotypes and their shallow-water counterparts, including opportunistic heterotrophic microbes belonging to the Gammaproteobacteria and the fastidious taxa SAR11 and Thaumarchaea. These comparisons are addressed in the context of culture-independent metagenomics and community diversity analyses on deep, oligotrophic pelagic communities. Both culture-dependent and—independent analyses suggest the presence of bathytypes as both isolates and whole communities are distinct from those found above them. While these studies show many attributes indicative of deep-ocean genomes, including genes for particle-association, heavy-metal resistance, the loss of a UV photolyase, and increased abundances of mobile elements, they also suggest that high-pressure adaptation seems to arise from the accumulation of many small changes, such as differences in gene expression or the accumulation of compatible solutes. Genomic analyses on a larger dataset of samples and piezophilic isolates are necessary to distinguish attributes specific to deep-sea adaptation.

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2.1 Introduction

With an average depth of 3800 m the deep ocean is one of the largest biomes on Earth. Microorganisms dominate these deep-sea pelagic and benthic environments in terms of biomass, metabolic activity and turnover. The deep sea is characterized by low temperatures, high hydrostatic pressures, no sunlight, and low abundances and recalcitrant forms of organic carbon. These conditions lead to total microbial biomass decreases per milliliter of seawater by approximately three orders of magnitude from epipelagic to abyssal habitats (Nagata et al. 2010). Still, the pelagic ocean deeper than 200 m and its underlying subseafloor sediment contain $\sim 6.5 \times 10^{28}$ and 2.9×10^{29} cells respectively, values similar to those in the entire soil and terrestrial subsurface environments (Whitman et al. 1998; Kallmeyer et al. 2012). Therefore, a large fraction of the total microbes in the biosphere must be adapted to deep-ocean conditions. Hydrostatic pressure, which increases by 1 MPa (megapascal) for every 10 m of depth and can reach ~110 MPa (16,000 lbs/in^2, 1100 atmospheres) in the deepest trenches, is one important selective parameter in the deep sea. Piezophiles, microorganisms that show optimal growth at hydrostatic pressures greater than 0.1 MPa (Yayanos 1995), have been successfully isolated from the deep ocean. They are closely related to shallow-water species but have adaptations to their in situ physical conditions mediated in part through changes in cell membrane composition (Allen et al. 1999; Allen and Bartlett 2000; Bartlett 2002), DNA replication and cell division (Yayanos and Pollard 1969; Welch et al. 1993; Ishii et al. 2002, 2004; El-Hajj et al. 2009, 2010) and protein synthesis (Kawano et al. 2004; Lauro et al. 2007; Lauro and Bartlett 2008). Advances over the past decade in cultivation-independent genomics have helped provide a more complete picture of deep-ocean microbial physiology and function by overcoming the small sample sizes and culturing challenges typically affiliated with the deep sea. Metagenomics and deep-sequencing community analysis, which shows community composition and the functional repertoire of a community, and single-cell genomics, which shows genomic content from one individual cell, can provide genomic insight into unculturable or rare members of a community. These studies, along with culture-dependent analyses, have revealed a number of distinct genomic and physiological features distinct to deep-ocean ecotypes. Here we briefly review genomic comparisons between cold, oligotrophic, deep-ocean microbial isolates and communities with their shallow-water counterparts.

2.2 The Culturable Community

To date at least 41 bacterial and 12 archaeal strains showing maximum growth at elevated hydrostatic pressure have been isolated (Jebbar et al. 2015). Almost all psychrotolerant or psychrophilic obligate piezophiles are members of the genera

Shewanella, *Colwellia*, *Moritella*, and *Psychromonas* within the Gammaproteobacteria (Jebbar 2015). In general, the psychrophilic piezophiles display a good correlation between their isolation depth and growth pressure optimum, a fact that has been used to infer that high pressure has selected for the evolution and distribution of distinct groups of microorganisms (Yayanos 1986). However, studies attempting to isolate microbes capable of growth above or below the pressures corresponding to their collection depth have not yet been reported. Genomes of some cultured deep-sea piezophiles, including *Shewanella benthica* KT99 (Lauro et al. 2013a), *Colwellia* sp. MT41 (Kyaw et al.; unpublished data), *P. profundum* SS9 (Vezzi et al. 2005), *Psychromonas* sp. *CNPT3* (Lauro et al. 2013b), *Moritella* sp. PE36, *Marinitoga piezophila* KA3 (Lucas et al. 2012), *Desulfovibrio piezophilus* (Pradel et al. 2013), *Desulfovibrio hydrothermalis* AM13 (Ji et al. 2013), *Carnobacterium* sp. AT7 (Stratton 2008), *Thermococcus barophilus* (Vannier et al. 2011), and *Pyrococcus yayanosii* (Jun et al. 2011) have been sequenced to date. 16S rRNA gene phylogenies show that piezophiles of the same genera are typically closely related to one another (Lauro et al. 2007; Miyazaki and Nogi 2014; Urakawa 2014; Satomi and Fujii 2014). Piezophilic clusters have radiated out from within multiple genera, separately evolving many times. Piezophiles that show optimum growth at pressures exceeding 40 MPa are either psychrotolerant/psychrophilic or thermophilic/hyperthermophilic (DeLong et al. 1997; Lauro et al. 2007). This likely reflects the habitats sampled as much of the deep ocean is low temperature and hydrothermal vent sites have been intensively sampled. Some microbes show better, and sometimes optimum, growth at higher pressures when grown at higher temperatures (Yayanos 1995; Kaneko et al. 2000; Martini et al. 2013). This may be due to elevated pressure compensating for temperature effects (Yayanos et al. 1983). Future attempts at isolating more mesophilic piezophilic microbes, such as from the deep marine subsurface, could yield success.

Despite the major role of elevated pressure in structuring the distribution of piezophilic deep-sea life, many pressure-sensitive microbes have also been obtained from the deep sea. Analogous to the enrichments for piezophiles, a select group of heterotrophic taxa are consistently isolated from the deep-sea when incubated at atmospheric pressure, including the genera *Pseudomonas*, *Pseudoalteromonas*, *Halomonas*, *Marinobacter*, and *Psychrobacter* and the phyla Actinobacteria and Firmicutes (Takami et al. 1999; Biddle et al. 2005; Batzke et al. 2007; Kaye et al. 2011; Orcutt et al. 2011). These taxa do not seem to be location or depth-specific as they have been isolated from many different oceanic basins and depths, including trenches (Yanagibayashi et al. 1999; Takami et al. 1997; Pathom-aree et al. 2006). While many of these microbes are likely present in low abundance, some culture-indepent studies also indicate their presence or even dominance (Kato et al. 1997; Li et al. 1999; Xu et al. 2005; Nunoura et al. 2015; Salazar et al. 2015a, b). Extreme conditions, food scarcity, or an ability to adapt to broad environmental conditions may select for these microbes and result in their widespread distribution. While specific strains of *Halomonas* (Kaye et al. 2011) and *Pseudomonas* (Tamegai et al. 1997; Sikorski et al. 2002) may be adapted to the deep sea, the lack of culturable members of these taxa at high pressure suggests they may originate in

more shallow waters but survive in a dormant state at depth. Dormancy, a microbial strategy to minimize energy requirements for long-term survival (Lennon and Jones 2011), can be accomplished in one manner through the formation of spores. Sporeformers are abundant in subseafloor sediments based on metagenome studies (Kawai et al. 2015), and this is even more apparent when spore levels are directly measured using the endospore-specific compound dipicolinic acid (Langerhuus et al. 2012; Lomstein et al. 2012).

Because so few distinct high pressure-adapted microbes are known and piezosensitive isolates are commonly obtained it is unclear what fraction of deep-ocean communities are adapted to high hydrostatic pressure. Measurements of microbial activity as a function of pressure, measured by comparing activity rates at both in situ and atmospheric pressure conditions, suggest that activity is dependent not only on depth but also collection location and sample type, with sediment, water column, and benthic boundary layer samples showing different levels of piezophily (Tamburini et al. 2013). These findings are likely due to the mixing of autochthonous communities with microbes attached to sinking particulate organic matter (POM) as less stratified and benthic boundary layer communities showed less in situ pressure adaptation than their counterparts. This issue could be addressed in future work by determining the taxonomic distribution of deep-sea microbial communities active at in situ pressures versus atmospheric pressure.

2.3 Insights from Deep Ecotypes

Ecotypes, or closely related microbial lineages adapted to specific environmental conditions, possess genomic adaptations that allow them to exploit different habitats or ecological roles. For example, different ecotypes of high- and low-light adapted *Prochlorococcus* strains show adaptive strategies unique to their respective depths (Ting et al. 2002; Rocap et al. 2003; Delong and Karl 2005; Martiny et al. 2009). Hundreds of coexisting subpopulations of *Prochlorococcus* have been identified, each with a distinct genomic backbone that provides differential fitness, leading to changes in relative abundances with varying environmental conditions (Kashtan et al. 2014). Similar ecotypes have been identified among deep-ocean microbes and their shallow-water counterparts. Depth-specific ecotypes, termed bathytypes, have been defined as a population of a species adapted to a certain water column depth (Lauro and Bartlett 2008). This classification of bathytypes has been expanded to include not only species but also distinct clades as many putative deep-ocean-specific microbes are members of groups with poorly defined phylogenies. Ubiquitous taxa with important ecological roles, such as the SAR11 clade (Field et al. 1997; Eloe et al. 2011c; Thrash et al. 2014) and the Thaumarchaea (Brochier-Armanet et al. 2008; Hu et al. 2011; Luo et al. 2014; Swan et al. 2014) show deep-specific clades distinct from those at shallower depths. Members of candidate bacterial phyla have also been suggested to be deep-specific. The ‘Gracilibacteria,’ ‘Microgenomates,’ and ‘Parcubacteria,’ members of the

"candidate phyla radiation" that show relatively reduced genome size and metabolic potential (Brown et al. 2015), potentially include piezophiles because of their identification at depth within the East Pacific Rise (Hedlund et al. 2014). Other uncultured groups, including SAR406 (Nunoura et al. 2015) and SAR324 (Brown and Donachie 2007) show depth-dependent distributions and likely have members that are specifically adapted to the deep-ocean. Here we briefly review the findings of comparisons between ten sets of sequenced bathytypes.

2.3.1 Bacteria: Alphaproteobacteria: SAR11

The SAR11 clade within the Alphaproteobacteria is one of the most numerically dominant bacterial lineages in the ocean (Morris et al. 2002). The clade's success has been attributed to a number of traits, including minimal cell size, genome streamlining (Giovanonni et al. 2005; Grote et al. 2012), and large population sizes to evade viral attack (Brown and Fuhrman 2005; Zhao et al. 2013). The SAR11 clade has been separated into subgroups, with some confined to warmer, temperate, or polar locations while others show a cosmopolitan distribution (Brown and Fuhrman 2005; Rusch et al. 2007; Brown et al. 2012), and many clades show variable responses to seasonal changes, suggesting ecological differentiation (Brown and Furhman 2005; Morris et al. 2005; Carlson et al. 2009; Brown et al. 2012). Deep populations have also been identified (Field et al. 1997; García-Martínez and Rodríguez-Valera 2000), including at abyssal and hadal depths (DeLong et al. 2006; Martin-Cuadrado et al. 2007; Konstantindis et al. 2009; Eloe et al. 2011c; Léon-Zayas et al. 2015). Single-cell amplified genomes (SAGs) of members of subclade Ic, a deep bathytype, were obtained from 770 m at Station ALOHA and were compared to surface SAR11 genomes, including those from subclade Ia, and metagenomic datasets (Thrash et al. 2014). Subclades Ia and Ic showed a rRNA sequence identity of 95% and an amino acid identity of 62%, suggesting they may be different genera. Relative abundances of subclades Ia and Ic estimated by read recruitment from metagenomic datasets showed that subclade Ia was dominant in the upper surface waters while subclade Ic represented more than half the reads taken from aphotic depths. The metabolism of many of the deep genomes suggested a metabolism similar to surface sucblade Ia strains, focused on the oxidation of organic acids, amino acids, and C1 and methylated compounds, but with the capacity for nitrogen salvage and sulfite oxidation. COG distribution was conserved except for enrichment in genes for cell wall/membrane/envelope biogenesis (M) and inorganic ion transport (P) in subclade Ic. Unique phage related genes were also identified, including a CRISPR region that showed preferential recruitment to the mesopelagic at Station ALOHA. Adaptations of this SAR11 bathytype are thought to be reflected in subtle differences, such as increases in genome size, larger intergenic spacer regions, and preferential amino acid-substitutions. Populations of *Pelagibacter* (a major genus within the SAR11 group) identified using metagenomics at 4000 m at Station ALOHA had nonsynonymous to synonymous (Dn/Ds) ratios two times higher when compared to

shallow-water representatives, suggesting decreased purifying selection (Konstantinidis et al. 2009). One gene conspicuously missing is that encoding deoxyribodipyrimidine photolyase (*phr*) (Vezzi et al. 2005). This gene, which uses blue-light energy to repair UV-mediated DNA damage (Todo 1999), is expected to be missing from microbes not exposed to light and may be a diagnostic tool to identify obligate deep-ocean microbes (Lauro and Bartlett 2008). Despite relatively good genome sequence coverage for the SAGs, ranging from 55 to 86%, none of the subclade Ic genomes had a DNA photolyase. However, genes for proteorhodopsins, which are light-driven proton pumps, were identified in two SAGs. Thrash et al. suggest that members of subclade Ic may occasionally circulate to the euphotic zone where proteorhodopsins would provide an energetic advantage. The work by Thrash et al. (2014) provides a framework for genomic adaptation to the deep ocean in one of the most-abundant microbes in the ocean.

2.3.2 Gammaproteobacteria: Alteromonadales: Alteromonas

Members of the genus *Alteromonas* are heterotrophic r-strategists typically found in nutrient-rich niches (López-López et al. 2005; Shi et al. 2012). One species, *A. macleodii*, is readily cultivable and has surface and deep-specific clades (López-López et al. 2005; Ivars-Martínez et al. 2008a), with members identified at high abundance in some deep-ocean samples (López-López et al. 2005; Quaiser et al. 2011; Smedile et al. 2013). When the genome sequence from a representative deep isolate, termed *A. macleodii* AltDE (Ivars-Martínez et al. 2008b), was compared to that of the shallow ecotype *A. macleodii* ATCC 27126 (Baumann et al. 1972), many similarities with other deep-sea genomic comparisons were found. 65 transposable elements and 63 insertion sequences (IS) were identified in AltDE while only three and one, respectively, were found in ATCC 27126. Transposases are abundant in the genomes of particle-attached microbial communities and those that are associated with surfaces (Burke et al. 2011; Ganesh et al. 2014). Genes involved in phage interaction, such as integrases and a CRISPR region, were also overrepresented in AltDE. These findings may be due to a reduced capacity to selectively purge mobile elements, such as within species of reduced effective population sizes, greater cell-cell interactions leading to increased mobile element transmission, or as a result of the adaptive value of greater transposon-mediated lateral gene transfer occurring in microbes encountering environmental change in association with sinking particles (Ganesh et al. 2014). AltDE also encodes for a cytochrome BD complex, which may function as an alternative respiratory chain during hypoxic conditions, such as those that could exist on POM. The piezophile *Shewanella violacea* DSS12 contains a gene for this same cytochrome and upregulates its expression at high pressure (Chikuma et al. 2007). The deep bathytype is also enriched in genes for extracellular polysaccharide biosynthesis and export,

consistent with the increased mucosity of AltDE and other deep *Alteromonas* isolates (Raguenes et al. 1996). While ATCC 27126 is enriched in genes for signal transduction and transcriptional regulation, environmental sensing and sugar and amino acid utilization, which may reflect a capacity for making use of a broader array of substrates, AltDE contains more dioxygenases and genes involved in urea use and transport, suggesting it is more adept at using recalcitrant organic matter.

Deep isolates of *A. macleodii* were experimentally shown to be more resistant to zinc, mercury and lead than their shallower strains, consistent with the identification of large numbers of genes for heavy metal resistance and detoxification in its genome. Heavy metals are thought to be associated with particulate matter (Puig et al. 1999) and therefore particle-associated microbes may require genes to deal with high, inhibitory concentrations of these metals. Based on these findings and the distribution of AltDE and ATCC 27126 relatives it was hypothesized by Ivars-Martínez et al. (2008b) that niche separation of these ecotypes is achieved by the preference of AltDE for large, fast-sinking POM present in both the deep sea and the upper water column, whereas ATCC 27126 associates with smaller, more slowly sinking POM present in shallow environments.

2.3.3 Gammaproteobacteria: Alteromonadales: Pseudoalteromonas

Thanks to versatile metabolic capabilities the genus *Pseudoalteromonas* shows widespread oceanic distribution and adaptability to dissimilar ecological habitats (Ivanova et al. 2014). While no piezophilic *Pseudoalteromonas* spp. are known, members of this genus are consistently isolated from the deep ocean, suggesting they may be important participants in these environments. One deep-sea *Pseudoalteromonas* that has been studied is the psychrophile *Pseudoalteromonas* sp. SM9913. SM9913 produces exopolysaccharides (Liu et al. 2013), a function typical of other *Pseudoalteromonas* spp. (Nichols et al. 2005; Zhou et al. 2009), which may facilitate colonization of particles and stabilize cold-adapted proteases for the degradation of POM (Chen et al. 2003; Qin et al. 2007, 2011). To identify the deep-sea adaptations possessed by *Pseudoalteromonas* sp. SM9913 its genome was sequenced (Qin et al. 2011) and compared to *Pseudoalteromonas haloplanktis* TAC125, a psychrophilic isolate from Antarctic coastal seawater (Médigue et al. 2005). Like *A. macleodii* AltDE and other deep-ocean microbes, SM9913 is enriched in transposases and integrases. A large number of SM9913-specific genes belong to COG categories involved in cell motility (N) and signal transduction (T), which are suggested to be involved in sensing and movement towards POM. Indeed, SM9913 has three gene clusters for flagellar biosynthesis, with one encoding a lateral flagellum similar to that found in *P. profundum* SS9 (see Sect. 2.3.6; Eloe et al. 2008). SM9913 also has more genes involved in heavy metal resistance and is

more resistant to zinc than TAC125. These findings, along with the presence of a complete glycolysis pathway and TCA cycle, suggest *Pseudoalteromonas* sp. SM9913 is adept at colonizing labile particulate organic carbon in the deep ocean.

2.3.4 Gammaproteobacteria: Alteromonadales: Shewanella

Members of the genus *Shewanella* are some of the most common deep-sea isolates under both high and low hydrostatic pressure conditions. The *Shewanella* genus forms distinct clades largely based on level of psychrophilicity and Na + dependence (Kato and Nogi 2001; Zhao et al. 2010; Satomi 2014). Genomic comparisons of 17 *Shewanella* species and other members of the Gammaproteobacteria indicated that cold-adapted species have similar amino acid compositions, with enrichments in isoleucine, lysine, and asparagine, and low alanine, proline, and arginine content, in part due to differences in GC content (Zhao et al. 2010). Reciprocal best blasts of cold-adapted strains of *Shewanella* also showed similarity to the cold-adapted *P. profundum* SS9 and *C. psychrerythraea* 34H (Zhao et al. 2010).

While similar analyses are yet to be performed for all piezophilic and non-piezophilic *Shewanella* species, studies comparing individual piezophiles to similar piezosensitive members have been completed. One study compared the genome of the psychropiezophilic microbe *S. piezotolerans* WP3 (Wang et al. 2008), isolated from sediment at a depth of 1914 m (Wang et al. 2004) and showing optimum growth at 20 MPa and 15–20 °C (Xiao et al. 2007), to that of the mesophile *Shewanella oneidensis* MR-1 (Heidelberg et al. 2002). WP3 showed moderately higher abundances of genes involved in cell wall/membrane/envelope biogenesis (M), energy production and conversion (C), intracellular trafficking, secretion, and vesicular transport (U), and inorganic ion transport and metabolism (P). The WP3 genome had higher numbers of duplicated genes, mostly involved in transport, secretion, energy metabolism, and transcriptional regulation. Consistent with the psychrophiles *S. halifaxensis* and *S. sediminis*, WP3 also showed higher abundances of cytochrome c oxidases when compared to MR-1 (Zhao et al. 2010). Like *P. profundum* SS9, WP3 has genes for both a polar and lateral flagellum (Wang et al. 2008), which are for swimming and swarming (i.e. movement along surfaces) respectively. The two sets of WP3 flagella were inversely regulated by low temperature and high pressure; the lateral flagellum was upregulated at low temperature but slightly repressed by high pressure (20 MPa) while the polar flagellum was repressed at low temperature but upregulated at high pressure. Further analysis showed that a mutant lacking a functional lateral flagellum showed no motility at 4 °C, indicating that this flagellum is essential for movement at low temperatures.

The genome sequence of the psychropiezophile *S. violacea* DSS12, isolated from sediment at a depth of 5110 m in the Ryukyu Trench and with optimum temperature and pressure of 8 °C and 30 MPa (Nogi et al. 1998a), has also been compared against that of the mesophile *Shewanella oneidensis* MR-1

(Aono et al. 2010). While most members of the *Shewanella* are capable of anaerobic growth using a number of electron acceptors, DSS12 contains only nitrate and trimethylamine N-oxide (TMAO) reductases and instead has more terminal oxidases for aerobic respiration. More proteases, polysaccharidases, chitinases, and cellulose hydrolases were identified in DSS12 than in MR-1, which may be useful for breaking down sinking POM-containing chitin exoskeletons. DSS12 also has genes for phosphatidylserine decarboxylase, an enzyme which catalyzes the conversion of phosphatidylserine to phosphatidylethanolamine (PE) and may play a role in cell division at low temperatures, and cardiolipin synthetase, which is thought to be important for maintaining the structural stability of the cytochrome c oxidase complex under high hydrostatic pressures. Both strain DSS12 and another *Shewanella* piezophile, *S. benthica* strain KT99, lack DNA photolyase genes. KT99 was isolated from amphipods collected at a depth of 9856 m in the Kermadec Trench and is obligately piezophilic, showing optimal growth at 90 MPa and no growth at 40 MPa (Lauro et al. 2013a). When COG abundances were compared against that of *Shewanella frigidmarina* NCIMB400, KT99 was enriched in genes for DNA replication, recombination, and repair, and transposases, all attributes of other deep-sea bacterial genomes.

2.3.5 Gammaproteobacteria: Mixed Orders (Oceanospirillales and Alteromonadales): Oceanospirillales and Colwellia

Two microbial taxa that have members adapted to high pressure and show interesting physiological plasticity include the order Oceanospirillales and the genus *Colwellia* within the Alteromonadales. Members of the *Colwellia* are some of the most common deep-sea isolates under high hydrostatic pressure conditions and have been isolated from at least four different trenches, including *Colwellia* sp. MT41 (Yayanos et al. 1981), *C. piezophila* (Nogi et al. 2004), and *C. hadaliensis* (Deming et al. 1988) (Fig. 2.1). Recently the first piezophile of the Oceanospirillales, *Profundimonas piezophila* YC-1, was obtained (Cao et al. 2014). YC-1 is a slow-growing, facultative anaerobic heterotroph most closely related to the uncultured symbiont of the deep-sea whale bone-eating *Osedax* worms. YC-1 has a doubling time of 41 h at its optimum pressure of 50 MPa, much slower than most piezophilic Gammaproteobacteria but similar to the 36 h seen for the piezophilic *Roseobacter* PRT1 also isolated from the Puerto Rico Trench (Eloe et al. 2011b), suggesting it might be adapted to an oligotrophic environment. Like other members of the Oceanospirillales YC-1 is capable of hydrocarbon utilization. Shortly after the Deepwater Horizon oil spill the associated microbial community was dominated by the Oceanospirillales (Mason et al. 2012). Metagenomics, single-cell genomics, and metatranscriptomics revealed that members of the Oceanospirillales were actively degrading alkanes (Mason et al. 2012). After the spill was capped, *Colwellia*

spp. began to dominate the plume and sediment communities in concomitance with changes in hydrocarbon composition and abundance, leading to the hypothesis that non-gaseous n-alkanes and cycloalkanes were degraded by Oceanospirillales followed by gaseous and aromatic hydrocarbon degradation by *Colwellia* (Valentine et al. 2010; Mason et al. 2014a, b). Stable isotope probing experiments have shown that *Colwellia* spp. are capable of incorporating ^{13}C from ethane, propane, and benzene and are therefore able to use a wide range of hydrocarbons (Redmond and Valentine 2012). However, a *Colwellia* SAG recovered from the Deepwater Horizon plume did not have complete pathways involved in hydrocarbon degradation (Mason et al. 2014a) and no isolate has been reported that degrades oil as the sole carbon source, although *Colwellia* sp. RC25 was shown to degrade hydrocarbons in the presence of Corexit (Bælum et al. 2012; Chakraborty et al. 2012). Corexit, a dispersant thought to make oil more bioavailable and which was used in the Deepwater Horizon oil spill, may allow some organisms to outcompete natural hydrocarbon degraders. Members of the *Colwellia* were enriched in dispersant-only and oil-Corexit mixtures but not in oil-only microcosms, which were dominated by the genus *Marinobacter* (Kleindienst et al. 2015). Rates of hydrocarbon oxidation were highest in oil-only microcosms and therefore Corexit may not support stimulation of oil biodegradation (Kleindienst et al. 2015). However, whether *Colwellia* outcompetes other hydrocarbon degraders in the presence of Corexit or if Corexit inhibits *Marinobacter* is unknown.

2.3.6 Gammaproteobacteria: Vibrionales: Photobacterium

The genus *Photobacterium*, composed of more than 20 species (Amaral et al. 2015), is a member of the Vibrionales order within the Gammaproteobacteria. Members of the genus *Photobacterium* are best known for their symbiotic relationships with marine eukaryotes (Amaral et al. 2015). One of the most studied piezophilic microbes is *P. profundum* SS9. SS9 was isolated at a depth of 2551 m from an amphipod in the Sulu Trough (DeLong 1986) and can grow over broad pressure (0.1–90 MPa) and temperature (2–20 °C) ranges, with optimum growth at 28 MPa and 15 °C (El-Hajj et al. 2010). At least three other strains of *P. profundum* have been isolated, including the piezophile DSJ4 from deep-ocean sediment (Nogi et al. 1998b) and the piezosensitive strains 3TCK and 1230sf1 (Campanaro et al. 2005; Lauro et al. 2014). Genomic comparisons of *P. profundum* bathytypes have revealed a number of attributes for a deep-sea adapted lifestyle (Campanaro et al. 2005; Lauro et al. 2014). SS9 has 15 copies of the rRNA operon that show variability in specific helices, which may confer ribosome stability under high-pressure conditions (Lauro et al. 2007). COG comparisons showed higher abundances of genes for motility and chemotaxis (N) and DNA replication, recombination and repair (L) but fewer genes involved in energy production (C) when SS9 was compared to 3TCK (Lauro et al. 2014). The overrepresentation of COG L was due to large numbers of transposable elements in SS9, which had 206 compared to 3 in

3TCK, while higher abundances of genes associated with motility were the result of a second cluster of genes for flagellum biosynthesis. These genes appear to encode for the production and function of a lateral flagella which also exist in related microbes, including piezophilic members of the Vibrionaceae (McCarter 2004) such as *P. phosphoreum* ANT-2200 (Zhang et al. 2014), *Moritella* sp. PE36 (Nagata et al. 2010), and *S. piezoterolans* WP3 as described in section IIID. Genetic experiments indicate that the SS9 surface motility system is primarily functional at high pressure, conditions that also favor the expression of the genes present within the lateral flagella gene cluster (Eloe et al. 2008). SS9 therefore regulates its lateral flagella genes differently than WP3. Regardless, in the surface ocean genes for chemotaxis and motility have been observed to be a characteristic of copiotrophic microbes, which possess the sensory and physiological capabilities to swim along the concentration plumes of organic matter, utilize it in high concentrations, and rapidly increase in cell numbers (Yooseph et al. 2010). These types of adaptations may also be important for deep-ocean microbes colonizing POM descending through the water column.

Pressure-related changes in regulation have also been found in genes affiliated with TMAO. SS9 has two *torS* genes which regulate the response to TMAO (Bordi et al. 2003). One of these genes, which is absent from 3TCK, is upregulated under high hydrostatic pressure conditions (Campanaro et al. 2005). SS9 also has multiple *torA* genes encoding TMAO reductase, one of which is upregulated at high pressure (Vezzi et al. 2005). The piezophile *P. phosphoreum* ANT-2200 has four copies of *torA*, including one homologous to the upregulated gene in SS9 (Zhang et al. 2014). These results suggest that TMAO reduction in deep-sea *Photobacterium* strains is an important respiratory adaptation at greater depths (Vezzi et al. 2005), perhaps within the anaerobic zones of particles or inside animal hosts. This is consistent with concentration measurements that show TMAO increasing in the tissues of some marine animals with depth of capture. This is thought to be an adaptation to high pressure (Yancey et al. 2014).

Unlike shallow *Photobacterium* spp., SS9 lacks a DNA photolyase (Vezzi et al. 2005; Lauro et al. 2014) and is extremely UV sensitive, a phenotype shared by many deep-sea microbes (Yayanos 1995). When the *phr* gene cluster was cloned from the shallow-water strain 3TCK into SS9 it dramatically enhanced UV-resistance. Because the *phr* gene cluster has a distinct codon usage in strain 3TCK it appears likely to have been acquired through horizontal gene transfer. *P. profundum* strains may be undergoing adaptive radiation driven by gene acquisition and loss and the horizontal gene transfer of the *phr* gene cluster into 3TCK could have been one of the evolutionary changes required for its adaptation from the deep-sea to shallower, sunlight-exposed waters (Lauro et al. 2014). This presents the intriguing possibility that the evolutionary path between shallow-water and deep-sea bacteria is not one-way, and shallow-water derivatives of piezophilic deep-sea bacteria can arise just as the reverse case. Horizontal gene transfer is also reflected in the genomes of deep-sea bacteria, with the *nar* gene cluster in the deep-sea isolate *Pseudomonas* sp. MT-1 being one example (Tamegai et al. 2004; Ikeda et al. 2009; Oikawa et al. 2015).

2.3.7 Gammaproteobacteria: Vibrionales: *Vibrio*

Despite being most well known for its pathogenic members, the genus *Vibrio* is composed of diverse species with representatives found in the deep sea (Ohwada et al. 1980; Tabor et al. 1981b; Reen et al. 2006). Recently the genome of *Vibrio antiquarius,* which was isolated from the East Pacific Rise hydrothermal vent system at 2520 m, was compared against other *Vibrio* spp. (Hasan et al. 2015). The genome of *V. antiquarius* suggests a unique ability to scavenge hydrogen peroxide and the potential for manganese oxidation and heavy metal resistance. *V. antiquarius* also encodes a fatty acid desaturase which may help maintain membrane fluidity under high pressures and low temperatures. Many psychrophilic and piezophilic bacteria produce unsaturated fatty acids or even polyunsaturated fatty acids (PUFAs) to help counter the effects of increasing pressure and decreasing temperature on their membranes (DeLong and Yayanos 1985; Allen et al. 1999; Bartlett 2002). Indeed, the benefits of polyunsatured fatty acid consumption on human health (Wall et al. 2010; Joffre et al. 2014) have led to interest in piezophile sources of PUFAs for use by humans or other animals. Hasan et al. note that the genome contains many homologs of virulence genes found in other *Vibrio* species, which they suggest may provide ecological functions outside of virulence, such as the establishment of host/cell relationships, provide a means of attachment to surfaces, signaling, or other interactions among aquatic communities. When recruited against metagenomic data *V. antiquarius* shows a ubiquitous distribution, identified in saltern, marine, coral, and human gut metagenomes. This distribution, along with the presence of a DNA photolyase, suggests that *V. antiquarius* may not be obligately adapted to the deep ocean.

2.3.8 Deltaproteobacteria: *Desulfovibrio*

Sulfate-reducing bacteria (SRB) contribute to the breakdown of organic matter and in some cases the coupling of anaerobic oxidation of methane to sulfate reduction in marine sediments (Hu et al. 2010; Stokke et al. 2012; Ruff et al. 2015). Members of the SRB within the genus *Desulfovibrio*, including *D. profundus* (Bale et al. 1997), *D. hydrothermalis* (Alazard et al. 2003), and *D. piezophilus* (Khelaifia et al. 2011), have been isolated from the deep sea. *D. piezophilus* C1TLV30, which was isolated from a wood fall at a depth of 1700 m and shows optimum growth at 30 °C and 10 MPa, was compared against other *Desulfovibrio* spp. and had its transcriptome analyzed at 0.1 and 10 MPa (Pradel et al. 2013). *D. piezophilus* has a cytochrome c gene that is overexpressed under high hydrostatic pressure conditions and is closely related to one found in *P. profundum* SS9. Amino acid composition, transport, and metabolism may be an important adaptive strategy for *D. piezophilus* as this bathytype showed an amino acid composition distinct from other *Desulfovibrio* strains and elevated expression of genes involved in alanine, histidine, and arginine

biosynthesis and glutamine/glutamate metabolism and transport at high pressure. Similarly, transcriptomes from *D. hydrothermalis*, which was isolated from an East Pacific Rise hydrothermal vent at 2600 m, at 0.1, 10, and 26 MPa also showed differential expression of genes involved in glutamate metabolism (Amrani et al. 2014). Glutamate was shown to accumulate in *D. hydrothermalis* cells under high hydrostatic pressure, suggesting it may act as a piezolyte, an osmolyte that accumulates at high pressure (Martin et al. 2002). Genes involved in glutamate metabolism in *P. profundum* SS9 and *S. violacea* DSS12 also show differential expression with pressure (Campanaro et al. 2005; Vezzi et al. 2005; Ikegami et al. 2000). Thus, glutamate could serve an important global role as an extrinsic factor modulating protein structure or activity as a function of pressure in multiple piezophiles.

2.3.9 Firmicutes: Carnobacterium

Members of the genus *Carnobacterium,* which belongs to the Lactobacillales within the Firmicutes, are lactic acid bacteria of wide interest in the food and aquaculture industries. Carnobacteria have been isolated from processed meat, fish, and dairy products and can produce antimicrobial peptides to stunt the growth of other microbes, leading to speculation that members of this genus may cause or inhibit food spoilage (Hammes and Hertel 2006; Leisner et al. 2007). Similarly, carnobacteria have been identified within fish and may have both probiotic and pathogenetic effects on their host (Hammes and Hertel 2006; Leisner et al. 2007, 2012). Isolates have also been obtained from many different environments, including permafrost (Pikuta et al. 2005), and some strains even show growth under the low temperature, low pressure, and anoxic conditions similar to those found on Mars (Nicholson et al. 2013). Two isolates from 2500 m in the Aleutian Trench, AT7 and AT12, show optimum growth at 15 MPa (Lauro et al. 2007; Yayanos and DeLong 1987) and are the only gram-positive piezophilic bacteria currently isolated. Comparative genomics of *Carnobacterium* sp. AT7 with other *Carnobacterium* spp. and *Enterococcus faecalis* have shown this microbe is distinct (Stratton 2008; Leisner et al. 2012; Voget et al. 2011). While AT7 does not contain a photolyase, it does contain two endonucleases that may be involved in UV damage repair (Stratton 2008). When compared to *C. maltaromaticum* ATCC 35586, a host-associated species, AT7 lacks genes affiliated with host colonization, invasion, and metabolism (Leisner et al. 2012). A unique characteristic of AT7 is the presence of two plasmids that contain genes putatively affiliated with cadmium, tellurium, and copper efflux which are likely to contribute to heavy-metal resistance (Stratton 2008). The identification of *Carnobacterium* spp. showing growth under high pressure may have important repercussions for foods that are sterilized using pascalization.

2.3.10 Archaea: Marine Group I Thaumarchaea

Marine Group I Thaumarchaea (MGI) are ammonia-oxidizing archaea recognized as important drivers of nitrification in marine environments. MGI can be found at all depths (Nunoura et al. 2015) and in some cases compose the majority of deep-ocean communities (Karner et al. 2001). The presence of their ammonia monooxygenase gene (*amoA*), which helps catalyze a key intermediate step in nitrification by converting ammonia to nitrite, can be identified throughout the water column (Sintes et al. 2013). Genes involved in the 3-hydroxypropionate/4-hydroxybutyrate (3H/4H) pathway for carbon fixation and ammonia oxidation seem to be conserved among members of the Thaumarchaea (Ngugi et al. 2015), highlighting the importance of these functions within this lineage. However, mixotrophy has also been suggested in these archaea based on substrake uptake (Ouverney and Fuhrman 2000; Teira et al. 2006; Seyler et al. 2014; Qin et al. 2014) and genomic data (Hallam et al. 2006; Agogué et al. 2008; Swan et al. 2014). Despite their abundance and important role in the global nitrogen cycle cultivation and isolation of Thaumarchaea has proven difficult (Könneke et al. 2005; Tourna et al. 2011; Qin et al. 2014, 2015). Current isolates are physiologically distinct, displaying different tolerances to light, pH, and salinity, and varying capacities for the use of certain carbon compounds (Qin et al. 2014). While no deep-ocean Thaumarchaea are currently in culture, phylogenetic analyses using 16S rRNA, *amoA*, and concatenated single-copy marker genes have shown that MGI fall into shallow- and deep-water clades (Hallam et al. 2006; Beman et al. 2008; Nicol et al. 2011; Ngugi et al. 2015). These clades likely represent distinct ecotypes as niche separation has been identified according to ammonia concentrations, among other factors (Sintes et al. 2013; Nunoura et al. 2015). Analysis of single-amplified genomes have also shown that deep-ocean MGI represent distinct bathytypes. While thaumarchaeal photolyase genes have been identified in surface waters, they were absent in mesopelagic SAGs, suggesting they are not exposed to light-induced damage (Luo et al. 2014). It has been proposed that sensitivity to photoinhibition may be partially responsible for depth distributions (Mincer et al. 2007; Church et al. 2010) as some Thaumarchaea are inhibited by light (Merbt et al. 2012; Qin et al. 2014). Analysis of MGI SAGs collected from mesopelagic depths at Station ALOHA and the South Atlantic identified nine phylotypes within the deep clade, with six containing SAGs from both regions, suggesting these members have a cosmopolitan deep-ocean distribution (Swan et al. 2014). COG distributions associated with signal transduction and urea utilization were also found to differ between bathytypes, with the former more abundant in the shallow ecotype and the latter in the deep one (Luo et al. 2014). A SAG related to *Nitrosopumilus* from the Puerto Rico Trench also has genes for urea degradation, along with genes involved in fatty acid and lipoic acid synthesis and glycine cleavage (Léon-Zayas et al. 2015). This SAG also has an aquaporin, which function in osmotic pressure

adaptation by effluxing water from cells exposed to hypotonic environments or help in the retention of small-molecule compatible solutes. This aquaporin may therefore play a role in high-pressure adaptation.

2.4 What Do These Deep-Sea Microbial Communities Actually Look like?

Deep-ocean bathytypes can provide insight into adaptations in specific microbial lineages but are these results ecologically important? While SAR11 and Thaumarchaea are widespread, dominant members of the deep-ocean community, many deep bathytypes are present in low abundance in deep ocean samples (Eloe et al. 2011c; Vandieken et al. 2012; Smedile et al. 2013). Many bathytypes are opportunistic heterotrophs, capable of rapid growth in nutrient-rich environments, and show increases in abundance when associated with enrichments, such as *Colwellia* and the *Oceanospirillales* at oil spills (Valentine et al. 2010; Redmond and Valentine 2012; Mason et al. 2014b) and *Pseudoalteromonas, Colwellia,* and *Shewanella* with polyaromatic hydrocarbons (Dong et al. 2015). Perhaps unsurprisingly, therefore, the same species are consistently isolated in rich media under high hydrostatic pressure conditions. These species have been isolated over long time scales and geographic distances, suggesting a widespread distribution in the deep ocean. Even strains within specific bathytypes, like those of *S. benthica*, can display distinct pressure optima and roughly cluster based on depth, suggesting they may colonize distinct depth-zones (Fig. 2.1). Comparisons of these strains may reveal small but significant differences required for adaptation to increasing depth. However, no studies thus far have assessed the in situ depth restrictions of piezophiles.

In contrast to culture-based studies, community analysis using deep 16S rRNA gene sequencing suggests deep-ocean microbial communities are composed of abundant, widely distributed species and a number of rare members with a more limited distribution (Sogin et al. 2006; Salazar et al. 2015a, b). Deep-ocean communities tend to be more similar to one another when compared to shallower samples as deep-sea datasets cluster together despite disparate environmental locations (DeLong et al. 2006; Eloe et al. 2011c; Thureborn et al. 2013; Jacob et al. 2013; Nunoura et al. 2015), a finding that also seems to be true with viral communities at depth (Yoshida et al. 2013; Winter et al. 2014; Hurwitz et al. 2015). Within these deep-sea communities composition has been shown to differ depending upon their size-fraction (DeLong et al. 1993; Moeseneder et al. 2001; Simon et al. 2002, 2014; Wilkins et al. 2013; Ganesh et al. 2014; Salazar et al. 2015a, b), which is thought to separate free-living from particle-associated taxa. Comparisons of bathypelagic communities have shown that free-living communities are dependent on temperature and depth and are similar between different sites, whereas particle-attached communities are more basin-specific and dependent on

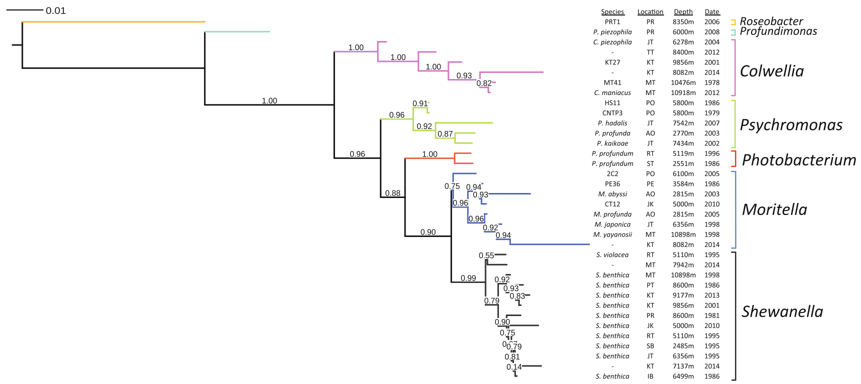

Fig. 2.1 Phylogenetic tree of piezophilic bacterial strains and their isolation location, depth, and date. Many piezophiles have been isolated from many different oceanic basins, suggesting they have a widespread and relatively consistent distribution, over time, in the ocean. Samples without a species name (-) are recent, unpublished isolates. *PR* Puerto Rico Trench, *JT* Japan Trench, *TT* Tonga Trench, *KT* Kermadec Trench, *MT* Mariana Trench, *PO* Pacific Ocean, *AO* Atlantic Ocean, *RT* Ryukyu Trench, *ST* Sulu Trough, *PE* Patton Escarpment, *JK* Japan/Kurile-Kamchatcka Trench, *PT* Phillipine Trench, *IB* Izu-Bonin Trench, *SB* Suruga Bay

dispersal limitation, water age, and mixing (Salazar et al. 2015a). Deep-ocean particles may be produced at depth and thus lack a surface ocean connection, leading to their distinctive basin-specific microbial community compositions (Herndl and Reinthaler 2013; Salazar et al. 2015a). A high proportion of taxa, including 7 of 15 identified phyla, were consistently found to be associated with either the free-living or particle-attached size fraction in the bathypelagic. The archaea, including the Thaumarchaea, SAR86, SAR324, SAR406, and SAR202 clades were all associated with the free-living fraction, while the Bacteroidetes, Firmicutes, and Planctomycetes were associated with the particle-attached fraction. These findings suggest that these size-fractions have distinct biogeochemical roles associated with differing lifestyles and are consistent with our understanding of the metabolism of many of these taxa (Salazar et al. 2015b). Future studies should focus on how mesoscale events, seasonal cycles, and decadal shifts may alter community composition due to the tight coupling between the deep ocean and the surface by sampling at the same sites over longer time scales.

The most abundant deep-ocean prokaryotic groups are the Gammaproteobacteria (López-García et al. 2001; Delong et al. 2006; Konstantinidis et al. 2009; Smedile et al. 2013; Wilkins et al. 2013; Nunoura et al. 2015; Salazar et al. 2015a, b), Alphaprotebacteria (Martin-Cuadrado et al. 2007; Eloe et al. 2011c), Deltaproteobacteria, (Salazar et al. 2015a, b), Actinobacteria (Salazar et al. 2015a, b), and Thaumarchaea (Martin-Cuadrado et al. 2009; Eloe et al. 2011c; Yakimov et al. 2011). Archaeal abundances have been seen to range from 2%

(Salazar et al. 2015a, b) to 39% (Karner et al. 2001) of deep-sea communities. In a variety of bathypelagic settings Salazar and colleagues found that the only abundant and cosmopolitan OTUs belonged to *Alteromonas*, MGI Thamaurchaea, and SAR324, despite the presence of similar microbial community compositions when assessed at broader phylogenetic classifications (Salazar et al. 2015a, b). The Deltaproteobacteria SAR324 clade can be divided into distinct clusters (Brown and Donachie 2007) and is specifically enriched within oxygen minimum zones (Wright et al. 2012) and in meso- and bathy-pelagic dark-ocean locales (Wright et al. 1997; Treusch et al. 2009; Wright et al. 2012; Nunoura et al. 2015). Other groups have been seen to increase with depth along depth profiles, including the Planctomycetes, Gemmatimonadetes, Acidobacteria, Alteromonadaceae, Nitrospina, SAR11 (Delong et al. 2006; Konstantinidis et al. 2009), SAR406 (Nunoura et al. 2015), and SAR202 of the Chloroflexi (Morris et al. 2004; Varela et al. 2008). The candidate phylum SAR406 (known as the 'Marinimicrobia' or Marine Group A) is one of the most consistent groups of microbes found preferentially at depth (Nunoura et al. 2015; Salazar et al. 2015a, b). More than 10 subgroups have been identified (Allers et al. 2013; Wright et al. 2014) and many members are prevalent in low oxygen environments. This is consistent with fosmid library gene studies suggesting that Marinimicrobia have adaptations to O_2 deficient conditions and may be capable of sulfur-based energy metabolism (Wright et al. 2014). Single-amplified genomes have been published (Rinke et al. 2013) but while SAR406 is consistently identified in deep-ocean samples (López-García et al. 2001; Wilkins et al. 2013; Salazar et al. 2015a, b), including trenches (Eloe et al. 2011c; Nunoura et al. 2015), little is known about the physiological properties of its deeper dwelling members.

Much more is known about community composition at bathyal and abyssal depths than of the hadal zone. Recently a community depth profile down to 10,257 m in the Challenger Deep portion of the Mariana Trench identified microbial assemblage stratification through the water column (Nunoura et al. 2015). An increase in heterotrophic microbial groups was present at depths exceeding 7000 m at the expense of chemoautotrophs, potentially as a result of higher availability of POC. Communities deeper than 9000 m were dominated by *Pseudomonas* spp. In addition, ammonia oxidizers and nitrifiers also showed distinct depth trends, with ammonia oxidizing bacteria (AOB) of the genus *Nitrospira* abundant in the surface and upper water column and in the hadal zone, while ammonia oxidizing archaea (AOA) and *Nitrospina* were abundant in the intermediate depths. These results are consistent with niche separation due to the increased availability of ammonia and nitrite resulting from the decomposition of organic matter in the upper water column and within the trench, as AOB prefers higher ammonia concentrations than AOA. These results add to a growing body of evidence that the hadal ocean may display increased rates of heterotrophy because of increased carbon deposition due to the funneling and concentration of POC into the deeper trench regions (Glud et al. 2013; Ichino et al. 2015; Nunoura et al. 2015). Interestingly, in contrast to the Challenger Deep pelagic waters most bacterial sequences obtained from the water column at 6000 m in the Puerto Rico Trench were members of the SAR11 clade (Eloe et al. 2011c). The difference in community

composition between these two hadal settings, even when considering depth and distance from the seafloor, suggests that trench communities can differ markedly from one another.

2.5 What Is the Effect of Decompression?

Sample handling is an especially important consideration when working with deep-ocean samples. One normal consequence following the collection of materials from within the deep ocean is sample decompression during recovery. Because sample collection can take upwards of four hours to return to the surface from the hadal zone, followed by time for sample processing, it is likely that community composition changes occur as a result of physiological stress. All piezophiles currently in culture are able to withstand moderate periods of decompression. This is evident by the decompression that is routinely employed during most sample recoveries, as well as during the preparation of piezophile subcultures and frozen stocks. While incubation at 0.1 MPa for <10 h did not lead to large decreases in CFUs for the obligate piezophile *Colwellia* sp. MT41 or the moderate piezophile *Psychromonas* sp. CNPT3, decompression of MT41 for 50 h led to CFU decreases by over five orders of magnitude and cell ultrastructural alterations (Yayanos and Dietz 1983; Chastain and Yayanos 1991). This suggests that given enough time at atmospheric pressure many obligately piezophilic deep-ocean microbes will lose viability.

While isolate viability may be maintained during short-term decompression the effects of decompression or decompression-recompression cycles on community composition and their activity is relatively unexplored. Some research groups have developed pressure-retaining samplers or equipment for in situ filtration and preservation (Yayanos 1977, Tabor et al. 1981a; Jannasch and Wirsen 1982; Bianchi et al. 1999; Kato 2006; Tamburini et al. 2009). One study developed and deployed a sampler to ~2200 m water depth and compared transcriptomes of seawater filtered in situ and after collection at the surface (Edgcomb et al. 2014). Almost two times more total and classifiable reads were recovered from the in situ filtered and fixed samples as compared with those filtered post-recovery, and changes in gene expression were also noted between the two sample collection methods. Members of the Thaumarchaea may be some of the microbes most affected by sampling methods that include decompression (La Cono et al. 2015). La Cono and colleagues collected samples from 500 m and 2222 m in the Mediterranean Sea and filtered immediately after collection or after incubation for 24 or 72 h at in situ temperatures. In samples left for three days archaeal populations decreased three-fold while their bacterial populations doubled. Thaumarchaea composition shifted over time, perhaps reflecting the existence of autochthonous and allochthonous ecotypes. Regardless of how communities and activities shift as a function of pressure, maintaining in situ pressure conditions should be an important consideration when sampling the deep ocean to obtain ecologically relevant results.

2.6 How Do Bathytype Features Compare with Results from Culture-Independent Metagenome Analyses?

Comparisons of deep-ocean bathytypes with shallow-water ecotypes have indicated the presence of a variety of characteristics that correlate with growth and survival at depth. These include larger genome sizes, increased abundances of transposable elements, the ability to colonize POM and break down recalcitrant DOC, heavy metal resistance, and the absence of genes for repairing UV-induced DNA damage (Table 2.1). A valuable complement to the genome comparisons of cultured deep and shallow ecotypes is the comparison of deep and shallow metagenomic sequence data. Metagenomic data can provide insight into broad community genetic and physiological characteristics, and when compared with specific bathytypes, can shed light on genomic changes required for deep-ocean lifestyles. Below these two approaches are compared with regard to (1) particle attachment, (2) mobile elements and genome size, (3) heavy metal resistance, (4) recalcitrant organic carbon utilization and (5) autotrophy and lithotrophy.

Particle attachment. Deep-ocean microbes are thought to be largely dependent on particulate organic matter (POM) sinking from the surface. Sinking POM are microbial "hot spots," harboring dense aggregations of bacteria that can be orders of magnitude higher than the surrounding seawater thanks to higher nutrient levels (Azam and Long 2001; Simon et al. 2002; Lyons and Dobbs 2012; Turner 2015). Despite the notion that the deep ocean is carbon-limited, large fluxes of organic matter into the deep ocean have been seen, showing that there are pulses of detritus to the deep-sea floor (Billett et al. 1983; Rice et al. 1986; Lochte and Turley 1988; Thiel et al. 1989; Danovaro et al. 2002; Wu et al. 2013; Agusti et al. 2014), and trenches may be enriched in organic matter when compared to shallower sites thanks to topography (Boetius et al. 1996; Danovaro et al. 2003; Gooday et al. 2010; Glud et al. 2013; Ichino et al. 2015). It is likely that deep-ocean communities are adapted to generally low and sporadic fluxes of organic matter and have methods to cope with resource scarcity, such as increased numbers of signal transduction pathways in the Puerto Rico Trench (Eloe et al. 2011a). They may also require movement for quick colonization when POM becomes available. Deep bathytypes are typically enriched in methyl-accepting chemotaxis proteins which may be involved in finding POM and DOM (Lauro and Bartlett 2008). For example, the piezophilic bathytype *Psychromonas* sp. CNPT3 and a Psychromonas SAG from the Puerto Rico Trench have genes involved in motility and chemotaxis not present in the psychrophile *Psychromonas ingrahamii* (Stratton 2008; Léon-Zayas et al. 2015). Genes for pilus synthesis, type II secretion systems, polysaccharide synthesis, and antibiotic synthesis, which are associated with microbes who live particle-attached lifestyles or are part of a biofilm, were enriched at 4000 m (DeLong et al. 2006), consistent with other deep-sea sites (Tringe et al. 2005; Martin-Cuadrado et al. 2007). In contrast, flagellar biosynthesis and bacterial chemotaxis proteins were more highly represented in photic zone samples than those in the deep water at Station ALOHA (Delong et al. 2006). Deep-ocean microbes may also colonize particles, reach high

Table 2.1 A list of bathytypes described in this review and genomic attributes identified that may confer adaptations to the deep ocean. NA, not described; ROM, refractory organic matter; ✓, present genomic or physiological attribute; -, presence currently undescribed

Bathytype	Comparison strain	Isolation depth (m)	P_{opt} (MPa)	P_{range} (MPa)	DNA photolyase	Heavy metal resistance	Genome size and mobile elements	Movement and attachment	Other unique attributes	References
Alteromonas macleodii AltDE	*A. macleodii* ATCC 27126 *Pseudoalteromonas atlantica* T6c	1000	NA	NA	✓	✓	Transposable elements, insertion sequences, phage interaction	EPS biosynthesis	ROM degradation, cytochrome BD complex	Ivars-Martinez et al. (2008a, b)
Desulfovibrio piezophilus	*Desulfovibro* spp.	1700	10	0.1–30	✓	–	–	–	Altered amino acid composition, differential abundances of COGs CDEOPQ, glutamate metabolism	Pradel et al. (2013)
Pseudoalteromonas sp. SM9913	*P. haoloplanktis* TAC125	1855	NA	NA	✓	✓	Transposable elements, phage interaction	lateral flagellum, COG T increased, EPS biosynthesis	Cold-adapted proteases	Qin et al. (2011)
Shewanellaa piezotolerans WP3	*S. oneidensis* MR-1	1914	20	0.1–50	×	–	Increased genome size, gene duplications	Lateral flagellum	Higher abundances of cytochrome c oxidases, COG CMPU increased, RNA modification genes	Wang et al. (2008)
Carnobacterium sp. AT7	*Comobatterium* spp. *Enterococcus faecalis*	2500	15	0.1–60	×	✓	–	–	RNA modification genes	Stratton (2008) and Voget et al. (2011)

(continued)

Table 2.1 (continued)

Bathytype	Comparison strain	Isolation depth (m)	P_{opt} (MPa)	P_{range} (MPa)	DNA photolyase	Heavy metal resistance	Genome size and mobile elements	Movement and attachment	Other unique attributes	References
Vibrio antiquarius	*Vibrio* spp.	2520	NA	NA	✓	✓	–	Lateral flagellum	Increased O_2/H_2o_2 tolerance, fatty acid unsaturation	Hasan et al. (2015)
Photobacterium profundum SS9	*P. profundum* 3TCK	2551	28	0.1–70	×	–	Transposable elements	Lateral flagellum	Higher rRNA operon abundances, TMAO reductase	Vezzi et al. (2005), Campanaro et al. (2005) and Lauro et al. (2014)
Shewanella violacea DSSU	*S. oneidensis* MR-1	5110	30	0.1–70	×	–	–	–	Higher abundance of terminal oxidases for aerobic respiration, TMAO reductase, secreted enzymes	Aono et al. (2010)
SAR11 clade lc	Epipelagic SAR11	Mesopelagic	NA	NA	×	–	Increased genome size and intergenic spacer regions, phage interaction, higher Dn/Ds ratio	–	Altered amino acid acomposition	Konstantinidis et al. (2009) and Thrash et al. (2014)
Thaumarchaea	Epipelagic Thaumarchaea	Mesopelagic	NA	NA	×	–	–	–	Higher abundances of urea utilization and signal transduction genes	Luo et al. (2014) and Swan et al. (2014)

densities, and produce bioluminescence in order to be engulfed by larger predators and colonize their gastrointestinal tracts (Zarubin et al. 2012; Martini et al. 2013). The moderate piezophile *Photobacterium phosphoreum* ANT-2200 showed three times more bioluminescence when grown at 22 MPa than at 0.1 MPa, forming aggregates under these high pressure conditions (Martini et al. 2013). Threefold more luciferase oxygenase homologs for bioluminescence production were found at 4000 m than in surface datasets (Konstantinidis et al. 2009). However, release of these microbes from larger organisms as fecal pellets in shallower waters, which then sink into the deep ocean, may also be responsible for increased numbers of genes involved in bioluminescence present at depth.

Genome size and mobile elements. Genome size estimates in both cultured deep-ocean microbes and metagenomic analyses show increases versus their shallow-water counterparts. Estimates of genome size at Station ALOHA showed a ~1.35 fold increase in the bathypelagic versus the surface ocean (Konstantinidis et al. 2009; Beszteri et al. 2010). One potential reason for increased genome size is an enrichment of transposases within deep-ocean microbes, such as in bathytypes of *Photobacterium, Shewanella*, and *Pseudoalteromonas*. A *Marinosulfonomonas* SAG from the Puerto Rico Trench also has a large number of transposases (Léon-Zayas et al. 2015). One of the most striking findings in the Station ALOHA metagenomes was increased abundances of transposases, integrases, and ratios 2–3 times higher of nonsynonymous to synonymous (Dn/Ds) mutations at depth (Delong et al. 2006; Konstantinidis et al. 2009). Transposase sequences were from different families and present in diverse microbial taxa suggesting this is not attributable to one specific group in high abundance. Similar abundances have been noted in the deep Mediterranean at 4908 m where transposases, phage integrases, and plasmids were ~10 fold more abundant than in the surface ocean (Smedile et al. 2013). Furthermore, deep-ocean bathytypes and metagenomes are enriched in genes involved in phage interaction, such as CRISPR regions (Ivars-Martínez et al. 2008b; Smedile et al. 2013; Thrash et al. 2014; Mason et al. 2014a). The ratio of viruses to prokaryotes typically increases with depth within the water column (Parada et al. 2007; De Corte et al. 2010; Nunoura et al. 2015) but not in the sediment (Corinaldesi 2015). Still, in one study almost half of deep-ocean sediment isolates harbored prophages (Engelhardt et al. 2011) and viral lysis may be responsible for most prokaryotic mortality in deep-sea sediments (Danovaro et al. 2008), highlighting the importance of viruses in these ecosystems. Protein clusters found exclusively in viruses in the aphotic deep Pacific paralleled those that function in adaptation to high hydrostatic pressure, including those involved in DNA replication, DNA repair, and motility, potentially boosting their hosts' fitness (Hurwitz et al. 2015). Taken together with increases in intergenic spacer regions, these findings suggest more relaxed purifying selection in deep-ocean microbes. Microbes with large genomes may also be more successful in environments with low and diverse resources and where there is no drawback for slow growth (Konstantinidis and Tiedje 2004), such as in SAR11 (Thrash et al. 2014). Both the ability to quickly colonize energy sources when available or grow slowly in an oligotrophic environment are niches found in the deep-ocean.

Heavy-metal toxicity. Another adaptation that may reflect particle attachment is the abundance of genes involved in heavy-metal efflux. Like other bathytypes the deep-ocean isolate *Halomonas zincidurans* contains heavy-metal resistance genes involved in copper homeostasis and tolerance, cobalt–zinc–cadmium resistance, mercuric reduction, and arsenic resistance, and was experimentally shown to tolerate elevated levels of zinc (Xu et al. 2013). Similarly, metagenomic data has shown enrichment in heavy-metal resistance genes at depth. Both the deep Puerto Rico Trench and Mediterranean metagenomes were enriched in genes for heavy metal efflux and detoxification (Eloe et al. 2011a; Smedile et al. 2013). Live sediment trap communities collected from 500 m at Station ALOHA, which were essentially enrichments of actively growing microbes associated with sinking particulate matter, had higher abundances of genes for heavy-metal resistance (*czcABCD*), copper efflux (*cusAB*) and copper two-component sensory systems (*cusRS*), and mercury resistance (*merABR*) and transport (*merTP*) (Fontanez et al. 2015). These genes were affiliated with *Alteromonas*, *Marinobacter*, and *Glaciecola*, genera typically associated with POM and which have been isolated from deep-sea sites. Further analysis of size-fractionated metagenomes, coupled with culture-based studies, will provide insight into the microbial groups and their underlying physiology that are responsible for enrichments in heavy-metal efflux genes and mobile elements at depth.

Recalcitrant organic carbon utilization. DOC export into the mesopelagic and bathypelagic is thought to represent between 10 and 20% of total primary productivity at the surface (Carlson et al. 2010, Giering et al. 2014), leading to concentrations of approximately 40 umol L^{-1} in the deep ocean (Arrieta et al. 2015). Refractory DOC is the dominant form of DOC present in the deep ocean because of its resistance to rapid microbial degradation and subsequent accumulation (Hansell 2013). However, a recent study showed that deep bathypelagic communities are capable of using in situ DOC but low concentrations make its use not energetically feasible (Arrieta et al. 2015), a finding that has been previously suggested (Kujawinski 2011; Hansell 2013). Surface water communities amended with high molecular weight DOM showed that *Alteromonas* spp. and *Idiomarina* spp. were the most highly represented taxa soon after addition (McCarren et al. 2010), consistent with the hypothesis that *A. macleodii* AltDE is capable of breaking down DOM (Ivars-Martínez et al. 2008b). The class *Dehalococcoidia* of the Chloroflexi, another microbial group identified in marine sediment communities at depth, may also be able to perform oxidation of complex organic compounds (Wilms et al. 2006; Fry et al. 2008) or carbon fixation via the Wood-Ljungdahl pathway (Wasmund et al. 2014). Metabolic genes related to the degradation of refractory DOC have also been identified in deep-ocean metagenomes. Genes for glyoxylate and dicarboxylate metabolism for degradation of oxidized and degraded DOM have been identified in deep samples (Delong et al. 2006; Eloe et al. 2011a) and the 3010 m Mediterranean metagenome was enriched in pathways for the breakdown of a number of recalcitrant forms of carbon (Martin-Cuadrado et al. 2007). Interestingly, whole-body extracts of the hadal amphipod *Hirondellea gigas* showed the ability to degrade plant-derived polysaccharides using amylase,

cellulose, mannanase, xylanase, and α-glucosidase (Kobayashi et al. 2012). Bacteria were not able to be isolated from these samples and bacterial or archaeal DNA was unable to be amplified, leading the authors to believe this activity is performed by the amphipod itself. However, dockerin type I repeats, which are involved in cellulose degradation, were more abundant in deep Station ALOHA samples (Konstantinidis et al. 2009) and *P. profundum* SS9 upregulates pathways to degrade chitin and cellulose under high pressure (Vezzi et al. 2005). Furthermore, piezophilic bacteria have been isolated from *H. gigas* previously (Yayanos et al. 1981), so microbes may be responsible for the breakdown of these hard-to-process recalcitrant materials in amphipods.

Autotrophy and lithotrophy. Because of the assumed reliance of deep-ocean communities on POC, estimates of POC flux should theoretically be balanced with community metabolism. However, the metabolic activity of deep-sea microbial communities can be up to 2 orders of magnitude higher than that which is sustainable through measured estimates of sinking POC (Reinthaler et al. 2006; Baltar et al. 2009; Giering et al. 2014). This mismatch highlights potential problems estimating microbial activity or POC flux or could reflect the presence of as yet unidentified sources of organic carbon, such as neutrally buoyant macroscopic particles (Bochdansky et al. 2010) or virus decomposition (Dell'Anno et al. 2015).

One alternative source of organic carbon could be the fixation of dissolved inorganic carbon in the dark ocean. The abundance of genes involved in autotrophy were comparable at 4000 m to those at the surface at Station ALOHA (Konstantinidis et al. 2009) and genes for autotrophy in the 6000 m Puerto Rico Trench metagenome showed comparable abundances to those at the surface, although some key enzymes were missing (Eloe et al. 2011a). These findings are consistent with activity measurements that have shown that carbon fixation in the deep sea is an important source of organic carbon, with estimates of dark primary production being similar to rates of heterotrophic production and equal to 15–53% of the carbon that is exported from the surface (Reinthaler et al. 2010). It is thought that Thaumarchaea may be predominantly responsible for autotrophy in many deep-sea settings (Herndl et al. 2005; Hallam et al. 2006; Ingalls et al. 2006; Wuchter et al. 2006; Yakimov et al. 2011; Smedile et al. 2013; Swan et al. 2014). However, bacteria may also perform DIC fixation in the dark ocean and in some situations could incorporate DIC at higher rates than archaea (Varela et al. 2011). Screening of 502 bacterial single-amplified genomes from 770 and 800 m showed that 12% were positive for RuBisCO, with 25% of the Gammaproteobacteria and 47% of SAR324 encoding the RuBisCO large subunit (Swan et al. 2014). In the case of the deep-sea clade SAR324, the consistent co-occurrence of RuBisCO and sulfur oxidation genes may mean that dissimilatory sulfur oxidation is used for energetic support of autotrophic carbon fixation, although other studies have concluded that members of SAR324 could also be heterotrophic (Chitsaz et al. 2011; Sheik et al. 2014). Sulfur oxidation and autotrophy have also been identified in the SUP05 clade, a subgroup within the Gammaproteobacteria that predominates at suboxic and anoxic sites (Walsh et al. 2009; Wright et al. 2012; Anantharaman et al. 2013). Interestingly, genomes of viruses associated with SUP05 contained genes for

subunits of reverse dissimilatory sulfite reductase and may represent a genetic reservoir responsible for conferring this ability to SUP05 (Anantharaman et al. 2014; Roux et al. 2014).

In addition to chemoautotrophy it has been noted that heterotrophic microbes could be responsible for some of the measured dark primary productivity by incorporating CO_2 during anaplerotic reactions, which provide no net gain of carbon. Heterotrophs, including *Alteromonas macleodii* AltDE, may actively take up CO_2 in nutrient replete conditions to replace necessary metabolic precursors or intermediates (Alonso-Sáez et al. 2010; Yakimov et al. 2014). Deep-ocean microbial communities are important drivers of carbon remineralization and sequestration and a greater understanding of their function, both in using and fixing organic carbon, will be fundamental to evaluating the role of the deep ocean as a carbon sink.

Electron donors in the deep ocean are scarce (Reinthaler et al. 2010) and some deep-ocean microbes may use unique sources. One such example is the prevalence of genes for aerobic carbon monoxide oxidation in the deep sea. The *coxL*, *coxM*, and *coxS* genes, which encode for carbon monoxide dehydrogenase, have been identified in many deep-ocean metagenomes (Martin-Cuadrado et al. 2007, 2009; Eloe et al. 2011b; Quaiser et al. 2011; Smedile et al. 2013). Carbon monoxide oxidation may provide an alternative or supplementary energy source (Martin-Cuadrado et al. 2007) and while the possible role of CO in the deep ocean has been questioned because the source is unclear (Quaiser et al. 2011), it is possible that it is produced during organic matter degradation (Eloe et al. 2011b). Most Thaumarchaea in the deep ocean are thought to be capable of oxidizing ammonia based on abundances of *amoA* genes (Konstantinidis et al. 2009; Yakimov et al. 2011) and their recovery from the majority of mesopelagic SAGs from the South Atlantic (60%) and North Pacific (81%) (Swan et al. 2014). However, low ammonia levels in the deep ocean may result in archaeal nitrification that is too low to support the measured rates of carbon fixation (Herndl et al. 2005; Reinthaler et al. 2010). The identification of urease genes within MG1 SAGs suggests they may also oxidize urea, providing an alternative energy production pathway (Alonso-Sáez et al. 2012; Swan et al. 2014).

2.7 Conclusions and Future Directions

Deep-ocean communities contain genotypic and phenotypic properties that confer adaptation to the deep ocean. Many attributes associated with deep-ocean bathytypes, such as genes for particle-association, heavy-metal resistance, and increased numbers of mobile elements are also found in metagenomes, suggesting they are relatively conserved across major groups of deep-sea taxa. Most of the bathytypes discussed here show growth at atmospheric as well as high pressure and more extreme comparisons with obligate piezophiles may allow for a better understanding of the adaptations to the deep ocean. One of the major challenges moving

forward will be to determine the taxonomic and functional traits that distinguish not just "deep" versus "shallow" pelagic prokaryotes, but "ultra-deep" or hadal prokaryotes from everything else, and to uncover the environmental conditions that lead to these differences. To accomplish these tasks it will be important to apply genomics technologies to a far larger number of piezophiles and hadal locations than the handful examined thus far, along with the collection of robust suites of associated biological, chemical, and physical data. Large sampling expeditions comparable to the Global Ocean Sampling Expedition or the TARA Expedition (Rusch et al. 2007; Karsenti et al. 2011) should be considered for global analyses of microbial communities and their genomic adaptations within hadal environments.

Another major emphasis must be to distinguish autochthonous from allochthonous microorganisms present at great depths. Because pressure is clearly a major driver of the vertical distribution of marine life, one way to address this question will be to determine the impact of retaining or changing the in situ pressure on the identities and activities of the microbes present. This objective becomes progressively more complicated, technically challenging and expensive with increasing depth and pressure. Another approach is to consider ways in which the microbiology to be performed can be accomplished in the deep sea prior to sample retrieval. This could involve the fixation of samples at depth for later processing topside or through analyses done remotely like that conducted with the Environmental Sample Processor (Scholin et al. 2009).

Another major research direction must include more mechanistic studies of the basis of life at great depth. In most cases this will involve pure cultures, but innovative interrogations of microbial communities present in microcosms might also yield new insights. These efforts would benefit from the collection of a much more diverse and representative collection of piezophiles than now exist. Enrichments with more complex organic sources over longer time scales may provide new species of microbes for study, and transitioning from batch to recirculating culturing approaches to maintain carbon sources, electron donors, and electron acceptors (notably dissolved oxygen) at constant concentrations under high-hydrostatic pressure should be pursued (Parkes et al. 2009; Deusner et al. 2010; Zhang et al. 2011; Sauer et al. 2012; Ohtomo et al. 2013; Foustoukos and Perez-Rodriquez 2015).

Important information already exists on the cellular changes needed for life at elevated pressure, most clearly in the case of the requirement for elevated unsaturated fatty acids in the membranes of psychrophilic or psychrotolerant piezophiles. However, despite the close phylogenetic relationship that exists between piezophilic and nonpiezophilic microbes, fundamental gaps remain in the understanding of the basis of high-pressure life. Thus, high-pressure growth appears to arise from a modest number of subtle changes. The application of systems biology approaches (transcriptomics, proteomics, lipidomics, metabolomics) in concert with metabolic flux analyses, osmolyte measurements, directed evolution, genetics, biochemistry, and modeling will all be needed for further progress. Genetic studies are currently possible for only a small number of piezophiles (Chen et al. 2011; El-Hajj et al. 2010; Thiel et al. 2014; Li et al. 2015).

There are many reasons to study deep-sea life in general and ultra-deep microbes in particular. Deep-sea microbes are considered important targets for the search for bioactive secondary metabolites and drugs (Hopwood 2007) or as sources of enzymes and metabolic pathways for the breakdown of complex hydrocarbons, plastics or other organics (Sekiguchi et al. 2011). The study of deep-sea microbes also makes it possible to examine life under different boundaries of pressure and temperature than is the case for virtually all other habitats, excluding the deep subsurface. In this regard they are useful as proxies for possible life forms existing in extraterrestrial habitats (Schrenk and Brazelton 2013). But, they should primarily be appreciated as the members of a major portion of the biosphere, responsible for much of the carbon mineralization and sequestration that takes place on Earth.

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Chapter 3
Adaptations of Cold- and Pressure-Loving Bacteria to the Deep-Sea Environment: Cell Envelope and Flagella

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Abstract Compared to terrestrial environments our knowledge of microorganisms inhabiting oceans, the largest ecosystem on Earth, is limited. Deep oceans contain bacteria that thrive at high pressure and low temperature. For them, as for all bacteria, the outer structures of the cell are the first point of contact with the environment, both sensing and being modified in response to it. The vast majority of studied cold- and pressure-loving bacteria are Gram-negative and so in this chapter, the adaptations of their cell envelope and flagella are presented. In deep-sea bacteria, the structure of phospholipids and lipopolysaccharides is modified in order

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to maintain membrane fluidity and enable membrane-localised proteins to perform their functions. Many of the membrane proteins involved in nutrient acquisition, transport, respiration, sensing and signalling are also specifically adapted to function at high pressure and low temperature. The ability to move towards nutrients or away from hostile environment is extremely important for bacterial survival and yet very vulnerable to increased pressure. Deep-sea bacteria are capable of swimming even at 150 MPa, which suggests their motility systems are specifically adapted to high pressure. Moreover, some bacteria have been shown to produce a second type of flagella (lateral flagella) in response to high pressure or low temperature. The findings presented in this chapter are a result of many techniques and analyses applied to whole microbial communities, single species as well as particular genes and proteins. Investigation of the adaptations to high pressure and low temperature not only expands basic knowledge but also identifies targets that could have biotechnological and industrial application. Deep-sea bacteria could be used for production of biofuels, secondary metabolites of value for drug development, and various pressure and temperature adapted enzymes.

3.1 Introduction

Oceans constitute the largest habitat on Earth, comprising over 90% of the biosphere by volume and yet, when compared to terrestrial ecosystems, the marine environment is understudied. Our knowledge of marine microorganisms is limited. Over the years, little effort has gone into the exploration of marine biodiversity (Duarte 2006). In fact, for a long time all bacteria collected from ocean water samples were regarded as terrestrial contamination (Williams 2009). Furthermore, in comparison to the number of colonies arising from studied samples with that obtained by microscopic observation it is evident that only 1% of bacteria can be cultivated (Stach and Bull 2005). Therefore, unless molecular techniques are used, a huge fraction of bacterial species present in marine samples remains undetected. Discoveries are also hindered by the fact that few countries have the equipment needed to explore ocean depths below 200 m (Duarte 2006).

The marine environment is exceptionally diverse and cannot be easily classified. The average pressure in oceans equals 38 MPa (which is 380 times more than the atmospheric pressure) and the average deep-sea temperature is 1–3 °C, but every local environment has its own characteristics (Abe and Horikoshi 2001). Hydrostatic pressure varies from 0.1 MPa on the surface to 110 MPa at the bottom of the Challenger Deep within the Mariana Trench, the deepest place in the ocean. This variety of habitats results in a diversity of bacterial adaptation to pressure. In the surface waters, pressure-sensitive (piezosensitive) microorganisms can be found, whose growth is inhibited by high pressure. Deeper waters abound in piezophiles, pressure-loving organisms that grow optimally at elevated pressures above atmospheric 0.1 MPa, and piezotolerant strains, able to grow both at atmospheric and high pressure (Abe and Horikoshi 2001). Deep-sea temperatures

can range from −2 °C in polar oceans and ~4 °C at temperate and tropical latitudes to over 300 °C near hydrothermal vents (Deming 1998). Organisms growing optimally at low temperatures are categorised as psychrophiles (cold-loving). However, the classical definition of a psychrophilic microorganism uses arbitrary limits to define its optimal growth temperatures as being under 15 °C with ~20 °C being the maximum temperature still allowing for growth. It has been postulated that a more relevant definition is necessary (Feller and Gerday 2003).

High hydrostatic pressure and low temperature often exert similar effect on bacterial cells. In fact a 16S rRNA-based phylogeny analysis revealed that cold-loving piezophiles appear to be descendants of polar psychrophiles (Lauro et al. 2007). However, when reactions are concerned, an increase in temperature simply accelerates the reaction, whereas pressure can accelerate, inhibit or exert no effect at all, depending on the change in reaction volume (Abe and Horikoshi 2001). A process is enhanced by elevated pressure if the system volume of its reaction decreases, and is inhibited if the associated system volume increases. The effect of pressure is especially complex as it also depends on other physicochemical factors such as temperature, pH and nutrient composition (Bartlett 2002). Moreover, when the marine environment is considered, it is noteworthy that the pressure range in the oceans varies 1000-fold, whereas temperature (excluding hydrothermal vents and cold seeps) does not change by more than 6 °C in the deep sea (Bartlett 1991). The complexity of the high pressure effect on bacterial cells has been shown in *Escherichia coli*, which increases rates of synthesis both of heat shock proteins and cold shock proteins in response to pressure (Welch et al. 1993). This unusual paradoxical response can be explained by the fact that some of the effects of elevated pressure resemble those of high temperature (e.g. destabilisation of protein quaternary structure) whilst others resemble those of cold temperature (decreased protein synthesis and membrane fluidity) (Bartlett 2002).

Most of the isolated piezophiles are psychrophilic Gram-negative bacteria that belong to the *Gammaproteobacteria* class and include species from the genera *Shewanella*, *Psychromonas*, *Photobacterium*, *Colwellia*, *Thioprofundum* and *Moritella* (Zhang et al. 2015). A few piezophilic bacteria have been obtained which belong to the *Alphaproteobacteria* and *Deltaproteobacteria* classes. Gram-positive psychropiezophiles are rare and the two isolated species belong to family *Carnobacteriaceae* (Lauro et al. 2007). Adaptations to life in the ocean depths, namely to the high pressure and low temperature, have been mostly studied in *Photobacterium* and *Shewanella* species. Investigating the adaptations of deep-sea microorganisms to their environment broadens our understanding of the processes occurring in nature, but can also have potential biotechnological and industrial applications such as production of new metabolites or low temperature- and high pressure-adapted enzymes for bioreactors and deep-sea waste disposal (Kato and Bartlett 1997). It has also been suggested that in the future obligate piezophiles could be used as a containment system for production of toxins, virulence factors and virus particles without posing a threat to the surrounding environment at atmospheric pressure (Zhang et al. 2015).

The cell envelope is the first point of contact of bacteria with their environment (Silhavy et al. 2010). This sophisticated and complex structure protects cells, but also allows selective passage of nutrients from the outside and waste products from the inside. Spanning all the layers of cell envelope and extending into the extracellular space is the flagellum, responsible for bacterial motility (Erhardt et al. 2010). Both the cell envelope and the flagella enable the deep-sea bacteria to adapt to their environment and are crucial for the survival at high pressure and low temperature. Deep-sea microorganisms and their adaptations to environmental conditions found in oceans are relatively recent research subjects. In contrast, the effect of high pressure and cold temperature on mesophilic bacteria with midrange temperature optima, such as *E. coli*, has been well documented over the years although not always understood.

Growth of mesophilic microorganisms is impeded within the range of several dozen MPa, is completely inhibited at approximately 50 MPa and pressures greater than 200 MPa kill most microorganisms (Abe 2007; Bartlett 2002). Motility is affected at 10 MPa, nutrient uptake at 15–20 MPa, membrane protein function at 25–50 MPa and protein oligomerization at 50–100 MPa. Pressure affects both lipids and proteins present in the membrane. The compression of phospholipid acyl chains causes a decrease in membrane fluidity and permeability (Abe 2007). Additionally high pressure can affect the functioning of membrane-bound enzymes. Pressure changes the conformation and activity of proteins, especially multimers, since these are stabilised by weak chemical bonds (Aertsen et al. 2009). Bacteria also become less motile, possibly due to perturbations of the cell membrane and flagellum apparatus (Meganathan and Marquis 1973). Low temperatures influence solute diffusion rates, enzyme kinetics, membrane fluidity and conformation as well as topology and interactions of proteins (Rodrigues and Tiedje 2008). The loss of membrane fluidity is the primary signal perceived by bacteria when exposed to low temperature. Although studying the response of mesophilic bacteria to high pressure and low temperature provides substantial knowledge, it is not sufficient to understand how psychropiezophiles adapt to the extreme environment.

3.2 The Effect of High Pressure and Low Temperature on the Membranes of Psychropiezophiles

The cell envelope of Gram-negative bacteria contains two membranes (Silhavy et al. 2010). The inner membrane is a phospholipid bilayer, while in the outer membrane phospholipids are confined to the inner leaflet. The outer layer of the outer membrane consists of lipopolysaccharide molecules. In addition to lipids, biological membranes contain also various proteins. In this section, the adaptation of membrane components to high pressure and low temperature in deep-sea bacteria will be discussed.

3.2.1 Phospholipids

Microorganisms need to maintain the structural and dynamic properties of their cell membranes at all times. Both high pressure and low temperature exert similar effects, leading to tighter packing and restricted motion of acyl chains (Winter and Dzwolak 2005). To counteract these changes and restore the membrane fluidity, many organisms adapt their membrane phospholipid unsaturated fatty acid content in a process known as homeoviscous adaptation (Sinensky 1974). It has been suggested that homeophasic adaptation is more physiologically relevant than the homeoviscous adaptation (McElhaney 1982). Homeophasic adaptation requires a certain percentage of lipid membrane to remain in the liquid crystalline phase rather than gel phase (a solid state). In response to high pressure and low temperature bacteria increase the ratio of unsaturated to saturated, *cis* to *trans* and short to long fatty acids and alter the size and charge of the polar groups (Shivaji and Prakash 2010). Hence, monounsaturated fatty acids (MUFAs), branched chain fatty acids (BCFAs) and polyunsaturated fatty acids (PUFAs) all play a role in bacterial adaptation to the deep sea environment. Unsaturated fatty acids pack less compactly and adopt a more expanded conformation due to the 30° bend introduced by the double bond (Hazel and Williams 1990). They also possess lower melting temperatures than their saturated equivalents, allowing for their less orderly alignment within membrane phospholipids when the temperature decreases.

Marine organisms are the major producers of omega-3 PUFAs with 20:5 (all-*cis*-5,8,11,14,17-eicosapentaenoic acid, EPA) and 22:6 (all-*cis*-4,7,10,13,16, 19-docosahexaenoic acid, DHA) acids being the most common. It has been shown that genes required for the synthesis of long chain PUFAs are often present in the genome even if bacteria do not produce PUFAs (Wang et al. 2009). Production of DHA is a characteristic trait of genus *Moritella* and *Colwellia* (Bowman et al. 1998; DeLong et al. 1997; Kato et al. 1998; Nogi et al. 1998b). Approximately 70% of *Moritella yayanosi* membrane lipids are unsaturated fatty acids, which is consistent with its adaptation to grow at high pressure (optimal 80 MPa) (Kato et al. 1998). The importance of PUFAs for low temperature- and high pressure-adapted growth is different among bacterial species. EPA-deficient strains have been analysed more thoroughly and it has been found that depending on the strain, the growth of the EPA-deficient mutant can be affected by low temperature or high pressure, by both or not affected at all (Allen et al. 1999; Kawamoto et al. 2009, 2011; Wang et al. 2009).

When *Photobacterium profundum* SS9 was grown at high pressure (28–50 MPa, 15 °C) or at low temperature (4 °C, 0.1 MPa) the amount of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid EPA 20:5, was increased compared to growth at atmospheric pressure and 15 °C (Allen et al. 1999). However, elevated pressure and low temperature growth conditions resulted in the greatest increase in levels of different MUFAs, 18:1 (*cis*-11-octadecanoic acid; *cis*-vaccenic acid) and 16:1 (*cis*-9-hexadecanoic acid; palmitoleic acid) respectively. Additionally, MUFAs seem to be more effective in maintaining optimum membrane fluidity, as the EPA-deficient *P. profundum* SS9

mutant was not affected by high pressure or low temperature (Allen et al. 1999). Despite the unperturbed growth of *P. profundum* SS9 in its absence, EPA constitutes a significant proportion of fatty acids present in piezophilic *P. profundum* strains (9% in SS9 and 13% in DSJ4) while it is absent in the piezosensitive *Photobacterium* species (Nogi et al. 1998a). Interestingly, the EPA biosynthesis operon is also present in the piezosensitive *P. profundum* 3TCK (Campanaro et al. 2005), but the fatty acid content of this strain has not been analysed. The *pfa* operon responsible for EPA biosynthesis appears as alien DNA present in all three isolates of *P. profundum* (SS9, DSJ4, 3TCK) (Campanaro et al. 2005). Although high pressure and low temperature exert similar effects on bacterial membranes, in *P. profundum* SS9 they seem to be perceived and responded to in a different manner. FabF, a β-ketoacyl-acyl carrier protein synthase II, was found important for the increase in 18:1 levels at both high pressure and low temperature but only essential for the piezophilic growth of *P. profundum* SS9 (Allen and Bartlett 2000). Based on the comparison of *P. profundum* SS9 gene expression at 0.1, 28 and 45 MPa, a previously unrecognised gene, encoding a putative delta-9 fatty acid desaturase (PBPRB0742), was found up-regulated with increasing pressure (Campanaro et al. 2005).

Shewanella violacea DSS12 phospholipid fatty acid content also changes with the increasing pressure. The shift from atmospheric pressure to 30 MPa affects the two major fatty acids present in the outer membrane of *S. violacea* differently, with 16:1 increasing and 20:5 decreasing in content (Kawamoto et al. 2011). Despite its decrease at high pressure, eicosapentaenoic acid is important for growth at 30 MPa and cannot be compensated for by the increased levels of 16:1 MUFA or iso-15:0 BCFA (iso-pentadecanoic acid). The EPA-deficient strain exhibits defects in the late division steps at 30 MPa (Kawamoto et al. 2011). Similarly to *P. profundum* SS9, the growth of EPA-deficient mutant of *S. violacea* DSS12 was not affected by low temperature. This is not always the case as in the psychrophilic *Shewanella livingstonensis* Ac10 EPA is required for growth at low temperatures (Kawamoto et al. 2009). However, it has been suggested that in this bacterium EPA plays a role in membrane organization and cell division, not maintenance of membrane fluidity.

In *Shewanella piezotolerans* WP3, EPA is necessary for growth at both, high pressure (20 °C, 20 MPa) and low temperature (4 °C, 0.1 MPa) (Wang et al. 2009). Contrary to *P. profundum* SS9, in *S. piezotolerans* WP3 MUFAs cannot complement for the absence of EPA. The dissimilarity between *P. profundum* SS9 and *S. piezotolerans* WP3 possibly stems from the different content of MUFAs in the cells. In the *P. profundum* SS9 EPA-deficient strain almost 70% of fatty acids are MUFAs, whereas in *S. piezotolerans* WP3, the MUFAs levels reach only 30%, which might not be sufficient to compensate for the absence of EPA. The differences between these genera could also reflect differences in physiology, i.e., fermentative versus respiratory growth.

Along with the unsaturated fatty acids, branched chain fatty acids also play a role in adaptation to low temperatures. *S. piezotolerans* WP3 was found to increase the BCFA content at low temperature mainly by importing precursors of BCFAs from the environment using the branched-chain amino acid ABC transporter LIV-I (Wang et al. 2009). The LIV-I transporter was identified only in the cold-adapted

Shewanella species and was assumed to supply an important strategy for the cold- but not pressure-adaptation.

Generally it is accepted that organisms inhabiting cold and high-pressure environments need to maintain greater membrane fluidity. However, a recent study suggests that retaining a certain level of membrane stability under a wide range of environmental conditions is more important. *S. violacea* DSS12 attains the required membrane rigidity at its optimal growth temperature of 10 °C due to the production of substantial levels of EPA that prevents the membrane from becoming hyperfluid (Usui et al. 2012). As a result, the membrane properties do not change drastically over a wide range of hydrostatic pressures (Abe 2013; Usui et al. 2012). Moreover, EPA and DHA have been found to possess an antioxidative function, possibly shielding cells against reactive oxygen species in vivo (Okuyama et al. 2008).

Research into the fatty acids produced in marine bacteria is not limited to understanding their role in adaptation to the deep-sea environment. Fatty acids produced by bacteria could be used as a renewable source for the production of commercially available biofuels. Currently, one of the main steps towards this goal is the increase in the fatty acid production by microbes. An active dehydratase tetradomain protein fragment from the PUFA synthase enzyme complex from *P. profundum* SS9 was shown to increase the production of fatty acids in *E. coli* as much as 5-fold (Oyola-Robles et al. 2014). This enhancement in fatty acid production was more pronounced at lower temperatures. Several other enzymes encoded in the *pfa* operon of *P. profundum* SS9 have also been studied. The solved structure of tandem acyl carrier protein (ACP) domains suggests that artificial linking of multiple ACP domains could increase the yield of fatty acids in bacterial cultures (Trujillo et al. 2013). Omega-3 fatty acids have beneficial effects on human health and in the future could be produced by microbes, since currently the fish-derived PUFAs cannot sustainably meet the human consumption demands. Cloning of the DHA synthesis operon into *E. coli* and optimisation of DHA production in *Moritella marina* MP-1 have already been carried out (Amiri-Jami and Griffiths 2010; Kautharapu et al. 2013; Orikasa et al. 2006).

3.2.2 Lipopolysaccharide

In Gram-negative bacteria, lipopolysaccharide (LPS) forms the outermost layer of the outer membrane with the hydrophobic lipid A situated in the interior of the outer membrane and hydrophilic O-antigen facing the outside of the cell. LPS is a fundamental component of the outer membrane and as such plays an important structural role. The phosphate groups in lipid A and the core region bind divalent cations and stabilise the outer layer of the outer membrane (Kumar et al. 2002). In marine bacteria, the O-antigen is very often anionic and also binds cations, which provides further stability towards external stressors such as high pressure (Nazarenko et al. 2011). Bonds forming between O-antigen moieties and cations stabilise the membrane but also play a role in interactions with other bacterial cells,

like in *V. cholerae* O139, where the O-antigen is required for the formation of Ca^{2+}-dependent biofilms (Kierek and Watnick 2003). O-antigen moieties help both with the aggregation of cells and with their attachment to surfaces. Atomic force microscopic analysis showed that LPS facilitates adhesion to negatively charged surfaces and longer O-antigen results in a higher force of adhesion, most likely due to formation of increased numbers of hydrogen bonds (Abu-Lail and Camesano 2003; Strauss et al. 2009). Additionally, the O-antigen acts as a surfactant and increases wettability of surfaces, promoting bacterial swarming (Toguchi et al. 2000). It is also required for successful colonisation of the host by symbiotic and pathogenic bacteria. The O-antigen ligase mutant of *Vibrio fischeri* has a motility defect and is delayed in colonisation of the light organ of the Hawaiian bobtail squid (Post et al. 2012). The O-antigen mutant of the pathogenic *E. coli* O157:H7 is cleared faster from the murine intestine and does not establish an as effective bovine intestine colonisation as the parent strain (Sheng et al. 2008). Additionally, O-antigen molecules also protect bacteria from recognition by the host's immune system and bacteriophages, since they can mask the bacteria's conserved epitopes and receptors (Whitfield et al. 1997).

When the temperature decreases or pressure increases, bacteria adapt their membranes to counteract the rigidification (Bartlett 1999). This is usually achieved by modifying the phospholipids, as discussed in the previous section. However, it has also been found that fluidity of the outer membrane increases when the concentration of LPS or the length of the polysaccharide side chain is reduced (Rottem and Leive 1977). Bacteria grown at low temperatures change the fatty acids present in lipid A or even produce new kinds of LPS. For example *Salmonella* species incorporate an unsaturated fatty acid into their lipid A when grown at low temperatures (Wollenweber et al. 1983). *Yersinia pestis* cells produce a new type of LPS at 6 °C with a number of significant structural modifications to the core and lipid A (Knirel et al. 2005). The surface polysaccharides of the Antarctic psychrophilic bacteria *Pseudoalteromonas haloplanktis* TAC 125 and *Pseudomonas syringae* Lz4W are less phosphorylated at low temperatures (Corsaro et al. 2004; Ray et al. 1994). Moreover, the hydroxylated fatty acid content of the *P. syringae* LPS increases at low temperatures (Kumar et al. 2002), but a role for these LPS changes in the cold-adapted growth of the bacterium has not been studied.

The LPS has not been studied extensively in psychropiezophiles. It is known that *P. profundum* SS9 contains both types of LPS, rough (R-LPS, consisting of lipid A and the core) and smooth (S-LPS, consisting of rough LPS and the O-antigen) (El-Hajj et al. 2009). Interestingly, the LPS of three *P. profundum* strains, two piezophilic (SS9 and DSJ4) and one piezosensitive (3TCK) differ significantly (Fig. 3.1a) (Myka 2013). All strains were grown at 15 °C, atmospheric pressure, because it has been shown before that *P. profundum* SS9 LPS PAGE profile does not change with temperature (Allcock 2009). The high molecular weight S-LPS of SS9 and 3TCK was primarily hydrophilic as it extracted into the aqueous phase, while the DSJ4 S-LPS partitioned into the phenol phase, which indicates its hydrophobicity. Moreover, the LPS profile of the piezosensitive 3TCK resembled the "ladder-like" LPS found in *Salmonella* species. The "ladder" LPS profile results

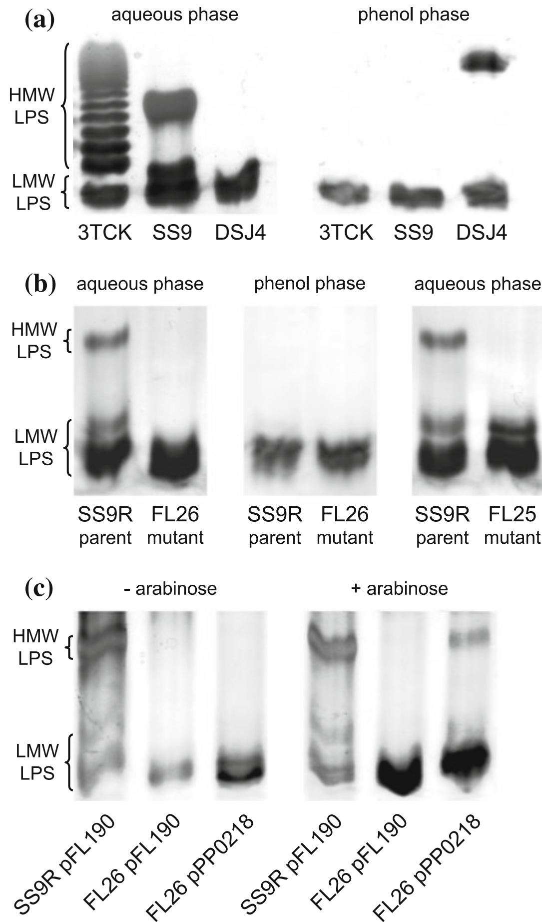

Fig. 3.1 The LPS of *Photobacterium profundum*. **a** LPS species from three *P. profundum* strains with different pressure and temperature growth optima were purified using the hot phenol-water extraction method (Carlson et al. 1978). Both aqueous and phenol phases were analysed by DOC-PAGE followed by silver staining (Tsai and Frasch 1982). SS9 grows optimally at 28 MPa, 15 °C; DSJ4 at 10 MPa with very little change up to 50 MPa, 10 °C; 3TCK is piezosensitive and grows optimally at atmospheric pressure, 20 °C (Campanaro et al. 2005; DeLong 1986; Nogi et al. 1998a). **b** The FL26 and FL25 mutants lack high molecular weight smooth LPS (HMW S-LPS) and produce only low molecular weight rough LPS (LMW R-LPS). FL26 and FL25 LPS species were purified as in (a) and analysed by DOC-PAGE followed by alcian blue-silver staining. (c) Plasmid encoded putative O-antigen ligase restores smooth LPS to the FL26 mutant. LPS species were analysed as in (b). Since S-LPS does not extract into the phenol phase, the phenol phases R-LPS species are not shown. The *P. profundum SS9 pbpra0218* gene was amplified using primers 5-ATGAATTCAGAGAATATTTTTAAACTACTGATGT-3 and 5-ATTCTAGATTA GCAATTGTTCTTCTTTGAAGTT-3 and cloned into pFL190 (Lauro et al. 2005) using EcoRI and XbaI to produce pPP0218

from the heterogeneity of LPS molecules containing O-antigens of a different chain length (Raetz and Whitfield 2002). Each band “up the ladder” in the PAGE LPS profile corresponds to the lipid A-core with an O-antigen with an additional O-unit. It is not known why only the 3TCK would have such variation in O-antigen polysaccharide length and more experiments would be necessary to test if this phenotype has a connection to its piezosensitivity and/or relatively high optimal temperature of growth.

A study of 16 *Shewanella* strains including at least 8 different species found that half possessed only R-LPS while the other half contained also S-LPS (Korenevsky et al. 2002). Most *Shewanella* species tested did not alter their LPS in response to temperature (Korenevsky et al. 2002). However, the LPS of *Shewanella frigidimarina* displayed a ladder-like pattern when visualised using SDS-PAGE only when it was extracted from a strain grown at 15 °C, which was not observed at higher or lower temperatures (Korenevsky et al. 2002). The change in acyl moieties of lipid A with temperature was tested in an obligatory psychrophile *Colwellia hornerae* and psychropiezophile *Colwellia piezophila* and compared with a facultative psychrophile, *Psychrobacter cryohalolentis* (Sweet et al. 2015). Both *Colwellia* strains did not display alterations in lipid A based on growth temperature, while *P. cryohalolentis* did. Lipid A of *Colwellia* species contains shorter acyl units compared to *E. coli* and *Vibrio*. It seems that in these obligatory psychrophiles the constitutive change of lipid A is the evolutionary adaptation to cold environments. On the other hand, the facultative psychrophile (psychrotolerant) *P. cryohalolentis* relies on metabolic responses to tune the fluidity of the outer membrane to a wider range of temperatures.

Bacterial survival in the deep sea very often relies on the attachment to surfaces, either mineral or of other organisms. The outer layer of the outer membrane has the most influence on the bacterium-surface interaction. The adhesion of *Shewanella* species is mostly governed by electrostatic interactions and depends primarily on the LPS, with membrane proteins and capsular polysaccharides playing minor role (Korenevsky and Beveridge 2007). LPS is the most abundant molecule present on the Gram-negative surface (Beveridge 1999) and its O-antigen not only extends considerable distances from the cell surface but can also possess more exposed electrostatic sites than proteins (Korenevsky and Beveridge 2007). On the other hand, it was suggested that the short LPS and high potential charge of the piezosensitive *Shewanella putrefaciens* CN32 cell surface allow for a good contact of the putative outer membrane iron reductase with the iron oxide present in the environment (Korenevsky et al. 2002).

The importance of LPS in the attachment to surfaces of *P. profundum* SS9 could also be inferred from the study performed by Lauro et al. (2008). Eight out of thirty-one transposon mutants of *P. profundum* SS9 were found to possess insertions in genes predicted to encode proteins involved in the cell envelope biogenesis (Lauro et al. 2008). All of these mutants were cold-sensitive, confirming the requirement for a correctly assembled cell envelope for the survival at low temperature. Three of the genes inactivated in cold-sensitive transposon mutants are located in a cluster involved in the cell envelope biogenesis that also contains genes

regulated by temperature or pressure at the transcription level (Campanaro et al. 2005, 2012) and at the level of translation (Le Bihan et al. 2013).

Interestingly, two of the cell envelope biogenesis mutants identified by Lauro and colleagues displayed the cold-sensitive phenotype only when grown on agar (Lauro et al. 2008). The *P. profundum* SS9 mutant, FL26, has a transposon insertion in the gene encoding a putative O-antigen ligase, *pbpra0218* (Lauro et al. 2008). O-antigen ligases are responsible for attaching the O-antigen sugar polymer to the rough lipopolysaccharide (R-LPS) consisting of lipid A and the sugar core, thereby creating a complete LPS molecule, called smooth LPS (S-LPS) (Raetz and Whitfield 2002). The second cold-sensitive mutant, FL25, has a disruption in a putative glycosyltransferase, *pbpra2700* (Lauro et al. 2008). Glycosyltransferases are essential for the biosynthesis of the LPS core oligosaccharide and the O-antigen (Raetz and Whitfield 2002). It is worth noting that the expression of the *pbpra0218* gene was shown to be temperature dependent and down-regulated at 4 °C compared to 16 °C in liquid cultures (Lauro et al. 2008). Dr. Gail P. Ferguson's group analysed the LPS present in the FL26 and FL25 mutants and found that both mutants lack the high molecular weight species corresponding to the S-LPS (Fig. 3.1b) (Myka 2013). The S-LPS of the FL26 mutant could be restored by a plasmid encoded putative O-antigen ligase (PBPRA0218) (Fig. 3.1c). Sequence analysis revealed that PBPRA0218 is 48% similar (30% identical) to an O-antigen ligase from *V. cholerae* O1 (GenBank accession no. AAL76923.1) and contains characteristic for the O-antigen ligase pfam04932 domain and two conserved motifs RX_3L and $HX_{10}G$ (Myka 2013; Schild et al. 2005). However, the complementation of the cold-sensitive phenotype of FL26 could not be achieved by the plasmid-encoded *pbpra0218*, as the presence of a plasmid conferring resistance to streptomycin masked the cold-sensitive phenotype of FL26 (Myka 2013). Chi and Bartlett observed that streptomycin exerted an inhibitory effect on the growth of *P. profundum* SS9, even in the presence of a plasmid encoding streptomycin resistance (Chi and Bartlett 1995). This inhibition was enhanced by elevated pressure and low temperature. Due to these technical difficulties, it cannot be definitely stated that the lack of S-LPS is directly responsible for the cold-sensitive phenotype of the *P. profundum* SS9 mutant. Nevertheless, the discovery of mutants that are cold-sensitive only on agar calls for research that would investigate adaptations of the deep-sea bacteria to both the temperature and the mode of growth (single cell/colony/biofilm). S-LPS might protect *P. profundum* SS9 against the effects of the low temperature during colony growth by promoting the attachment of cells to the marine agar. It has been shown that surface association is important for Arctic microbes activity at sub-zero temperatures (Junge et al. 2004). The putative selective advantage of surface association that we observe for *P. profundum* SS9 in the laboratory could also extend to the environment. *P. profundum* SS9 was isolated from an amphipod homogenate (DeLong 1986) and in the cold deep sea the attachment to surfaces of marine organisms could increase its environmental fitness and enhance its survival.

Studies of the LPS in marine bacteria not only provide information about the strategies that bacteria adopt to survive in the deep sea, but could also have

biotechnological applications. The LPS of marine bacteria often differs from the well-studied mesophilic organisms. Marine bacteria were found to possess a substantial heterogeneity in their lipopolysaccharides, which contain uncommon monosaccharides, higher sugars and derivatives with non-sugar substituents (Nazarenko et al. 2003). *Shewanella oneidensis* (a piezosensitive strain) contains an unusual modification to its LPS core sugar, Kdo, which is phosphorylated and with one hydroxyl group converted to a primary amine (Kdo8N) (Gattis et al. 2013). Deletions of the biosynthetic genes led to the sensitivity or *S. oneidensis* to compounds perturbing the outer membrane, suggesting that Kdo8N increases membrane integrity. It is hypothesised that marine bacteria from high pressure and low temperature environments produce lipid A structures of possible pharmacological interest (Solov'eva et al. 2013). LPS and lipid A obtained from several marine bacteria display low toxicity and exhibit properties of endotoxin antagonists, suggesting they could be promising candidates for treating Gram-negative sepsis.

3.2.3 Membrane Proteins

High pressure and low temperature affect membrane proteins in several ways. Changes in the fluidity and physical state of the membrane lipids can affect the activity of membrane enzymes and transport systems (McElhaney 1982). Pressures up to 100 MPa induce a reversible change in the structure of transmembrane proteins, 100–220 MPa cause dissociation and conformational changes in protein subunits, and pressure higher than 220 MPa causes irreversible protein unfolding and interface separation (Kato et al. 2002). Additionally it has been shown that protein synthesis is inhibited by pressure in the range of 50 MPa (Abe 2007; Bartlett 2002). High pressure affects porins, transport proteins, membrane proteins involved in sensing and signalling and respiratory system and other proteins, as discussed below. Most of the research has been performed in *P. profundum* SS9 and the advance of transcriptomics and proteomics allowed for more insight into the high pressure and low temperature induced changes in gene expression and protein abundance (Campanaro et al. 2005, 2012; Le Bihan et al. 2013). However, future proteomic studies could benefit from a membrane enrichment strategy, as in the available analysis most differentially expressed proteins were found in the cytoplasm with membrane proteins being largely underrepresented (Le Bihan et al. 2013).

3.2.3.1 Outer Membrane Porins

P. profundum SS9 was shown to modulate its outer membrane protein content in response to pressure. OmpH increases its abundance with increasing pressure

(10–100 times) and is maximally expressed at 28 MPa, optimal for SS9 growth (Bartlett et al. 1989; Chi and Bartlett 1993). Conversely, OmpL, encoded by one of the first pressure-regulated genes identified in SS9, is preferentially expressed at 0.1 MPa and decreases with increasing pressure (Le Bihan et al. 2013; Welch and Bartlett 1996, 1998). Both OmpL and OmpH encode outer membrane porins, but it has been suggested that the OmpH provides a larger diffusion channel (Bartlett and Chi 1994). *ompH* and *ompL* are each the fourth most expressed protein-encoding gene at high and atmospheric pressure, respectively (when genes coding for rRNA transcripts are not considered) (Campanaro et al. 2012). Despite the fact that OmpH is one of the most abundant proteins in the outer membrane of *P. profundum* SS9 at elevated pressure, an *ompH* mutant is not impaired in its growth at high pressure (Chi and Bartlett 1993). Moreover, the expression of the *ompH* gene can also be induced under low pressure under the conditions of increasing cell density and energy starvation (Bartlett and Welch 1995; Chi and Bartlett 1993).

The *P. profundum* SS9 genome contains 9 additional porin genes (Campanaro et al. 2012). Other porins influenced by high pressure include OmpC (PBPRB1639) and two hypothetical maltoporins PBPRB2004 and PBPRB0413 (Campanaro et al. 2012). The PBPRB2004 maltoporin transcript was found to be more abundant at 28 MPa, while another gene belonging to a porin superfamily, *pbpra2139*, was expressed more at atmospheric pressure. The various expression levels of genes encoding porins in response to high pressure are a clear example of the complexity of high-pressure adaptation. In a nutrient-scarce environment such as the deep sea, an increase in abundance of porins involved in diffusion of large compounds could enhance piezophile growth and survival.

3.2.3.2 Regulation by ToxR

The *P. profundum* SS9 *ompH* and *ompL* genes are transcriptionally regulated by the inner membrane proteins ToxR and ToxS (Welch and Bartlett 1996, 1998). ToxR has been mostly studied in *V. cholerae* and is an oligomeric transmembrane protein localised in the inner membrane (Dziejman and Mekalanos 1994; Ottemann and Mekalanos 1995). It functions together with ToxS, a membrane bound periplasmic effector protein (DiRita and Mekalanos 1991). ToxR is an environmental sensor that regulates gene expression in response to changes in osmolarity, pH, temperature and levels of certain extracellular amino acids (Gardel and Mekalanos 1994; Miller and Mekalanos 1988). In *V. cholerae* ToxR controls the expression of over 150 genes, including those responsible for virulence (Bina et al. 2003). It binds directly to genes under its control via a cytoplasmic DNA binding domain (Miller and Mekalanos 1988). ToxS modulates ToxR activity and protects ToxR from premature proteolysis at late stationary phase, under starvation conditions and at alkaline pH (Almagro-Moreno et al. 2015a, b; DiRita and Mekalanos 1991). All of these are associated with the entry of *V. cholerae* into a dormant state, which enables its survival in the aquatic environment.

In *P. profundum* SS9, ToxR protein levels and its activity decrease at high pressure (Le Bihan et al. 2013; Welch and Bartlett 1998), while the ToxS level of expression in relation to pressure could not be significantly evaluated (Le Bihan et al. 2013). ToxR is required for *ompL* expression and *ompH* repression, since the *toxR* mutant does not possess OmpL, but OmpH is maintained constitutively at a high level (Le Bihan et al. 2013; Welch and Bartlett 1998). The *toxR* mutant has no growth defects at a high pressure, which suggests that ToxR/S is not required for high pressure adaptation. However, the overexpression of the *toxRS* genes leads to pressure-sensitive growth, either because genes necessary for survival at high pressure are repressed or genes deleterious to high pressure growth are activated (Simonato et al. 2006). ToxR functions only as a pressure sensor. At increased temperatures, the ToxR abundance drops, but its activity increases, which maintains the same level of overall ToxR activity (Bartlett 2002). Moreover, ToxR/S pressure sensing depends on the physical state of the membrane (Bartlett 1999). The increase in *P. profundum* SS9 membrane fluidity with local anaesthetics resulted in a low pressure ToxR/S signalling phenotype (high OmpL, low OmpH abundance) even when the cells were grown at high pressure (Welch and Bartlett 1998).

ToxR controls the expression of outer membrane porins, but also other genes involved in membrane structure and starvation response (Bidle and Bartlett 2001; Campanaro et al. 2012). It is possible that its primary function is to homeostatically control membrane structure and energy flow under diverse environmental conditions as well as to cope with various nutrient stresses (Bartlett et al. 2008). Genes regulated by ToxR are just a fraction of those influenced by high pressure (Campanaro et al. 2012). An independent transcriptional regulator, the OmpR-2 protein, was recently found to be strongly up-regulated at 28 MPa (Campanaro et al. 2012). The comparison of the putative ToxR regulon in *V. cholerae* and *P. profundum* SS9 revealed that only four genes were shared between these two microorganisms, which suggests that there is not much overlap between adaptations to pathogenesis (*V. cholerae*) and deep-sea conditions (*P. profundum* SS9).

3.2.3.3 Membrane Transport

Transport is one of the processes in the bacterial cell most influenced by hydrostatic pressure (Vezzi et al. 2005). Changes to the fluidity of the membrane at high pressure most likely affect the efficiency of transporters embedded in the membrane (Campanaro et al. 2005). High pressure inhibits reactions that are accompanied by an increase in volume and so transport of amino acids such as tryptophan, lysine, histidine and leucine is reduced at high pressure due to the volume change of activation of the transport process (Abe and Horikoshi 2000). In marine bacteria the rates of uptake of many substrates, including glutamate and acetate, were shown to be greater at atmospheric pressure than at increased pressure (Jannasch and Taylor 1984). Somewhat counterintuitively, in *P. profundum* SS9 genes for amino acid and ion transport were up-regulated at 0.1 MPa and not at high pressure (Vezzi et al. 2005). This might reflect the adaptation of *P. profundum* SS9 transporters to high

pressure. A number of iron, phosphate, amino acids and sugar ABC transporters were found to have different isoforms that function at different pressure and temperature conditions, one being up-regulated at 0.1 MPa and the other at 28 MPa (Campanaro et al. 2005; Le Bihan et al. 2013). The differently regulated transporters could be an adaptation of *P. profundum* SS9 to grow over a large range of pressures. The sensing of hydrostatic pressure might allow *P. profundum* SS9 to detect its position (depth) in the ocean and adapt to the particular nutrient limitations. Transport was also identified as the most notable process that underwent positive selection based on amino acid substitution rates calculated between two deep-sea microorganisms, *P. profundum* SS9 and *Shewanella benthica* KT99, and their respective shallow water relatives (Campanaro et al. 2008). Higher frequencies of substitution occurred preferentially in extracellular regions of membrane proteins.

3.2.3.4 Other Membrane Proteins

Insertions in genes localised in the *rpoE* locus of *P. profundum* SS9 also lead to high pressure and cold sensitivity (Chi and Bartlett 1995). *E. coli rpoE* encodes the alternative RNA polymerase sigma factor σ^E that responds to extracytoplasmic stimuli and controls the expression of genes involved in the extracytoplasmic stress response (Missiakas and Raina 1998). It is maintained in its inactive state in the cytoplasmic membrane by the products of adjacent genes, *rseA* and *rseB* (Hayden and Ades 2008). *P. profundum* SS9 *rseB* mutants have been isolated from pressure and cold sensitivity screens (Chi and Bartlett 1995; Lauro et al. 2008). However, complementation experiments demonstrated that their sensitive phenotype occurs due to polar effects on the downstream gene, *rseC* (Chi and Bartlett 1995). *rseB* insertion mutants synthesised very low levels of numerous OMPs, including OmpL, but they were able to induce OmpH (Chi and Bartlett 1995). It seems that *rseB* is necessary for proper regulation of outer membrane proteins while *rseC* is necessary for psychro- and piezoadaptation. RseB in *E. coli* has been suggested to detect mislocalised lipoproteins in the cell envelope and induce the σ^E response (Wollmann and Zeth 2007). Moreover, many genes under the control of σ^E encode proteins involved in the biosynthesis, refolding and degradation of outer membrane proteins. The importance of genes in the *rpoE*-locus for growth at elevated pressure suggests that high pressure might ultimately lead to an increase in the accumulation of misfolded proteins in the periplasm, which is toxic for bacterial growth (El-Hajj et al. 2010). Hence, activation of the *rpoE*-locus might be important for maintaining the cell envelope integrity at high pressure. RseC in *E. coli* is believed to have a negligible effect on RpoE activity but functions as an inner transmembrane protein affecting electron transport (Beck et al. 1997). Perhaps the *P. profundum* SS9 *rseC* mutants are high pressure and cold-sensitive because of the effects of those growth conditions on membrane-based electron transport (Bartlett et al. 2008).

3.2.3.5 Respiratory Chain

Bacterial respiratory chains can be quite diverse and adaptable to environmental conditions. It has been proposed that two types of electron transport systems are present in the inner membrane of the piezophilic deep-sea bacterium *S. benthica* DB172F, depending on the growth pressure (Kato and Qureshi 1999). At atmospheric pressure complex I (NADH-dehydrogenase) oxidizes $NADH_2$ to NAD and two electrons are transferred to quinone Q, which is then reduced to quinol QH_2. Complex III (cytochrome bc_1-complex) then transfers the two electrons from quinol to the membrane-bound cytochrome *c*-551. The electrons are then passed onto the soluble cytochrome *c*-552, which transfers them to complex IV (terminal cytochrome *c* oxidase). The terminal oxidase reduces oxygen to water and pumps protons into the periplasmic space. Protons are also pumped by the bc_1-complex. The proton flow back into the cytoplasm enables the ATP synthase to produce ATP. At 60 MPa, the respiratory chain is more compact and electrons from quinol are passed onto the terminal *ccb*-type quinol oxidase, which reduces the oxygen supplied by the membrane bound cytochrome *c*-551 and pumps protons into the periplasmic space (Kato and Qureshi 1999; Qureshi et al. 1998a). The soluble cytochrome *c*-552 is not produced at high pressure (Qureshi et al. 1998b).

S. violacea DSS12 has also been proposed to utilise different electron transport systems depending on the growth conditions (Chikuma et al. 2007). The respiratory system of *S. violacea* DSS12 is branched, with 60% activity depending on the cytochrome bc_1-complex and 40% being independent, regardless of pressure. There are two types of soluble cytochrome *c* present in *S. violacea* DSS12, taking part in the bc_1-complex dependent electron transfer (Yamada et al. 2000). Cytochrome c_A (belonging to group c_5) is constitutively expressed, regardless of pressure, and c_B (group c_4) is repressed at high pressure. There are three terminal cytochrome *c* oxidases (one of cbb_3-type) and two quinol oxidases, *bo*- and *bd*-type in the *S. violacea* DSS12 genome (Ohke et al. 2013). The expression of *bd*-type quinol oxidase genes was unaffected at low oxygen and high pressure conditions, while the expression of all other four terminal oxidases genes decreased. However, the expression of genes encoding a glutathione transporter required for the assembly of the *bd*-type quinol oxidase, *cydC* and *cydD*, was up-regulated at high pressure (Ohke et al. 2013; Tamegai et al. 2005). It has been suggested that the *bd*-type quinol oxidase might be dominant at high pressure, but the cytochrome *c* oxidase also contributes to the respiration process. The respiratory terminal oxidase activity of the membrane of *S. violacea* DSS12 grown under high pressure showed piezotolerance in comparison with that of cells grown at atmospheric pressure (Ohke et al. 2013). In conclusion, *S. violacea* DSS12 has been shown to adapt its respiration system to environmental conditions.

Genomic analysis revealed that *P. profundum* SS9 encodes three putative gene sets for cytochrome *c* oxidases (including a cbb_3-type), one set for quinol oxidase and one for glutathione transporter, one gene for cytochrome c_5 and one for c_4 (Tamegai et al. 2012). The total amount of cytochromes decreased when

P. profundum SS9 cells were grown microaerobically but was not influenced by increased pressure. The expression of representative genes from each set of cytochromes was analysed by RT-PCR and shown not to be altered by increased pressure. Increased aeration elevated only expression of *pbpra0168*, encoding cytochrome *c* oxidase subunit. RT-PCR showed that the expression of ccb_3-type cytochrome *c* oxidase subunit I was not affected by pressure. However, proteomics analysis identified the putative CcoP subunit of the same oxidase as up-regulated at 28 MPa (Le Bihan et al. 2013). These contradictory results could stem from discrepancies between the mRNA levels and protein abundance. Cytochrome c oxidase ccb_3-type has a reduced proton pumping ability, but higher catalytic activity at low oxygen concentration, which supports an enhanced requirement for this protein in low oxygen environments, such as deep-sea (Buschmann et al. 2010). However, *P. profundum* SS9 can grow by both respiration and fermentation, and most likely uses the latter when oxygen is limited (Nogi et al. 1998a). The piezotolerance of the terminal oxidase activity of the membrane of aerobically-grown *P. profundum* SS9 at optimal temperature was lower than that of *S. violacea* DSS12 and increased when *P. profundum* SS9 was grown at high pressure (Tamegai et al. 2012). It seems that the pressure adaptation of the respiratory system in *P. profundum* SS9 is different than in *S. violacea* DSS12. It has been suggested that the activity of terminal oxidases could be affected by membrane lipid composition or other genes up-regulated at high pressure.

Possibly due to insufficient functioning of the membrane-based cytochrome respiratory system at 28 MPa, both the Stickland reaction (a pathway previously found only in anaerobic bacteria, responsible for amino acid fermentation using an amino acid reductase containing selenocysteine) and the TMAO (trimethylamine-*N*-oxide) reductase respiratory systems were up-regulated at 28 MPa (Le Bihan et al. 2013; Vezzi et al. 2005). Additionally, nitrate reductase and cytochrome c552, involved in the anaerobic respiration pathway were also found to be up-regulated at high pressure in a proteomic study (Le Bihan et al. 2013). In contrast, several proteins involved in the oxidative phosphorylation pathway, typical of aerobic respiration were up-regulated at low pressure. These included NADH dehydrogenase, cytochrome *d* ubiquinol oxidase subunit I and subunits of the F_0F_1 ATP synthase. Combined, these results suggest that pressure may regulate two different modes of respiration in *P. profundum* SS9, with aerobic respiration being up-regulated at atmospheric pressure and anaerobic respiration and fermentation being up-regulated at high pressure.

Bacterial membrane-bound F_0F_1 ATP synthases catalyse ATP synthesis from ADP and inorganic phosphate using the proton motive force or, in a few species, sodium motive force (Deckers-Hebestreit and Altendorf 1996). In anaerobic conditions they function as ATPases, generating a transmembrane ion gradient at the expense of ATP hydrolysis. The expression of genes encoding for F_0F_1 ATP synthase localised on the *P. profundum* SS9 chromosome II was higher at 0.1 MPa than 28 MPa (Campanaro et al. 2012). In contrast, expression of the chromosome I ATP synthase was not influenced by pressure and its absolute expression level was very high with respect to the chromosome II ATP synthase. The F_0F_1 ATP synthase

is duplicated in a small number of *Vibrionaceae* species and the two copies are different. It is possible that they play distinct roles in *P. profundum* SS9 adaptation to high pressure. The chromosome II ATP synthase possibly compensates for the reduction of functionality of the main chromosome I ATP synthase at suboptimal environmental conditions.

To summarise, high pressure and low temperature affect all elements of the cell envelope of deep-sea bacteria. The chemical structures of phospholipids and lipopolysaccharides, fundamental components of membranes, need to be modified in order to maintain membrane fluidity at high pressure and low temperature and enable membrane-localised proteins to perform their functions. Proteins present in the cell envelope allow for nutrient diffusion and active transport, respiration and take part in sensing and signalling. All of these processes are indispensable for the survival of bacterial cells and also subject to pressure and temperature adaptation.

3.3 The Role of Flagella in High Pressure- and Low Temperature-Adapted Growth of Deep-Sea Bacteria

Motility is a very important process for bacterial survival as it allows cells to escape unfavourable conditions and move towards environments abundant in nutrients. The locomotion is possible due to flagella, which are embedded in the cell envelope and extend into the extracellular space (Erhardt et al. 2010; Schuhmacher et al. 2015). Flagella of different bacterial species have a similar structure that can be divided into three parts: basal body, hook and filament. The basal body consists of the cytoplasmic C-ring, structures embedded in the membranes and the rod. The flagellum is built of approximately 25 different types of proteins and the type III secretion system is used to assemble the rod, hook and filament. The filament is constructed from 20,000 to 30,000 flagellin subunits, which are added at its distal end (Erhardt et al. 2010; Schuhmacher et al. 2015). Flagellar synthesis is complicated and requires many genes that are organised in a hierarchical manner with early, middle and late genes temporally expressed under the control of specific sigma factors and transcriptional regulators (Merino et al. 2006).

The flagellum can fulfil its role in propelling the bacterium through the rotation of its filament due to the activity of the motor embedded in the membrane. Motor-generated rotation is passed onto the rod and hook and then the filament which results in cell movement (Schuhmacher et al. 2015). The energy for that rotation is derived from the transport of protons or sodium ions. Most flagellar motors can rotate clockwise (CW) and counterclockwise (CCW). In species with peritrichous flagella (multiple flagella projecting in all directions), such as *E. coli*, the CCW movement of filaments allows them to bundle and propel the cell (Erhardt et al. 2010). When a bacterium encounters unfavourable conditions the signal from the chemosensory pathway is relayed to the motor and in response, the motor changes its rotation to CW. This causes the bundle to fall apart and as a result, the cell tumbles and changes the swimming direction. In *Vibrio alginolyticus*, which possesses a

single polar flagellum, the CCW rotation of the motor drives the cell forward and the CW rotation pulls it backwards (Xie et al. 2011). When the cell resumes forward swimming, it reorients itself and the new swimming direction is chosen at random. Depending on the environment bacteria are found in, they can either display swimming or swarming. Both movements are powered by rotating flagella, but swimming occurs as individual cells move in a liquid environment, while swarming is defined as rapid multicellular movement across surfaces (Kearns 2010).

Motility could be the most pressure-sensitive process in bacteria. Swimming of *E. coli* cells is affected by pressures lower than 10 MPa (Bartlett 2002; Meganathan and Marquis 1973). Taking into account that the inhibition of cell growth and protein synthesis happens at higher pressure (50–200 MPa), it has been suggested that the flagellum assembly process is more sensitive to high pressure compared to the synthesis of the flagellum components (Abe 2007; Bartlett 2002; Meganathan and Marquis 1973). Hence, deep-sea bacteria must possess specific adaptations that allow them to swim at high pressure. The bacterial motility is not only affected by high pressure, but also changes with temperature. The rotational speed of the sodium driven flagellum motor was found to decrease with a drop in temperature (Baker et al. 2011; Yuan and Berg 2010).

The flagellum propels the cell, but also plays the role of a mechanosensor, allowing for adhesion to surfaces and formation of a biofilm, which in pathogens can contribute to virulence (Belas 2014; Merino et al. 2006). In *Vibrio parahaemolyticus* surface sensing occurs via the polar flagellum. There are less than 70 surface-responsive genes and most of them are positively regulated (Gode-Potratz et al. 2011). Almost two thirds are involved in swarming motility, several in sensing and/or transducing signals (encoding chemotaxis receptor proteins, enzymes modulating levels of secondary messengers) and some in colonisation and virulence (encoding chitin-binding proteins, type III secretion system components, proteases, collagenases, etc.). Thus, surface sensing in *V. parahaemolyticus* induces swarming but also prepares cells for the changed environmental conditions and potential encounter with a host. In the deep-sea environment, where nutrients are limited, the ability to adhere to particles or surfaces of animals may provide a more sustainable source of organic matter for bacteria.

The flagella have been studied in more detail in two piezophiles, *P. profundum* SS9 and *S. piezotolerans* WP3. Both species contain two flagellar systems, polar (PF) and lateral (LF). It is noteworthy that the LF cluster is present in the *P. profundum* SS9 and the other pressure-loving isolate, DSJ4, but missing in the piezosensitive *P. profundum* 3TCK strain (Campanaro et al. 2005). The lateral flagellum is a very complicated structure, encoded by almost 40 genes with considerably higher GC content, which suggests they were horizontally acquired (Campanaro et al. 2005; Eloe et al. 2008). The polar and lateral flagellum gene clusters of *P. profundum* SS9 and *S. piezotolerans* WP3 are very similar to the flagellum clusters found in *V. parahaemolyticus* BB22. Both *P. profundum* SS9 and *V. parahaemolyticus* strains possess two kinds of motors for the propulsion of flagella: sodium-driven for the polar and proton-driven for the lateral flagellum (Eloe et al. 2008). The LF proton-driven system was also identified in

S. piezotolerans WP3, while the polar flagellum gene cluster does not seem to contain motor protein genes (Wang et al. 2008). Two candidate genes were found away from both flagellar clusters that could be responsible for the movement of the polar or both, polar and lateral flagella. *S. piezotolerans* WP3 contains two flagellin genes (Wu et al. 2011) and *P. profundum* SS9 three. In the latter, *flaA* and *flaC* encode the flagellins of the polar flagellum and *flaB* the lateral (Eloe et al. 2008).

The polar flagellum of *P. profundum* SS9 is required for swimming at high pressure and lack of *flaA* or *flaC* or a component of the sodium-driven motor (*motA2*) leads to an inhibition of motility (Eloe et al. 2008). The lateral flagellum is necessary for swarming and is induced at high pressure and increased viscosity conditions. Mutants Δ*flaB* and Δ*motA1* (component of a proton-driven motor) are non-motile under these conditions. All tested mutants unable to swim, with non-functional polar flagellum (Δ*motA2*) or missing polar flagellum (Δ*flaA* and Δ*flaC*) were also unable to swarm. Moreover, it has been found that expression of the *flaB* gene increases under high pressure and high viscosity conditions in the parent but not in the mutants with inhibited swimming motility. Together, this suggests that the production of lateral flagella is dependent on a functional polar flagellum. The opposite has been observed in *V. parahaemolyticus*, where physical or genetic disruption of the polar flagellum results in induction of lateral flagella (McCarter et al. 1988).

The regulation of the polar and lateral flagella gene cluster in *S. piezotolerans* WP3 is different from *P. profundum* SS9 and does not depend on the pressure and viscosity but on pressure and temperature (Wang et al. 2008). As shown using microarrays and quantitative RT-PCR, the LF genes of *S. piezotolerans* WP3 are up-regulated at 4 °C compared to 20 °C and are slightly repressed by high pressure (20 MPa vs. 0.1 MPa). The genes of PF show the opposite relationship, being up-regulated at high pressure but down-regulated at low temperature. Mutants lacking the polar or lateral flagella displayed decreased swimming motility at 20 °C on 0.3% agar. The swimming motility of PF mutants was not inhibited at 4 °C, while mutants in the lateral flagellum gene cluster displayed no motility and their growth decreased. Hence, it was concluded that the lateral flagellum is required for growth and motility at low temperatures. The swarming motility seems to also be controlled by the temperature-dependent function of LexA, a protein involved in the SOS response (Jian et al. 2015).

The expression of genes belonging to the lateral flagellum gene cluster in *S. piezotolerans* WP3 is not only affected by temperature, but also by the filamentous phage SW1 present in the WP3 strain both on the chromosome and as a plasmid (Jian et al. 2013). The phage is cold-active, but does not influence the growth of the WP3 strain at 4 °C or at optimal 20 °C. The phage has been shown to affect the expression of 49 genes at 4 °C, among which 16 belonged to the lateral flagellum gene cluster. The phage has no effect on swimming motility but inhibits swarming motility at 4 °C. It is still unclear how the phage affects the lateral flagellum. SW1 regulates a variety of host's genes and clearly has an evolutionary advantage since it has not been lost. Both phage production and motility are energy consuming. 2% of total energy produced by a cell is necessary for flagellum

synthesis and functioning (Soutourina and Bertin 2003). At low temperature *S. piezotolerans* WP3 must balance the energy spent on motility and on production of the phage, which inhibits motility.

The swimming velocity of *P. profundum* strains was measured by Eloe and colleagues using a high-pressure microscopic chamber (Eloe et al. 2008). Not surprisingly, 3TCK and SS9 displayed highest swimming velocity at their optimal pressures for growth, 0.1 and 30 MPa, respectively. *P. profundum* SS9 increased the swimming velocity up to 30 MPa and was able to swim up to the maximum measured pressure of 150 MPa. On the other hand, increased pressure resulted in gradual reduction of the swimming velocity of the piezosensitive 3TCK up to 120 MPa. In comparison, the swimming velocity of *E. coli* immediately decreased at elevated pressure and was completely abolished at 50 MPa.

Defects in motility can affect cell growth and survival under some conditions, as shown by the phenotype of a cold-sensitive transposon mutant with insertion in the polar flagellum gene cluster (Lauro et al. 2008). The gene disrupted in the *P. profundum* SS9 FL24 mutant encodes a putative FliS protein (Lauro et al. 2008). FliS prevents the premature folding or inappropriate association of newly synthesised flagellin subunits and postpones their filamentation until they are translocated through the narrow, hollow core of the growing flagellum (Muskotal et al. 2006). FliS is the most widely conserved flagellar chaperone in bacteria. The gene disrupted in the FL24 mutant encodes a protein which is 77% identical (91% similar) to *V. cholerae* FliS (GenBank accession no. YP_129130.1 and NP_231769.1). The absence of a putative FliS protein in *P. profundum* SS9 results in a cold-sensitive phenotype, but only when the mutant strain is grown on agar (Lauro et al. 2008). The connection between surface-growth and the flagellum has been studied before in *Vibrio* species. As mentioned previously, in *V. parahaemolyticus* the polar flagellum acts as a surface sensor and induces the expression of genes encoding proteins required for swarming motility, virulence factors and sensory enzymes (Gode-Potratz et al. 2011). The role of the FliS protein has not been studied before in the context of surface sensing or adaptation to cold temperature.

Dr. Gail P. Ferguson's group found that the FL24 mutant is non-motile at high pressure (Fig. 3.2a) (Myka 2013). As expected, the FL24 mutant lacked the polar flagellum when analysed using fluorescence microscopy (Emiley Eloe, in Myka 2013). Plasmid-encoded *fliS* restored the swimming motility of the FL24 mutant (Fig. 3.2b), but the complementation of the cold sensitivity was unsuccessful (Myka 2013). The presence of the plasmid conferring resistance to streptomycin masked the cold-sensitive phenotype of FL24, similarly to the situation with the LPS mutant FL26 described above and as observed by Chi and Bartlett (Chi and Bartlett 1995). Since the *P. profundum* SS9 Δ*flaA* mutant (EAE1) lacking the polar flagellum (Eloe et al. 2008) did not display cold-sensitive phenotype on marine agar (Fig. 3.2c), we inferred that merely the absence of the polar flagellum in FL24 could not account for its cold-sensitive colony growth (Myka 2013). We hypothesise that the cold-sensitive phenotype could be caused by the intracellular accumulation of flagellin in the absence of FliS chaperone. Further experiments will be needed to assess the basis of FL24 cold sensitivity.

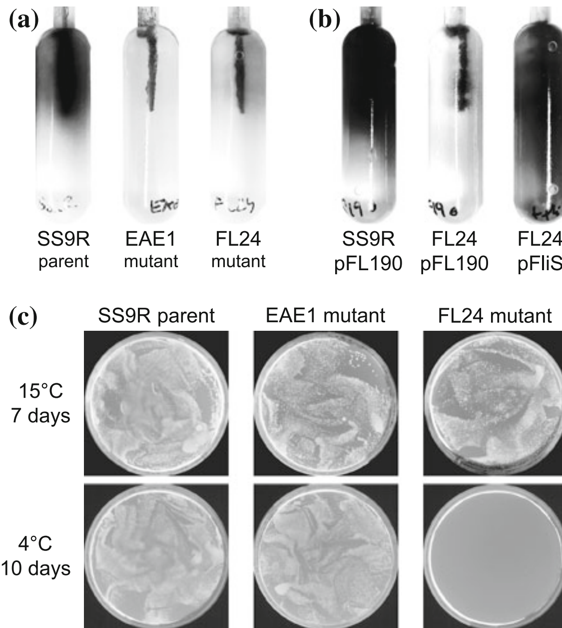

Fig. 3.2 The cold sensitivity of the *P. profundum* SS9 putative *fliS* mutant (FL24) does not result from the lack of polar flagellum. **a** The FL24 mutant displays a non-motile phenotype. The parent strain SS9R and the non-motile EAE1 mutant, lacking the FlaA flagellin (Eloe et al. 2008) were used as controls. Late exponential phase cultures (OD600 = 0.6–0.8) grown anaerobically at 15 °C, 0.1 MPa were inoculated in a straight line using a thin metal rod into the plastic Pasteur pipette bulb containing marine 0.3% agar with 0.05 mg ml^{-1} tetrazolium violet. Bulbs were incubated at 28 MPa for 48 h. **b** Plasmid-encoded putative FliS chaperone restores swimming motility of the FL24 mutant. Strains containing plasmids were pre-grown aerobically at 15 °C, 0.1 MPa and 100 μg ml^{-1} streptomycin and 1% (w/v) arabinose (for the expression of plasmid-encoded gene) were added to the media throughout the experiment. The strains containing plasmids grow slower, hence the incubation time at 28 MPa was extended to 72 h. The *P. profundum* SS9 *pbpra0917* (*fliS*) gene was amplified using primers 5-GTAGAATTCG CTCGATGCAGGCTTATAAT-3 and 5-TGCTCTAGAGATTATCTCTCGCTACACACCA-3 and cloned into pFL190 (Lauro et al. 2005) using EcoRI and XbaI to produce pPPfliS. **c** 100 μl of an early stationary phase culture adjusted to an $OD_{600} \sim 0.2$ was spread on marine agar plates. Growth was assessed at 15 and 4 °C after 7 and 10 days, respectively

To conclude, motility is extremely important for bacteria as it allows them to find a better-suited nutrient-abundant environment and escape unfavourable conditions. In mesophilic bacteria, motility is one of the processes most sensitive to increased pressure. This suggests that the motility systems of deep-sea bacteria must be specifically adapted to high pressure and low temperature. Indeed, the pressure optima for *P. profundum* SS9 and 3TCK swimming matched those for growth (Eloe et al. 2008). Interestingly, both strains were also capable of short-term swimming under pressures higher than the known growth limit of microbial life. The induction of lateral flagella required for swarming at high pressure or low temperature

(*P. profundum* SS9 and *S. piezotolerans* WP, respectively) suggests that for deep-sea marine bacteria planktonic swimming might not be the best survival strategy. Association with particles or surfaces of animals could provide a more stable nutrient source. Future studies investigating the precise role of proteins involved in polar and lateral flagella assembly are likely to reveal temperature- and pressure-sensitive assembly steps.

3.4 Conclusions

This chapter focused on the adaptations of the cell envelope and flagella of deep-sea bacteria to high pressure and low temperature. Psychropiezophiles adjust their fatty acid content to optimize membrane fluidity and many of their membrane proteins involved in nutrient acquisition, transport, respiration, sensing and signalling are specifically adapted to function at high pressure and low temperature. Deep-sea bacteria swim at elevated pressures (even up to 150 MPa), which suggests that their motility systems differ from mesophilic bacteria, since the motility of mesophiles is affected even by 10 MPa. Moreover, some deep-sea bacteria produce a second type of flagella, lateral flagella, in response to increased pressure or low temperature.

The findings presented in this chapter rely on a plethora of techniques and analyses applied to whole microbial communities, single species as well as particular genes and proteins. The use of metagenomics can shed light on the deep-sea microbial communities regardless of the ability to grow the organisms in the laboratory. However, future research into cultivation approaches will be highly beneficial to our understanding of the bacteria found in the oceans' depths. In order to fully understand the adaptations of bacteria to the deep-sea environment we need to obtain a more phylogenetically-diverse set of reference piezophiles, since all species discussed here belong to Gram-negative *Gammaproteobacteria*, mostly to the genera *Photobacterium* and *Shewanella*. Whole-genome sequencing and comparative genomics of piezophilic and piezosensitive isolates of the same bacterial species can better pinpoint genes likely to be involved in the high pressure adaptation (Campanaro et al. 2005), which can be further analysed using molecular genetics. The analysis of the bacterial transcriptome in response to different growth conditions (Vezzi et al. 2005) and the comparison of genes expressed in the parent strain and a particular mutant are also invaluable, especially when complemented by the quantitative proteomics studies (Campanaro et al. 2012; Le Bihan et al. 2013). The development of genetic tools for large scale mutagenesis, gene deletion, recombineering and protein expression in psychropiezophiles is important for the research aiming to understand the effects of pressure and temperature on biological systems, but also for possible biotechnological applications. Similarly, the biotechnology sector could greatly benefit from biophysical and biochemical characterisation of structure and function of high pressure-adapted proteins. Deep-sea bacteria could be used as a renewable source of fatty acids for the production of biofuels, PUFAs for human consumption, secondary metabolites and

chemical compounds for drug development, and various pressure and temperature adapted enzymes. Research on the adaptations of deep-sea microorganisms to high pressure and low temperature has the potential not only to broaden our understanding of the processes occurring in nature, but also identify targets that could have biotechnological and industrial application.

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Chapter 4
Microbial Electron Transport in the Deep Subsurface

Jamie Hinks, Mi Zhou and Jan Dolfing

Abstract The deep ocean may be one of the largest microbial habitats on the planet. Hence, high hydrostatic pressure is a feature of microbial life. We know very little about the deep biosphere because simulating deep ocean conditions in the laboratory whilst simultaneously monitoring microbial processes is difficult. Changes in pressure can inhibit some reactions, whilst simultaneously accelerating others. Assumptions about how biochemical reactions proceed under ambient conditions may lack validity in the deep biosphere. In extreme environments, microbes often exploit metabolic strategies that yield slim energetic margins. How these occur under pressure is an interesting thermodynamic puzzle. Extracellular electron transfer (EET) is a process whereby microbes respire solid substrates in their surrounding environment. For an electron to move outside of the cell, it must transit the microbial envelope through a series of membrane bound electron carriers each of which will have a unique pressure response. EET most likely evolved in the deep biosphere and therefore makes an excellent model system for studying microbial energetics in high pressure environments. In this chapter, the reader can explore the fundamentals of thermodynamics, the discovery of EET, theoretical implications of pressure effects on the relevant biochemical apparatus, and learn about a proposed system for studying the interesting phenomenon of EET under high pressure.

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4.1 Introduction

Covering two thirds of the Earth's surface and with an average depth of 3800 m the ocean exhibits tremendous diversity and activity and represents a considerable proportion of the biosphere yet it remains largely unexplored. The piezosphere, the proportion of the ocean that is below 1000 m water depth and where pressures are therefore greater than 10 megapascals (10 MPa), is the largest microbial habitat on the planet in terms of volume, perhaps accounting for 30% of total global biomass and representing *c.a.* 60% of the earth's surface (Meersman et al. 2013; Picard and Daniel 2013). Whilst other physiochemical conditions, such as temperature, are recorded at extremes in deep oceans, away from hydrothermal vents the temperature in the deep ocean below the thermocline is uniformly close to 2–3 °C (Picard and Daniel 2013; Daniel et al. 2006). Thus high hydrostatic pressure is the ubiquitous and defining feature of life in deep marine habitats.

For each km of water depth the hydrostatic pressure increases by 100 atmospheres (10 MPa). Lithostatic pressure in the sedimentary column increases by 15–25 MPa for each km of depth and in the oceanic crust by 27–32 MPa dependent on the prevailing geology. The average hydrostatic pressure in the ocean is 38 MPa and because the deepest recorded depth in the ocean is 11 km, the current known maximum pressure at the sediment water interface is around 110 MPa. Confirmation exists of prokaryotic life extending down to depths of at least 1.6 km in sediments, and oceanic crusts represent a rich deep-biosphere (Roussel et al. 2008; Salas et al. 2015). The depth and pressure maxima of the deep biosphere have yet to be constrained, but based on collective observations of high carbon turnover in the Mariana Trench, evidence of life in both deep sediments and ocean crusts, and confirmed growth of *Moritella yayanosii* at 130 MPa, it is likely that hydrostatic pressures approaching 200 MPa are biologically relevant (Meersman et al. 2013; Picard and Daniel 2013; Roussel et al. 2008; Salas et al. 2015; Yayanos et al. 1981). Evidence that microbes can survive under hydrostatic pressures up to 80 GPa exists although it is not clear if microbes can grow under these conditions.

The majority of the carbon input into marine systems is derived from primary production that occurs in the photic zone. Immediately below the photic zone, heterotrophic bacteria aerobically degrade the majority of organic carbon with only 1% being deposited in the sea floor as either dissolved or particulate organic matter (Picard and Daniel 2013). The refractory nature of dissolved organic matter (DOM) is known to increase with depth in marine and sedimentary environments hence, the main energy source and the building blocks for prokaryotic benthic lifeforms is difficult to degrade (Aparicio et al. 2015). Carbon turnover in bathypelagic (200–4000 m) and abyssopelagic (>4000 m) sediments necessarily includes the recycling of dead microorganisms or necromass (Meersman et al. 2013). Additionally, in benthic environments, oxygen is quickly depleted, sometimes in the upper few centimetres of sediment, making the job of breaking down available DOM less energetically rewarding (Picard and Daniel 2013). However, microbes

are versatile and anaerobic benthic organisms can exploit energy margins of only −4.5 kJ mol^{-1} (the oxidation of glucose to CO_2 yields −2870 kJ mol^{-1}) by growing slowly (Meersman et al. 2013; Willey 2014; Jackson and McInerney 2002). Accordingly, organic carbon turnover in sedimentary environments occurs over millennia suggesting that there is sufficient organic material in the marine subsurface to drive microbial metabolism for millions of years. However, the turnover rate of volatile acids like acetate happens much more quickly; on a decadal timeframe (Wang et al. 2010). Like oxygen, alternative anaerobic terminal electron acceptors (e.g. nitrate) are depleted quickly in the upper layers of sediment in a thermodynamically predicable order with those that are the most energetically rewarding being utilised first (Meersman et al. 2013). However, this textbook-case of thermodynamic stratification does not reflect the reality in deep benthic systems where sulphate, iron and inorganic carbon simultaneously undergo microbial reduction (Wang et al. 2010).

Despite these constraints, there is surprising activity and diversity in the deep ocean and microbial respiration can be coupled to oxidised iron and manganese containing minerals which are abundant on the seafloor (Liao et al. 2011). Iron reduction has been shown to occur over a range of pH but biological acidophilic iron reduction is poorly understood. At circumneutral pH, iron speciation in marine sediments is varied but even though iron is mostly present as solid oxides or oxyhydroxides, like goethite or haematite, it is still biologically available (Bird et al. 2011). In this chapter we will look at a special case of how organisms can obtain energy by using iron as a terminal electron acceptor—a process known as extracellular electron transfer (EET)—to thrive in marine benthic environments. We will describe what is currently known about EET under pressure extremes concluding that the pressure element has largely been overlooked in the study of an exciting group of organisms which evolved from benthic lifeforms and which deploy a metabolic strategy that likely has primordial origins.

4.2 Electron Acceptors and Microbial Respiration

Most lifeforms obtain energy from coupling the oxidation (i.e. loss of electrons) of an electron donor to the reduction (i.e. gain of electrons) of a terminal electron acceptor. The compound that is oxidised (also called reductant or fuel) is often organic carbon although certain specialist prokaryotes, known as chemolithotrophs, can use inorganic reductants such as hydrogen and sulphur. The terminal electron acceptor is also known as the oxidant and for many organisms, including mammals, this is molecular oxygen which, being ubiquitous in air and water as a by-product of photosynthesis, is the most oxidised, widely-available terminal electron acceptor. For chemical reactions to proceed spontaneously (with the release of energy), the reductant must have a lower reduction potential than that of the oxidant. The free energy available to microbes when catabolising a given reaction is directly proportional to the difference in reduction potential between the reductant and that of

the oxidant and is commonly known as Gibbs free energy and annotated in biochemical treatise as ΔG′—the prime symbol denotes biological conditions that are assumed to be representative of those prevailing in the cytoplasm. If the activities of all chemical species are known then ΔG′ can be calculated with precision.

The electron tower is commonly used in microbiology to illustrate the concept of bioenergetics (Fig. 4.1). The first thing to note is that reductants or electron donors have a negative reduction potential ($E^{\theta\prime}$) whilst compounds that have a tendency to accept electrons have a positive reduction potential ($E^{\theta\prime}$). Microbes can obtain the most energy from coupling the oxidation of reduced organic compounds (such as glucose) to oxygen because the potential difference between the electron donating reductant ($E^{\theta\prime}$ − 0.43 V for glucose) and the accepting oxidant (+0.82 V for oxygen) is large ($\Delta E^{\theta\prime}$ = 1.24 V). Electrons spontaneously flow from carriers having a negative potential to those with a positive potential and the greater the potential difference between the reductant and the oxidant, the larger the ΔG′ is for a given reaction and the more metabolic energy the microbe can extract.

Note from the electron tower that the reduction potential for the ferric iron (Fe (III)) to ferrous iron (Fe (II)) redox couple has a large positive value (E^{θ} = +0.77 V). In a thermodynamic sense, ferric iron is a good oxidant and therefore potentially a good biological terminal electron acceptor. The reduction potential for Mn (IV) to Mn (II) (E^{θ} = +0.47) is close to that of the nitrate (NO_3^-) nitrite (NO_2^-) redox couple ($E^{\theta\prime}$ = +0.42 V) suggesting that Mn (IV) makes as good a candidate terminal electron acceptor as nitrate based purely on its reduction potential (Logan 2008). The free energy of coupling acetate oxidation to iron and manganese reduction ranges between −712 and −814 kJ mol^{-1} at neutral pH which is very close to the energy that can be obtained from coupling acetate degradation to the oxygen reduction reaction

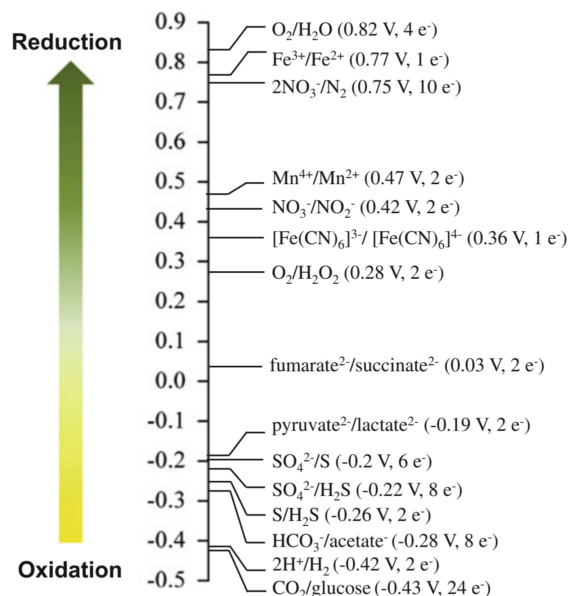

Fig. 4.1 The electron tower concept is useful to illustrate energy yielding redox reactions in biology. Theoretically, any reaction at the *bottom* of the electron tower can be coupled to a reaction at the top. The amount of energy (ΔG′) available from a given reaction is directly proportional to the difference in the reduction potential of the redox couples in question

($\Delta G' = -849$ kJ mol^{-1}), meaning microbes can gain significant energy from using iron or manganese as terminal electron acceptors (Lovley and Phillips 1988).

However, ferric iron and manganese (IV) are present primarily as insoluble oxides and oxyhydroxides in marine sediments which complicates the thermodynamic rationale for their suitability as terminal electron acceptors when compared to dissolved species like oxygen or nitrate (Bird et al. 2011; Nealson and Myers 1992). Additionally, iron is polymorphic, with structural order ranging from amorphous ferric oxides to highly crystalline goethite (α-FeOOH) and akaganeite (β-FeOOH) each of which have considerably different kinetic properties from dissolved terminal electron acceptors. The range of crystallinity apparent in iron containing minerals gives rise to different midpoint reduction potentials ranging from $E^{\theta'} = +0.38$ V for ferric citrate to −0.31 V for magnetite (Lovley and Phillips 1988). It not surprising therefore that the significance of metal oxides as biological terminal electron acceptors was not fully appreciated until the late 1980s when, almost simultaneously, Lovley and Phillips (1988) and Myers and Nealson (1988) reported the phenomenon of dissimilatory iron and manganese reduction by benthic isolates (Lovley and Phillips 1988; Myers and Nealson 1988). This was a watershed in microbiology because, until that point, biological terminal electron acceptors where though to be soluble species that diffused freely in and out of the cell and that cellular redox reactions occurred intracellularly. The in situ free energy of coupling acetate oxidation to haematite reduction at neutral pH is −41.6 (±12.4) kJ mol^{-1}, much smaller than the $\Delta G'$ determined from standard conditions (−738) meaning that the energetic margins in the deep biosphere are curtailed by prevailing environmental conditions (Wang et al. 2010; Lovley and Phillips 1988; Roden 2003). The reduction of iron and manganese oxides yields sufficient energy to support microbial life allowing organisms that adapt this metabolic strategy to thrive as is evident from the extent of metal oxide reduction in some sedimentary systems which has been reported to account for up to 78% of anaerobic organic carbon degradation (Canfield et al. 1993).

4.3 Extracellular Electron Transfer

4.3.1 Background

The thermodynamic case for biological metal oxide reduction is strong and evidence that several microbial genera can mediate extracellular transfer of electrons from the cytoplasm to a solid acceptor is unequivocal.

Much of what we know about EET in microbes comes from the field of bioelectrochemical systems (Wang et al. 2013; Harnisch and Schröder 2010; Logan et al. 2006; Allen and Bennetto 1993). Bioelectrochemical systems have been a benchtop curiosity for over a century and EET has been applied in microbial fuel cells to recover waste from sewage, in microbial electrosynthesis and, more recently, in bioelectroanalytics (Seviour et al. 2015; Hinks et al. 2016; Kim et al. 2002; Potter 1911).

The first dissimilatory metal oxide reducing isolates, GS-15 reported by Lovley and Phillips (1998) and MR1 reported by Myers and Nealson (1998), were later designated as the novel species *Geobacter metallireducens* and *Shewanella oneidensis* MR1 (formerly *Shewanella putrefaciens* and *Alteromonas putrefaciens*) respectively (Nealson and Myers 1992; Lovley et al. 1993; Venkateswaran et al. 1999). The unusual nature of a solid phase terminal electron acceptor invited speculation as to how the terminal electron acceptor was rendered biologically available. Hypotheses included: (1) direct electron transfer exchange via cellular attachment to the solid phase electron acceptor, (2) a method of solubilising the solid substrate, and a mechanism to transport solid particles into the cell where they could be reduced (Nealson and Myers 1992). The role of membrane bound electron transport chains in carrying out dissimilatory Fe (III) reduction was soon appreciated, but the exact mechanisms were still not completely understood (Gorby and Lovley 1991).

We now know that EET can occur either directly through contact between the microbe and the solid terminal electron acceptor or indirectly through redox carriers called electron shuttles (Gorby et al. 2006; Roller et al. 1984). EET is well documented although certain aspects of direct EET, particularly the role of and conductive nature of nanowires, are still controversial (Yan et al. 2015; Malvankar et al. 2011, 2012; Strycharz-Glaven and Tender 2012). EET may occur through a number of mechanisms and is not limited to *Geobacter spp*. and *Shewanella spp.* but appears to be a relative common phenomenon. However, in this chapter we will focus on EET mechanisms known in *Shewanellaceae* since EET has been well studied in this group. In addition, one of its members, *S. oneidensis*, was the first dissimilatory metal reducing organism whose genome was fully sequenced (Heidelberg et al. 2002).

4.3.2 Mechanisms of EET

Like many organisms, the most efficient energy generation in *Shewanella* is achieved by establishing a proton motive force across the biological membrane during oxidative metabolism. A proton motive force is established by transferring electrons from reduced carriers such as NADH and $FADH_2$, produced during the cytoplasmic catabolism of organic compounds, to the quinone pool. The reduced quinones must be continually reoxidised through a number of plasma-membrane bound electron carriers (quinone dehydrogenases) with sequentially increasing reduction potential in a manner analogous to the electron tower (Richardson et al. 2012). The protons generated during this process are translocated across the membrane and sequestered in the periplasmic space setting up a chemiosmotic gradient that is used in the oxidative phosphorylation of ADP to ATP, with a free energy change proportional to the difference in potential of the quinone oxidase and the terminal oxidase (Richardson et al. 2012; Mitchell 1961). The specific details of generic electron transport chains (ETCs) can be found in any good standard introductory text to microbiology (Willey 2014).

The electron transport chains of *Shewanellacea* and other dissimilatory metal reducers, and indeed many organisms capable of anaerobic respiration, can be highly branched. This means electrons can enter and exit at different points in the ETC effectively permitting interactions with a broad range of electron donors and acceptors with different midpoint reduction potentials. The branched nature of *Shewanella*'s ETC underpins its metabolic versatility as it allows electrons to exit the ETC at different reduction potentials, explaining why the organism can utilise a number of different terminal electron acceptors (Heidelberg et al. 2002).

Extracellular electron transport is achieved in *Shewanella oneidensis* via the metal reduction (Mtr) pathway—a modular, multicomponent protein system that creates an electron conduit between the cytoplasm and the outer membrane to effect the reduction of an extracellular terminal electron acceptor (Fig. 4.2). Electrons from cytoplasmic electron carriers, which have been reduced by cytoplasmic catabolic processes, are captured by menaquinone or ubiquinone and then transferred to CymA, a plasma-membrane bound c-type cytochrome with four haem units. In the periplasmic space, another c-type cytochrome, MtrA, is the next electron carrier in sequence that finally passes the electron to the outer membrane associated c-type cytochrome, MtrC. Both MtrA and MtrC are multi-haem proteins containing 10 haem units. Fully functioning Mtr pathways also contain an additional component, a β-barrel protein (MtrB), which functions as a pore and which ensures the steric accessibility and transmembrane arrangement necessary for MtrA and OmcA to conduct electrons through the outer membrane to directly reduce metal oxides or intermediate shuttling compounds such as a riboflavins (Coursolle and Gralnick 2012).

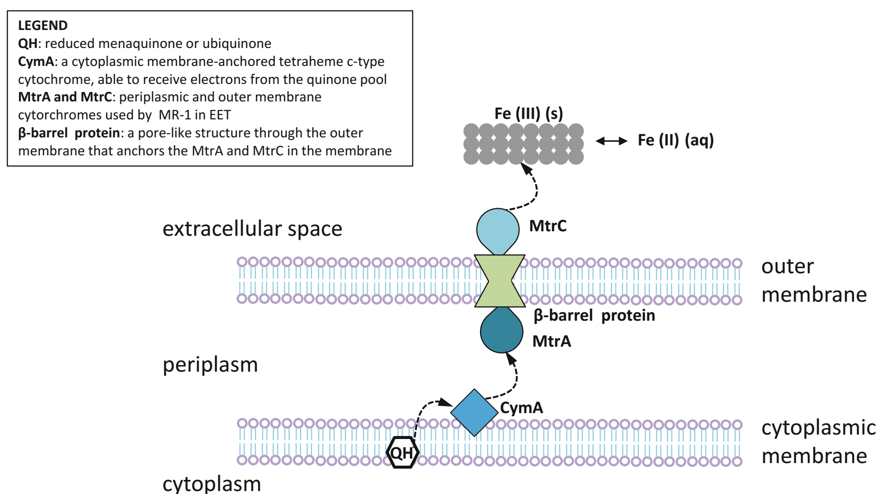

Fig. 4.2 A schematic of the Mtr pathway in Shewanellacae, the conduit through which EET is achieved. Note there are number of paralogs of each of the components in this transmembrane electron conduit

The majority of c-type cytochrome in *S. oneidensis* (80%) is located on the outer membrane; there are at least 39 different multi-haem cytochromes in its genome (although reports have suggested the genome encodes for 42 different multi-haem containing cytochromes) whereas *S. piezotolerans* WP3 reportedly contains 55 c-type cytochromes (Richardson et al. 2012; Heidelberg et al. 2002; Meyer et al. 2004). The outer membrane association of c-type cytochromes is consistent with the concept of EET and the large number of cytochrome variants is consistent with functional redundancy of the classical Mtr pathway described above. Each unit in the Mtr pathway, with the exception of CymA, contains a number of paralogs in many species of *Shewanellacea* (Coursolle and Gralnick 2012). In *S. oneidensis*, there are four paralogs each of both MtrA and MtrB and three of MtrC giving 11 putative Mtr components and 48 possible Mtr pathways (Coursolle and Gralnick 2012). However, only nine pathways have been confirmed to function in Fe (III) reduction (Coursolle and Gralnick 2012). This is because in *S. onedenis*, mutants with Mtr pathways reconstructed from different combinations of MtrABC paralogs, functional Fe (III) reduction was not demonstrated with two of the β-barrels, two of the MtrA paralogs, and for one outer membrane associated MtrC paralog. Given that the Mtr paraglogs are highly conserved in *Shewanella* species, it is likely that they have distinct functions that are yet to be discovered (Coursolle and Gralnick 2012).

The outer membrane associated MtrC has been show to bind ionically to iron oxides in a manner that is dependent on ionic strength and pH. Additionally, MtrC may interact with a number of intermediate electron carriers before depositing an electron to solid oxides (Richardson et al. 2012). Crystallographic analysis of *Shewanellacea* multi-heam cytochromes shows that they have a conserved CX_8C disulphide motif that alters its conformational state in response to redox conditions which in turn governs its binding affinity for riboflavin (Edwards et al. 2015). Experimental studies suggest that under anaerobic conditions, MtrC is configured as an outer-membrane flavocytochrome that is important for Fe (III) reduction, and that upon exposure to oxygen the disulphide bridge reforms, causing riboflavin to dissociate, presumably so that the reductive unit can utilise oxygen (Edwards et al. 2015). In silico reconstruction of the MtrC protein structure showed the spatial arrangement of haems in a ‘staggered cross’ formation with 4–10 Å intermolecular spacing flanked by β-barrel hydrophobic domains, suggesting that precise spatial arrangement is maintained by an equilibrium between the proteins and the supporting lipid bilayer (Edwards et al. 2015).

Conductive pili play a putative role in long range EET of *Shewanellacea* (Strycharz-Glaven et al. 2011). Whilst the mechanism of electron transport in nanowire based EET is unknown, the nanowires are thought to be an adjunct to the Mtr pathway rather than a separate conductive pathway. Nanowires are thought to directly interface with the outer membrane associated cytochromes and to be rendered conductive through a series of π–π couplings in the aromatic residues of the main structural protein, pilin (Yan et al. 2015). Alternatively, the length of the nanowires may be studded with multi-haem containing cytochromes which conduct electrons, and it has been show that two outer membrane associated deca-haem

c-type cytochromes, MtrC and OmcA are crucial in maintaining nanowire conductivity. However these details and the electron tunnelling mechanism are still the subject of fierce debate (Yan et al. 2015; Strycharz-Glaven et al. 2011; El-Naggar et al. 2010).

EET in *Shewanellacea* is, therefore, achieved through a specific spatial relationship of at least four proteins that are associated with both the plasma and outer membrane as well as a diffusible shuttle such as riboflavin and, putatively, a conductive appendage and possibly bound flavins in the form of outer membrane associated flavoproteins. The primary function of the β-barrel MtrB is thought to be in maintaining the orientation of these proteins with one another and within the lipid bilayer via hydrophobic interactions between the hydrophobic residues in the β-barrel and the surrounding lipid bilayer. Pressure exerts known effects on biomacromolecules, in particular lipids and proteins, yet the pressure effect of this essential respiratory pathway in benthic organisms remains largely unexplored. Before exploring what is known about EET and dissimilatory Fe (III) reduction under pressure, it is necessary to revise general considerations regarding the effect of pressure on biopolymers.

4.3.3 Pressure Effects

Temperature exerts a predictable and monotonic effect on the reaction rates and equilibria in chemical systems. Pressure, on the other hand, can accelerate, inhibit or have a neutral effect on chemical reactions depending on the sign and magnitude of a fundamental parameter, the volume change, which is described by Le Châtelier's principle, when pressure is exerted on a system. The equilibrium of a reaction with a positive volume change is shifted to the left (inhibited) when pressure is exerted whilst reactions that yield a negative volume change will be shifted to the right (facilitated). In the absence of a volume change the equilibrium remains unaffected as does the rate constant for a given reaction, and can be described as follows (Meersman et al. 2013; Abe 2007; Bartlett 1999):

$$K_p = K_1 exp(-P\Delta V/RT) \tag{4.1}$$

$$k_p = k_1 exp(-P\Delta V^{\neq}/RT) \tag{4.2}$$

where K and *k* represent the equilibrium and rate constants respectively with subscript '1' denoting the constant at atmospheric pressure and subscript 'p' its value at elevated pressure. The volume change established during equilibrium is ΔV and the volume change associated with the formation of the activation products of a given reaction is $\Delta V^{\neq}$, where R is the gas constant and T is the absolute temperature. It is interesting to note that the relationship between pressure and rate constants is exponential, therefore small changes in volume may exert an effect on the

rate constant that is large in magnitude (Bartlett 1999). Volume changes in the order of 20–100 cm^3 mol^{-1} are biologically relevant. A reaction with a 100 cm^3 increase in volume at atmospheric pressure would be inhibited by 35% at 10 MPa and by 99% at 100 MPa. We know that microbes thrive in environments exceeding 100 MPa and must conclude that their biochemistry favours reactions with low volume changes or that they have developed strategies that counter this fundamental thermodynamic constraint.

The implications of pressure effects on various macromolecules has already been covered in Chap. 3 but we will briefly review the implications of pressure for the biochemistry relevant to EET in this chapter, namely: Fe (III) reduction, membrane phospholipids, and membrane proteins.

4.3.4 Fe (III) Reduction

The ionic radius of the more oxidised ferric (Fe (III)) ion is smaller (≈0.64 Å) than the radius of the reduced ferrous ion (≈0.74 Å) the larger ionic radius resulting from the additional electron carried by Fe (II) (Slichter and Drickamer 1972). Le Châtelier's principle therefore predicts that, given the apparent positive ΔV associated with iron reduction, equilibrium favours a larger ratio of Fe (III) speciation to Fe (II), and—assuming that this positive ΔV translates into a positive $\Delta V^{\neq}$—that the rate constant would decrease under increasing hydrostatic pressure. However, the ΔV of various Fe (III)/Fe (II) systems has been experimentally determined to be smaller than expected based on the ionic radii alone. Whilst ΔV for Fe (III)/Fe (II) redox couples is positive in many instances, it is usually less than 10 cm^3 mol^{-1} in aqueous solutions (Giovanelli et al. 2004). While volume changes are invariably positive for dissimilatory Fe (III) reduction under high pressure the ΔV is within the lower range expected for biochemical reactions (Slichter and Drickamer 1972; Sachinidis et al. 1994). The specific ΔV and $\Delta V^{\neq}$ vary depending on the redox couple in question and in many instances of Fe (III)/Fe (II) couples with organic ligands, the observed ΔV is negative, which points towards strategies to counter thermodynamic constraints at high pressure. Given the multicomponent nature of EET pathways as described above, predicting volume changes at pressure is complicated. To date no sufficiently quantitative treatment of EET volume changes at high pressure has been experimentally determined and therefore the response of the ETC to increasing pressure and therefore the thermodynamics of the system in benthic environments is not well described. In studies conducted over a lithostatic gradient of 900 m (between 25 and 28 MPa) the ΔV was considered as a constant in determining ΔG although modelling studies have shown that of all possible microbial respiratory strategies, Fe (III) reduction was the only one when which became energetically more favourable with increasing hydrostatic pressure (Macdonald 1997; Fang et al. 2010; Fang and Bazylinski 2008). However, the problem is sufficiently multifactorial and situation depended that it would benefit

from experimental clarification that will shed light on the energetics of these processes under environmentally relevant conditions and for a range of organisms.

4.3.5 Lipids

By virtue of their ability to maintain the cell membrane in the homeoviscous state necessary for biological function across a range of physicochemical conditions, lipids are the most compressible of the common biopolymers. Hence their pressure response is large (Daniel et al. 2006). When subject to increased pressure, lipids undergo an increase in order (a more closely packed and linear arrangement) and a decrease in rotational motion which is manifest by a phase transition from the fluid-like liquid crystalline mesophase to a gel state (Winter and Jeworrek 2009). As a general rule, many biologically relevant lipids respond to pressure in a similar way as they do to temperature and a 100 MPa increase in pressure is equivalent to an 18–21 °C drop in temperature (Bartlett 1999; Watanabe et al. 2009). Thus at a depth of 10 km and a temperature of 3 °C, the lipid mesophase state will be equivalent to that predicted at −15 to 18 °C under ambient pressure conditions.

The increase in lipid order brought about by pressure means the membrane tends toward the solid gel phase at elevated pressures and microbes respond by increasing the proportion of both mono-unsaturated and poly-unsaturated fatty acids in their membranes at elevated pressures (Bartlett 1999). The double bonds in the acyl chain are associated with a 30° bond angle which increases both the apparent volume and the packing parameter of the phospholipid resulting in less membrane order and an increased tendency towards liquid crystalline mesophases. The liquid crystalline to gel transition temperature of mono-unsaturated and saturated fatty acids observed in the pressure response of microbes is low. The transition temperature for stearoyl-arachidonoylphosphatidylcholine, for example, is −13 °C and will therefore remain in a physiologically relevant gel mesophase in deep sea conditions (Daniel et al. 2006; Usui et al. 2012). Additional lipid based adaptations include increased fatty acid chain length and branching which, in combination, serve to counter the pressure related tendency towards increased ordering of the acyl chains (Bartlett 2002). Additionally, hopanoids have been postulated to extend the liquid crystalline to gel transition of lipid mixtures over a pressure gradient and are analogous in function to cholesterol found in eukaryotic membranes (Daniel et al. 2006).

It has been shown that various *Shewanellacea* respond to pressure by increasing the proportion of eicosapentaenoic acid in their membranes (Usui et al. 2012; Sato et al. 2008). Eicosapentaenoic acid is a 20 carbon polyunsaturated fatty acid with five double bonds (C20:5) and was shown to be critical for maintaining membrane fluidity close to that expected at atmospheric pressure across a pressure gradient. However, it is not fully understood whether fluidity must be globally maintained across the membrane or only in localised regions, called rafts, to maintain biological function (Usui et al. 2012).

4.3.6 Proteins

Pressure has a tendency to denature proteins in a specific way compared to temperature. Elevated pressure favours hydrogen bonding compared to hydrophobic interactions. Hence pressure denatured proteins exhibit a globular conformation compared to the completely unravelled state that occurs upon temperature denaturation. Pressure induced protein denaturation is, therefore, thought to occur through incursion of water into the protein interior because of impaired hydrophobic interactions and results in preferentially denatured secondary structures like β-sheets and α-helices. The volume change for protein unfolding is estimated to be in the region of $\Delta V = -80\ cm^3\ mol^{-1}$ indicating their tendency to unfold at elevated pressure (Daniel et al. 2006). Monomeric proteins are more stable than oligomeric proteins and typically undergo unfolding at 200 MPa compared to 100 MPa for many multimeric proteins (Daniel et al. 2006; Bartlett 1999). However, solute effect plays a major role in membrane stability and pressure adapted organisms have been shown to modulate the solute concentration of their membranes in such a manner that ΔV becomes independent of pressure over a physiologically relevant range (Daniel et al. 2006). Cytochromes undergo a negative volume change upon oxidation and therefore their tendency to reduce an electron acceptor would be favoured at high pressure (Giovanelli et al. 2004). Studies with horse heart cytochrome c determined ΔV to be $-24\ cm^3\ mol^{-1}$ accompanied by a positive shift in midpoint reduction potential of around 25 mV (Cruanes et al. 1992). Incidentally, the redox activity of cytochrome c could be maintained at 500 MPa suggesting that the tertiary protein structure may not be pressure sensitive (Cruanes et al. 1992).

Hydrophobic interactions are important in governing the spatial arrangement and stability of membrane inserted components including proteins like c-type cytochromes (Hinks et al. 2014, 2015). Molecular insertions into the membrane have been shown to alter membrane fluidity and microbes will respond to hydrophobic mismatch by altering their fatty acid profile. By extension, a pressure induced change in, for example, acyl chain length would result in a hydrophobic mismatch for proteins whose hydrophobic secondary structure usually results in a stable spatial configuration at atmospheric pressure such as that described for the Mtr pathway (Fig. 4.2). Positive mismatch (where the inserted component has a hydrophobic region that is greater than the thickness of the membrane), between a membrane protein and the cell membrane can be well tolerated and is achieved by a small rotation around the protein axis, effectively shortening its length, so that it fits obliquely into the bilayer (Hinks et al. 2015; Strandberg et al. 2012). Instances of negative mismatch, when the transmembrane domain is shorter than the lipid acyl chains, can induce membrane thinning and a tendency towards excessive membrane disorder or poration.

As described above, microbes respond to increased pressure by increasing the proportion of long chain fatty acids in their membranes. As a result, negative mismatch between the hydrophobic transmembrane regions that are stable at

atmospheric pressure and the lengthened fatty acids chains in the pressure adapted membrane will occur (Bartlett 2002). Indeed, the archetypal piezotolerant organism, *Photobacterium profundum* SS9, differentially expresses two outer membrane porins: OmpL is important at low pressure whereas OmpH is expressed preferentially at elevated pressure (Bartlett 1999). Neither OmpH nor OmpL are crucial for growth but it has been experimentally determined that OmpH has a larger transmembrane channel than OmpL (Bartlett 1999).

The ΔV determined for various reduction processes was shown to be highly dependent on the ordering of the supporting lipid bilayer in a model system because of its effect on the spatial arrangement of the redox active moieties supported within it (Cruanes et al. 1995). This means that steric considerations can also influence the thermodynamics of electron transfer processes under pressure further reinforcing the need for precise arrangement of the Mtr components within the membrane.

4.4 Implication of Biochemical Pressure Effects on EET Apparatus in *Shewanella*

As discussed earlier, there is speculation that the branched nature of the *Shewanella* EET chain is configured for it to respond quickly to changing conditions thus conferring a survival advantage in both stratified and dynamic environments (Coursolle and Gralnick 2012). This branching of the EET is loaded towards the terminal end, meaning that *Shewanellaceae* exhibit relative metabolic diversity in terms of electron acceptors but not electron donors when compared with many other species. Indeed the absence of paralogs of the membrane bound quinone oxidoreductase, CymA, means that the branched ETC in *Shewanellacea*, and therefore a choice of terminal electron acceptors, can be regulated by just this single protein (Coursolle and Gralnick 2012). In contrast, *E. coli* has up to eight paralogs of the CymA equivalent, NapC. This difference is in agreement with the notion of *Shewanellaceae* being biochemically equipped to respond quickly to changing environmental conditions (Jensen et al. 2010). The suggested advantage of the redox active motif in MtrC that forms a flavoprotein complex under anaerobic conditions is that it could allow rapid transition from an aerobic to an anaerobic lifestyle pointing towards transcriptionally independent ways of modulating redox pathways (Edwards et al. 2015).

Under high pressure, we can infer a negative volume change occurring when the outer membrane cytochromes become oxidised upon reducing Fe (III); therefore this reaction is likely to be stimulated by high pressure (Fig. 4.3). The ΔV for mammalian c-type cytochrome oxidation is around $-27\ cm^3\ mol^{-1}$ at ambient pressure and around $-23\ cm^3\ mol^{-1}$ at 100 MPa. Taking into account the ΔV for Fe (III) reduction of around $10\ cm^3\ mol^{-1}$, it likely that the net ΔV for biological Fe (III) reduction via c-type cytochromes is negative. This is because of the larger negative volume change associated with cytochrome oxidation relative to that of the iron reduction (Fig. 4.3). Experimental data is lacking to make this assertion with

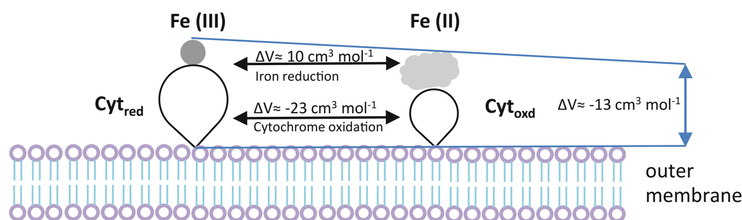

Fig. 4.3 Theoretical volume changes relevant to microbial Fe (III) reduction. The oxidation of cytochrome c can reasonably be assumed to undergo a negative volume change upon releasing an electron to Fe(III). Conversely, upon accepting an electron Fe(III) undergoes a positive volume change. Overall, the terminal step in EET undergoes a negative volume change negative because ΔV for cytochrome c has a greater magnitude than that of Fe(III) reduction

confidence although it appears to be supported by at least one modelling study of benthic environments (Fang et al. 2010). Assuming that a similar pressure-induced positive shift in midpoint redox potential as observed for mammalian cytochrome c also occurs in the membrane bound cytochromes of *Shewanellacea*, then more energy would be available to cells respiring Fe (III) under pressure. This is because the energy available to the cell is proportional to the difference in redox potential of the substrate and that of the terminal cytochrome. However, since the pressure response of the redox potential of Fe (III) minerals will more stable than cytochromes the electron transfer rate will slow as the redox potential of the cytochrome and the Fe (III) terminal electron acceptor eventually converge. Precise midpoint potentials are difficult to assign to Mtr cytochromes since the multiple redox centres mean they are active over a redox potential spanning 250 mV. Assuming the pressure response in terms of redox potential of *Shewanellaceae* cytochrome c is similar to that observed in mammalian equivalents (a positive shift of 20 mV at 100 MPa) (Giovanelli et al. 2004) we can infer that pressure is not likely to have a large effect on the kinetics of Fe (III) reduction at high pressure.

However, changes in membrane composition and, to a lesser degree, conformational protein changes would be expected to disrupt the precise spatial arrangements of the Mtr pathway observed at ambient conditions. This spatial disruption could be overcome biochemically by the maintenance of lipid raft arrangements—localised areas of lipids having a specific, an presumably desirable, fluidity that favour a particular biochemical function (Usui et al. 2012). It could also be the case that the spatial arrangement is inconsequential or even favourable with a subtle shift bringing another of the many redox-active haem centres into service. Such passive ways of responding to pressure are consistent with the idea put forward by Edwards et al. (Edwards et al. 2015) of a similar passive cytochrome based response of *Shewanella* to changing oxygen concentrations. This does not explain the conserved functional redundancy observed in the Mtr pathway however and the need for numerous paralogs for each component of the Mtr pathway (Edwards et al. 2015).

Indeed, changes in pressure may not happen as quickly as changes in the redox conditions in the deep biosphere so a rapid coordinated response to pressure may

not be necessary. Based on documented changes in fatty acid expression in *Shewanella*, the spatial hydrophobic distribution of the membrane at elevated pressure would be expected to be different to that at ambient conditions. Evidence for pressure adapted porins in *P. profundum* SS9 exists, a response that may be to maintain a desirable spatial arrangement of the membrane inserted porin. The β-barrel paralogs in *S. oneidensis* MR1 differ in predicted size by around 55 amino acid residues. The two β-barrel paralogs that have demonstrated functionality in the Fe (III) reducing unit of *S. oneidensis*, MtrC and MtrE, are slightly larger (687 and 712 a.a respectively) than dmsF (662 a.a) and SO4359 (652 a.a)—the two putative β-barrel paralogs with hitherto unknown function in *S. oneidensis* MR1. It may be that these paralogs are expressed under pressure and that they have an organisational function in pressurised Mtr pathways. Chikuma et al. (2007) observed a high pressure respiratory component in *S. violacea* that may be membrane dependent (Chikuma et al. 2007). A systematic transcriptional analysis of *Shewanellacea* under pressure would be immensely useful in exploring such phenomena.

An additional aspect of the pressure response of EET concerns the expression of nanowires, which are thought to be membrane extruded pilus type structures (Pirbadian et al. 2015). Flagella expression is particularly sensitive to pressure and can be inhibited at pressures as low as 10 MPa. Drawing once again on observations of *P. profundum* SS9 which expresses a pressure dependent lateral flagella under elevated pressure and a polar flagellum under ambient conditions, it is likely that cell appendages exhibit specific pressure adaptations. The same lateral flagellar arrangement as on SS9 has been observed on *Shewanella benthica* DB21m2-2 (Fang et al. 2010). Pili expression has not been studied under high pressure but owing to the superficial structural similarities of type IV pili to some flagella it may be that this appendage requires specific pressure adaptations too. Recent evidence suggests that conductive nanowires in *Shewanellaceae* are not proteinaceous pili or flagella like appendages as previously thought, but rather extensions of the membrane (Pirbadian et al. 2015; Malvankar et al. 2015). To date, all studies on the conductive nature of bacterial nanowires have been under atmospheric pressure.

Finally, an unrelated high pressure study of a conductive film determined the ΔV to be negative for electron hopping conductivity (of the type that us which is thought to occur between adjacent carriers of sequentially increasing potential) and that diffusive conductivity was determined to have a positive activation volume suggesting that electron hoping would be the preferred conductive mechanism at high pressures. Again, high pressure studies may prove useful to experimentally describe mechanisms of electron transfer at high pressures (Cruanes 1995).

4.5 Experimental Studies of EET Under High Pressure

Few studies have been conducted on EET at high pressure. A study by Wu et al. (2013) with *S. piezotolerans* at pressures ranging from 0.1 to 50 MPa showed that the Fe (III) reduction rate decreased with pressure in a linear fashion and predicted

that Fe (III) reduction would terminate at 68 MPa (Wu et al. 2013). Picard et al. (2011) have shown a similar trend for Se (IV) and Fe (III) reduction at high pressures with *S. oneidensis* MR1; these reactions appeared to terminate at 150 MPa for Se (IV) and at 100 MPa for Fe (III) respectively (Picard et al. 2011, 2012). Additionally, a Fe (III) reduction rate maxima at 30-40 MPa was observed. In a similar study but with S. *profunda* LT13a Picard et al. (2015) showed Fe (III) reduction proceeding until about 110 MPa although the study was carried out using two techniques each with different starting inoculum densities (Picard et al. 2012). In one experiment with a 'low' staring inoculum (10^8 CFU ml^{-1}) a linear decrease in Fe (III) reduction rate was observed which terminated above 50 MPa. With an higher initial cell density (10^9 CFU ml^{-1}), the Fe (III) reduction rate did not appear linear but was instead maintained in steps between 0–40 and 60–80 MPa and continued to around 100 MPa (Picard et al. 2014). This stepping is consistent with different Mtr modules being deployed over a range of pressures; this hypothesis could be tested by repeating the experiment and extending it with transcriptomic data.

These pioneering experiments have shown the magnitude and limits of Fe (III) reduction at elevated pressure and will undoubtedly be improved in future iterations with more mindful consideration of thermodynamic considerations and with more sophisticated experimental design. One of the main problems with these data is, on account of their rarity, there is little to compare findings with and the absence of standardisation between techniques. Accordingly, the observed reduction rates, for Fe (III) at least, vary over an order of magnitude from between c.a. 80–1500 μMol h^{-1}. Additionally, for each of these experiments, it is very difficult to decouple the effect of pressure induced killing from the observed reduction rates and relate them to in situ conditions. This is due to experimental constraints and the fact that the measurements relied on extremely high inoculum densities of between 10^7 and 10^9 CFU ml^{-1} and therefore growth would not be expected in these circumstances and Fe (III) reduction could therefore only really be correlated with cell maintenance and death. In all experiments, dissimilatory metal reduction proceeded without any delay. This observation was explained by the fact that the respiratory chain was not affected by pressure. However, evidence suggests that the respiratory chains of *Shewanellaceae* are pressure sensitive and that a genetic response would be expected over the experimental pressure ranges reported (Bartlett 2002; Chikuma et al. 2007). It remains to be seen if the application of pressure to actively growing cultures would yield different Fe (III) reduction phenomena in the *Shewanellacea* family.

4.6 Horizons in the Study of High Pressure EET

Apart from the cumbersome nature and expense of high pressure equipment, one of the main problems in traditional high pressure microbiology is the difficulty of continuously monitoring cultures. To access samples grown in traditional pressure

vessels to perform measurements, the pressure vessel needs to be depressurized and opened periodically (Picard et al. 2011). This is less than ideal, as it introduces at least two unknowns into the system: (1) what happens upon depressurisation until the time of the measurement, and (2) what would the state of system be at a given point had it not been subject to continual depressurisation and repressurization events during the experimental run (Picard et al. 2011). Given enough pressure vessels individual vessels can simply be sacrificed at each time point, which while helpful, offers only a partial solution to these issues (Wu et al. 2013) (Fig. 4.4).

Two main experimental systems have been reported which allow direct measurements on pressurised cells, the diamond anvil system and an autoclave system. Both are optically accessible, the diamond anvil system by virtue of the natural properties of diamond and the autoclave system because it can be fitted with a beryllium window (Picard et al. 2011). The technique for assessing these systems has been based on X-ray Absorption Near-Edge Structure (XANES) spectroscopy, which although immensely useful, has some drawbacks. XANES requires use of a synchrotron, and beam-time is expensive. Furthermore, the radiation energy that is generated using XANES can kill bacteria, which means that continuous monitoring is not advisable. Both the diamond anvil and the autoclave system have limited experimental volumes, in the low nL and mL range respectively (Picard et al. 2014).

A recent advance at the Singapore Centre for Environmental Life Sciences Engineering that will likely prove essential in high pressure microbiology studies, in particular to study high pressure EET, is the development of a high pressure bioelectroanalytical system.

High pressure electrochemistry is a century old field, high pressure bioelectroanalytical systems are built on this experience (Giovanelli et al. 2004). They combine a compressible bioelectrochemical cell that is simply put inside a

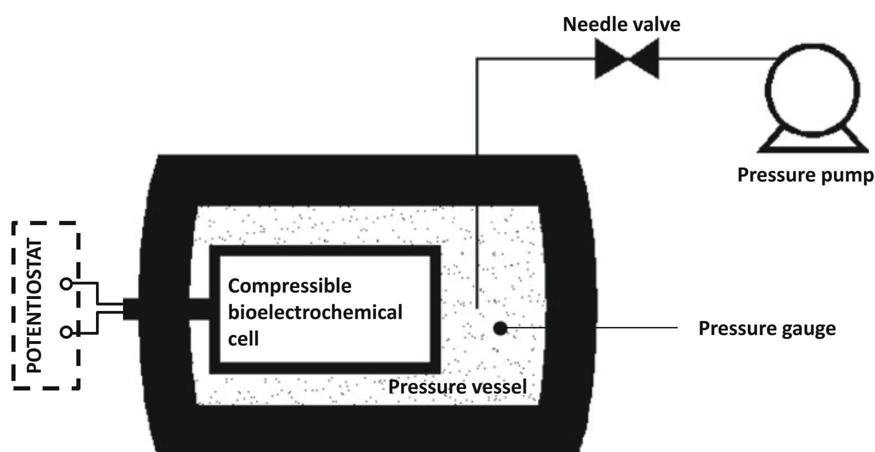

Fig. 4.4 A schematic of a high pressure bioelectrochemical reactor

traditional pressure vessel with wires passing through sealing glands in the pressure vessel wall. The compressible bioelectroanalytical cell contains either two or three electrodes and can be connected to a potentiostat, a power source or a data logger. Potentiostatic control poises the electrodes at a given potential allowing a number of amperometric, voltammetric or impedimetric analyses to be performed. Early experiments have already demonstrated a shift in redox potential at high pressure that is thought to be related to outer membrane cytochromes.

High pressure bioelectroanalytical reactors have several advantages over existing techniques. Firstly, they allow continuous, real time monitoring of microbes over durations only limited by an experimenter's budget. Several quantitative techniques, such as cyclic voltammetry or differential pulse voltammetry, can be applied in situ. The reactors are sufficiently low cost that replication can be achieved and they are simple enough they can be used in both batch and continuous flow pressure systems (Foustoukos and Pérez-Rodríguez 2015). Finally, volumes up to one litre are practical for most laboratories allowing predetermined volumes to be collected for ex situ analyses like proteomics and lipidomics. As well as fundamental questions about EET, the reactors could monitor growth and other microbial phenomena continuously and in real-time, not just of electrogenic taxa like *Shewanellaceae* or *Geobacteraceae* but, with the careful use of redox mediators, other genera such as *Photobacterium profundum*. Over the coming years these systems will be applied to explore the following:

(1) The nature of conductive nanowires and the mechanism of conductivity at high pressures
(2) The state of the membrane at high pressure using lipidomic techniques
(3) The transcriptomic response of EET under pressure including differential expression of the components of the Mtr pathway
(4) The volume changes of biologically important reactions such as Fe (III) reduction
(5) Data for a full thermodynamic analysis of EET systems under environmentally relevant conditions
(6) Performance of microbial fuel cells at high pressure
(7) High pressure biocorrosion in the deep sea.

4.7 Conclusions

There are a number of open questions in the field of microbial EET. With the development of new tools, some of which are at a stage where they can be productively used, we are now in a position to address these questions. This will give not only more insight in the effect of (high) pressure on the ecophysiology of bacteria and archaea in the deep subsurface in general and of EET in particular, but also result in a better understanding of EET at atmospheric pressure conditions too.

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Chapter 5
Metagenomics of Hyperthermophilic Environments: Biodiversity and Biotechnology

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Abstract The field of thermophilic microbiology was born in the late 1970s with the pioneering work of Brock (Thermophiles biodiversity, ecology, and evolution. Springer, Boston, pp. 1–9, 2001) and dramatically expanded through the '80s with the isolation of hyperthermophiles by Stetter (FEMS Microbiol Rev 18:149–158, 1996). The development of SSU rRNA phylogenetics revealed the complexity and diversity of prokaryotic phylotypes on biotopes widely differing in extreme conditions (e.g. spanning gradients of pH between 0 and 10 and temperatures from 60 °C to over 120 °C, respectively). Sites of volcanic activity all over the Earth's surface and under the sea provide a variety of different environments for extremophilic microorganisms. Hot springs populated by hyperthermophiles (T_{opt} > 65 °C), the majority of which belonging to the domain of Archaea, are very diverse and some of them show combinations of other extreme conditions, for example, acidic, alkaline, high pressure, and high concentrations of salts and heavy metals (Cowan et al. in Curr Opin Microbiol 25:97–102, 2015). Archaea inhabiting hot springs are considered to be the closest living descendants of the earliest living forms on Earth and their study provide insights into the origin and evolution of life (Woese et al. in Proc Natl Acad Sci USA 87:4576–4579, 1990; Olsen et al. in J Bacteriol 176:1–6, 1994). As with all studies of environmental microbiology, our understanding of the function of (hyper)thermophilic microbial consortia has lagged substantially behind. However, recent advances in 'omics' technologies, particularly within a system biology context, have made significant progresses into the prediction of in situ functionality (Cowan et al. in Curr Opin Microbiol 25:97–102, 2015). Most extremophilic microorganisms are recalcitrant to cultivation-based approaches (Amann et al. in Microbiol Rev 59:143–69, 1995; Lorenz et al. in Curr Opin Biotechnol 13:572–577, 2002); therefore, culture-independent metagenomic strategies are promising approaches to assess the phylogenetic composition and functional potential of microbial communities living in extreme environments (López-López et al. in Life 3:308–320, 2013). In addition, these approaches

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implement tremendously the access to enzymes from (hyper)thermophilic microorganisms that have important potential applications in several biotechnological processes. We report here on the state-of-the-art of the metagenomic surveys of different hot springs (T > 65 °C) (Table 5.1) and on the recent advance in the discovery of new hyperthermostable biocatalysts of biotechnological interest from metagenomic studies of these extreme environments.

5.1 Yellowstone National Park

The Yellowstone geothermal complex in the Yellowstone Caldera area is one of the most studied geothermal locations that includes more then 10,000 thermal sites such as geysers, mud pools, hot springs and vents showing a broad range in pH, temperature and geochemical properties. All of these conditions make of the entire geothermal area a natural laboratory for the study of the hyperthermophilic microbial communities since 1953 shedding light on their evolution, metabolic potential and adaptation to high-temperature environments (Marsh and Larsen 1953).

In 2005, Meyer-Dombard and co-workers reported a detailed environmental and microbiological survey of three different hot springs in Yellowstone National Park (YNP): Obsidian Pool (ObP) (80 °C, pH 6.5), Sylvan Spring (SSp) (81 °C, pH ~ 5.5), and Bison Pool (BP) (83 °C, pH ~ 8.0) (Table 5.1) (Meyer-Dombard et al. 2005). By the analysis of 16S rRNA genes, they reported that Thermocrinis, followed by Geothermobacterium and different proteobacteria, dominate the bacterial community in ObP and BP. In addition, the archaeal community was mostly composed by different groups of uncultured Crenarchaeota in ObP, while several members belonging to family Desulfurococcaceae were found in BP. By contrast, in SSp they identified *Hydrogenothermus* as the most abundant bacterial genus and, among the archaea, observed the dominance of the families Desulfurococcaceae and Thermoproteaceae (Meyer-Dombard et al. 2005).

A first wide metagenomic survey of the microbial species in the YNP was addressed by Inskeep and co-workers in 2010 (Inskeep et al. 2010) aiming to identify the predominant microbial populations of five geochemically different high-temperature environments, their metabolic potentials and the genes probably related to the different geochemical conditions across these sites (Table 5.1). Crater Hills (CH) (75 °C, pH 2.5) is a turbid acidic pool with dissolved oxygen lower than 3 μM, low concentrations of dissolved H_2S, H_2 and CH_4 and containing particles in suspensions basically composed by elemental S and SiO_2. The acidic sample collected in Norris Geyser Basin (NGB) (65 °C, pH 3.0) from an oxygenated outflow channel is rich in FeIII-oxides and showed dissolved O_2 concentration in a range of 30–100 μM. Previous studies report that NGB, as well as CH, contain significant numbers of Crenarchaea of the order Sulfolobales (Inskeep et al. 2005; Young et al. 2005; Kozubal et al. 2008). Joseph's Coat (JCHS), Calcite (CS), and Mammoth Hot Springs (MHS) are all sulfidic hot springs with pH > 6.1 and lower concentration of dissolved O_2, which differ geochemically from one another and show significant

Table 5.1 State-of-the-art of the metagenomic surveys of different hot springs

Abbreviation	Location	Coordinates	Temperature (°C)	pH	Dominant microorganisms	References
ObP	Yellowstone National Park, USA	44.60302 N, 110.86519 W	80	6.5	Aquificales (*Thermocrinis*) Uncultured Crenarchaeota	Meyer-Dombard et al. (2005)
SSp		44.69886 N, 110.76827 W	81	~5.5	Aquificales (*Hydrogenothermus*) Desulfurococcaceae–Thermoproteaceae	
BP		44.56969 N, 110.86519 W	83	~8.0	Aquificales (*Thermocrinis*) Uncultured Crenarchaeota	
DS		42.46313 N, 110.82983 W	68–72	3.1	Aquificales (*Hydrogenobaculum*)	Inskeep et al. (2013b), Beam et al. (2014), Takacs-Vesbach et al. (2013)
OSP_14		44.73304 N, 110.70899 W	72–74	3.5	Aquificales (*Hydrogenobaculum*)	Takacs-Vesbach et al. (2013), Inskeep et al. (2010), (2013b)
MHS		44.96942 N, 110.70983 W	70–72	6.5	Aquificales (*Sulfurihydrogenibium*)	Inskeep et al. (2010), (2013b), Takacs-Vesbach et al. (2013)
CS		44.90485 N, 110.40402 W	74–76	7.8	Aquificales (*Sulfurihydrogenibium*)	Inskeep et al. (2013a, b), Takacs-Vesbach et al. (2013)
OS		44.53401 N, 110.79781 W	80–82	7.9	Aquificales (*Thermocrinis*)	Schoenfeld et al. (2008), Pride and Schoenfeld (2008), Takacs-Vesbach et al. (2013)
BCH		44.2829 N, 110.9053 W	80–82	7.8	Aquificales (*Thermocrinis*)	Inskeep et al. (2010), (2013b), Takacs-Vesbach et al. (2013)
CH		44.65328 N, 110.48474 W	76	2.6	Crenarchaeota (*Sulfolobales*)	Inskeep et al. (2013a, b), Menzel et al. (2015)

(continued)

Table 5.1 (continued)

Abbreviation	Location	Coordinates	Temperature (°C)	pH	Dominant microorganisms	References
NL		44.75232 N,	88–92	3.0–4.0	Crenarchaeota (*Sulfolobales*)	Inskeep et al. (2013a), Menzel et al. (2015) Bolduc et al. (2012)
MG		44.72429 N, 110.70561 W	70–80	4.0	Crenarchaeota (*Desulfurococcales*, *Thermoproteales*)	Inskeep et al. (2013a, b)
CIS		44.72308 N, 110.70402 W	78–80	4.4	Crenarchaeota (*Desulfurococcales*, *Thermoproteales*)	Inskeep et al. (2010), (2013a, b)
JCHS		44.73916 N, 110.32449 W	80	6.1	Crenarchaeota (*Desulfurococcales*, *Thermoproteales*)	Inskeep et al. (2013a, b)
WS		44.76489 N. 110.43035″ W	76	6.4	Crenarchaeota Aquificales	Inskeep et al. (2013a, b)
OSP_8		44.73304 N, 110.70899 W	72	3.4	Crenarchaeota Mixed Novel Archaeal	Inskeep et al. (2013a, b)
NGB		44.7315 N, 110.71136 W	65	3.0	Crenarchaeota (*Sulfolobales*)	Inskeep et al. (2010)
Is2-5S	Iceland	64.03167N, 21.1966 W	85–90	5.0	Aquificales (*Thermocrinis*) Proteo bacteria	Menzel et al. (2015)
Is3-13		63.90416 N, 22.05805 W	90	3.5–4.0		
Kam37	Kamchatka	54.50 N, 159.97 E	81–85	5.5–7.4	Aquificales Euryarchaeota Crenarchaeota	Eme et al. (2013), Wemheuer et al. (2013)
Not assigned		52.453 N, 158.195 E	70	3.5–4.0	Miscellaneous Crcnarchacotic Group	Wemheuer et al. (2013)

(continued)

Table 5.1 (continued)

Abbreviation	Location	Coordinates	Temperature (°C)	pH	Dominant microorganisms	References
					Enterobacteriaceae Miscellaneous Crenarchaeotic Group	
AI	Furnas Valley, Azores	37.7725 N, 25.303889 W	51	3.0	Proteo bacteria	Sahm et al. (2013)
AII			84	2.5–3.0	*Not reported*	
AIII			85	8.0	*Not reported*	
AIV			92	8.0	Aquificae, Dictyoglomi	
BII			65	7.0	*Not reported*	
BIII			70	7.0	*Not reported*	
CII			76	8.0	*Not reported*	
SK	Perak, Malaysia	3.99663 N, 101.39310 E	80	8.0	Firmicutes	Chan et al. (2015)
Dgg	Hot Springs of Rehai region in Tengchong, China	24.95344 N. 98,43780 E	84.5	7.2	Aquificae	Hou et al. (2013)
Drty-l		24.95396 N, E98.43829E	85.1	2.6	Crenarchaeota	
GmqS			93	9.3	Aquificae	
GmqC		24.57060 N, 98.43615 E	89	9.4	Aquificae (water) Crenarchaeota (sediment)	
GmqP			82.5	9.3	Aquificae Aquificae (water)	
JmqL		24,95112 N, 98 43600 E	93.6	9.2	Crenarchaeota (sediment)	

(continued)

Table 5.1 (continued)

Abbreviation	Location	Coordinates	Temperature (°C)	pH	Dominant microorganisms	References
JmqR		24.95115 N, 98 43596 E	83.2	9.4	Aquificae	
Zzq			89.1	4.8	Crenarchaeota	
HtjL		24.95089 N, 98 43664 E	90	8.1	Crenarchaeota Crenarchaeota	
HtjR			92.3	8.0	Aquificae	
SrbzU		24.95002 N, 98.43728 E	79.8	8.0	Aquificae	
SrbzD			78.2	8.3	Aquificae	
Gxs	Hot Springs of Ruidian region in Tengchong, China	25.44012 N, 98.44081 E	73.8	7.3	Bacteria	Hou et al. (2013)
Jz		23.44138 N 98.46004 E	81.6	6.7	Bacteria Crenarchaeota	
CPc	Taupo volcanic zone, New Zealand	38.3591 S, 176.36990 E	75	5.8	Aquificales (*Sulfurihydrogenibium*)	Hug et al. (2014)
CPp			75	5.5		
CPr			68	5.5		
AP			45	6.9		
It6	Phlegraean Fields, Italy	40.82690 N. 14.13914 E	76	3.0	Proteobacteria (*Acidithiobacillus*)	Menzel et al. (2015)
It3		40.82920 N, 14,14712 E	86	5.5	Crenarchaeota (*Acidianus*)	

differences in the microbial communities. The sediment sampled at JCHS (80 °C, pH 6.1) is rich of sulfides and elemental S while the aqueous phase contains high concentration of CH_4, H_2, NH_4^+, arsenite and thiosulfate showing several reduced chemical species that could serve as electron donors for the chemolithotrophic metabolism with low concentration of O_2. CS (75 °C, pH 7.8) and MHS (71 °C, pH 6.6) samples were collected from high-velocity, highly-sulfidic outflow channels and have been reported to be dominated by microorganisms of the order Aquificales (Fouke et al. 2000, 2003; Reysenbach et al. 2005). Using binning and fragment recruitment approaches, Inskeep and collaborators observed that high-temperature springs with acidic pH were dominated by Archaea with distantly related organisms whose genomes have been sequenced and that can be assigned to the orders Sulfolobales (CH and NGB) and Thermoproteales (JCHS). Moreover, while a significant number of contigs were assigned to novel populations of Desulforococcales in all the three archaeal-dominated sites, just a modest number of sequences were assigned to Sulfolobales in JCHS. These results suggest that the members belonging to the class Thermoprotei are common in the archaeal communities of YNP and that their relative abundance is modulated by differences in pH and/or by the concentration of dissolved O_2. Conversely, Bacteria belonging to the order Aquificales dominated the two microbial communities inhabiting CS and MHS with pH > 6.0. In particular, about 90% of the reads found in MHS showed a nucleotide identity greater than 90% with *Sulfurihydrogenibium* sp. Y03AOP1 isolated in Obsidian Pool (Reysenbach et al. 2009) while the reads obtained from CS were assigned with high identity to *Thermus aquaticus* and Sulfurihydrogenibium yellowstonensis.

The more recently presented “YNP metagenome project” (Inskeep et al. 2013b), comparing metagenomes and geophysical parameters of 20 different geothermal sites in the YNP, represents one of the most complete studies of the communities populating high-temperature environments. Thirteen of them with T > 65 °C have been grouped in two different ecosystem types based on primary environmental factors, such as pH, temperature, presence of dissolved sulfide and elemental S, and additional physiographic parameters. The first ecosystem groups six sites populated by Aquificales-rich “filamentous-streamer” communities (Table 5.1): Dragon Spring (DS; 68–72 °C, pH 3.1); 100 Sping Plan (OSP_14; 72–74 °C, pH 3.5), Octopus Spring (OS; 74–76 °C, pH 7.9) and Bechler Spring (BCH; 80–82 °C, pH 7.8) together with MHS and CS described above (Takacs-Vesbach et al. 2013; Inskeep et al. 2013b). Takacs-vesbach and co-workers, by using a phylogenetic and functional analysis of the metagenomic samples from the “filamentous-streamer” communities, reported three lineages of Aquificales with a potential metabolism related mainly to pH and sulfide and/or elemental sulfur. DS and OSP_14, showing low-pH (pH 3.1–3.5) and high-sulfide concentration, contained *Hydrogenobaculum* spp., whereas higher-pH sites were dominated by *Sulfurihydrogenibium* spp., as in MHS and CS, or *Thermocrinis*-like populations, as in OS and BCH. Among these sites, CS is the only one in YNP hosting *Thermocrinis*-like population together with *Sulfurihydrogenibium* spp. as expected by previous 16S rRNA surveys that reported only minor overlap in the distribution of different Aquificales species across the

YNP geothermal environments (Reysenbach et al. 2005; Hall et al. 2008; Hamamura et al. 2009). In addition, the authors used an automated phylogenomic inference pipeline for bacterial sequences to detect the number of single-copy genes and to evaluate the sequence heterogeneity within the Aquificales populations. In this way, it was observed that although the community from MHS was dominated by a quite homogeneous population of *Sulfurihydrogenibium* spp., the sites OS, BCH and CS, carrying higher numbers of single-copy genes, showed greater heterogeneity of *Thermocrinis*-like populations in situ. Differently, the acidic sites DS and OSP_14 contained, together with the primary populations *Hydrogenobaculum* spp., different archaeal populations such as *Metallosphaera*-like, Thaumarchaeota and Thermoplasmatales-like, consistently with the analysis reported by Pride and Schoenfeld (2008), Schoenfeld et al. (2008). In particular, although both the higher-pH sites OS and BCH with low-sulfide concentration (<1 μM) contained similar *Thermocrinis*-like Aquificales, Takacs-vesbach reported that the microbial community of OS, which contains at least three additional novel bacterial assemblies related to unclassified 16S rRNA genes described previously (Reysenbach et al. 1994; Blank et al. 2002; Hall et al. 2008), is considerably different if compared to BCH populations. Even though, the inorganic constituents of these two springs were similar enough. On these bases, the authors suggested that the observed differences across these apparently similar sites could be addressed to additional geochemical and geophysical factors, such as the total dissolved organic carbon (DOC) and the amount of solid-phases of carbon, that have not been deeply characterized in their study and that could play a key role in the determination of the influence of the organic constituents on the microbial community structure (Takacs-Vesbach et al. 2013). The linkages among geochemistry, specific phylotypes and their metabolisms have been accounted by the global protein analysis of the "streamer communities" with TIGRFAMs specific to electron transport coupled with principal component analysis (PCA) and hierarchical clustering (HC). Focusing on the geochemistry of the community (in particular pH and sulfide content) Takacs-vesbach indeed observed that the samples from DS (sulfur rich) and OSP_14 (FeIII-oxide rich), although contain a quite similar *Hydrogenobaculum* population, show different archaeal co-communities composed by anaerobic/microaerophilic populations and oxidizing Sulfolobales in DS and OSP_14 sites, respectively. These primary differences among the populations of the sites reflect specific metabolic pathways and are strictly related to the different geochemical habitats. In particular, by the metagenomic sequence of the Aquificales, especially in the *Hydrogenobaculum* and *Sulfurihydrogenibium*-like organisms present in YNP, Takacs-vesbach and colleagues observed a variety of S-oxidation pathways coupled with cbb3-Type C heme-copper oxidases (HCOs) or bd-ubiquinol terminal oxidase complexes, indicating a clear potential for the oxidation of reduced sulfur species. By contrast, they observed that the *Thermocrinis* organism, present in the oxic samples from OS and BCH, contain Type A-HCOs, indicating a functional divergence from the other Aquificales genera, which have copies of both the HCOs type (Takacs-Vesbach et al. 2013).

The second ecosystem in the "YNP metagenome project" includes seven archaeal-dominated sediments (Table 5.1): Nymph Lake (NL; 88 °C, pH 4), Monarch Geyser (MG; 78–80 °C, pH 4.0), Cistern Spring (CIS; 78–80 °C, pH 4.4), Washburn Spring (WS; 76 °C, pH 6.4), 100 Spring Plan (OSP_8; 72 °C, pH 3.4), and including CH and JCHS described above (Inskeep et al. 2013a, b). By following the approach applied for the analysis of the Aquficales-dominated sites, the authors have investigated in detail the thermal sites (70–85 °C and pH range 2.5–6.4) that represent some of the major chemotrophic habitats in YNP (Inskeep et al. 2013a). As for the Aquificales "filamentous-streamer" communities, the archaeal-dominated sites were classified primarily on the base of pH and the presence of dissolved sulfide and elemental sulfur, while the temperature was not taken as a major variable. The abiotic consumption of oxygen by reduced sulfur contributes to the hypoxic conditions observed in CH, NL, MG, JCHS, CIS, and WS. The authors observed that pH was a major factor controlling the distribution of Sulfolobales *versus* Thermoproteales and Desulfurococcales. The combination of acidic pH, reduced sulfur and high-temperature acts as severe constrain on the microbial community diversity; indeed, CH and NL sites with these properties were dominated by only two major Sulfolobales populations. The acidic NL site, together with CH previously described, is a highly turbulent pool that contains suspended particles of elemental sulfur and SiO_2, and low concentration of dissolved sulfide (<5 μM). According to Menzel and collaborators, which analyzed CH and NL sites (Menzel et al. 2015), CH is almost exclusively populated by Archaea with 96.4% of reads (Roche/454 Titanium FLX), which are mostly Sulfolobaceae (85%) made up of *Sulfolobus* and *Acidianus* species. Only 0.2% of the CH reads have been assigned to bacterial species such as *Hydrogenobaculum* spp. and *G. thermoleovorans*. By contrast, the same authors report that NL site shows a higher abundance of Bacteria (12.4% of reads assigned), prevalently Aquificae, and just 58% of reads are assigned to Archaea. As well as CH, the most abundant archaeal family in NL was Sulfolobaceae (32%), followed by Thermoproteales and Acidobales (9 and 2% respectively). In addition, an elevated abundance of archaeal viruses (21% of reads) was found, consistent with those reported previously by Bolduc and co-workers: this was the first time that the presence of putative RNA viral genomes in high-temperature acidic hot springs was found by using a metagenomic approach (Bolduc et al. 2012).

Inskeep et al. reported that also the sulfur sediments from MG, CIS, JCHS, with pH between 4.0 and 6.0, contain a variable amounts of sequence assigned to Sulfolobales populations while the Fe-oxide mats of the sample OSP_8, together with the Fe-oxide "streamer" community in OSP_14, were the only sites with sequences assigned to *M. yellowstonensis*-like populations (Inskeep et al. 2013a). They observed that the main sequence clusters of MG, CIS and JCHS were related to members of the orders Desulfurococcales and Thermoproteales corresponding to *Caldivirga/Vulcanisaeta*-like and *Pyrobaculum*-like organisms. On the basis of nucleotide word frequencies and principal component analysis (NWF PCA), sequence similarity, and GC content, similar populations were also observed in the

non-sulfidic sample OSP_8. This suggests that, although these represent the main community members detected in sulfur-rich sediments from pH 4.0 to 6.0 (Jay et al. 2011) they inhabited also Fe-oxide mats in OSP_8 where sulfide and elemental sulfur are absent. In addition, at last four major archaeal populations were identified in OSP_8: *M. yellowstonensis*, *Vulcanisaeta* spp., *Acidilobus* spp. and a "novel archaeal Group I" (NAG1) (Inskeep et al. 2013a). Regarding the latter, successively Kozubal and colleagues proposed that, representing the most abundant member of the community in OSP_8 with about 20–55% of the total sequence reads, it was a new phylum named Geoarchaeota. The abundance of Geoarchaeota in OSP_8 allowed Kozubal and colleagues to obtain a contig coverage of average ~6x, and a total scaffold length of ~1.7 Mb in only eight scaffolds (Kozubal et al. 2013). Moreover, Inskeep and colleagues, reported that the microbial community of OSP_8 is composed also by several less abundant Archaea including relatives of the Euryarchaeota, Nanoarchaeota, and Crenarchaeota (as other Sulfolobales) (Inskeep et al. 2013a). In addition to these phyla, in the oxic iron mat from OSP_8, Beam and co-workers identified a new candidate phylum of Thaumarchaeota showing a different respiratory machinery respect to the Thaumarchaeota population present in the hypoxic sulfur sediments of DS sample (Beam et al. 2014).

JCHS sites showed evidence of subdominant bacterial populations and do not contain significant numbers of methanogens, while the sediment from WS (80 °C, pH 6.4) contained a significant amounts of sequences related to Bacteria. Inskeep reported that they were more diverse and composed mainly by *Sulfurihydrogenibium* and *Thermodesulfobacteria*-like (~15 and 10% of the assigned reads respectively). Instead, several Thermoproteales populations composed the WS archaeal community, and <1% of the sequences are related to Methanococci and/or Methanosarcinales, which is consistent with the increased abundance of these phylotypes at high pH. In addition, they reported that the PCA of the sequences from these samples, specific to electron transport TIGRFAMs, highlighted the differences in OSP_8 *versus* WS compared to the other sites. They suggest that these differences could be ascribed to the higher abundance of bacterial pathways in WS (*Thermodesulfobacteria*, *Sulfurihydrogenibium*), showing respiratory processes considerably different than the dominant Archaea present in other sites (Inskeep et al. 2013a). Moreover, it was found that WS was the only habitat containing a significant population of Korarchaeota. This result is in line with recent studies on the distribution of korarchaeotal sequences in Kamchatka and YNP showing that these organisms have a narrow pH range of growth (<5.0–7.0) (Auchtung et al. 2011; Miller-Coleman et al. 2012).

The YNP project and the related studies represent a remarkable effort toward understanding the relationship between the microbial community structures and the metabolic potential across the different extremely hot environments in the Yellowstone Caldera. The systematic selection of geochemically distinct sites indeed provides a real guidebook to link specific phylotypes with peculiar physico-chemical properties of the different habitats.

5.1.1 Iceland

Iceland has a high concentration of active volcanoes due to its location on the mid-Atlantic Ridge, a divergent tectonic plate boundary. The island has 30 active volcanic systems most active/volatile. Among these sites, Menzel and coworkers have analyzed through metagenomic approach two sites: Krísuvík and Grensdalur. The total genomic DNA was extracted from both samples (sediment/water), sequenced by Illumina Hiseq and analyzed with MEGAN (Huson and Weber 2013) for estimating species abundances (Table 5.1).

The first site, Krýsuvík (Is3-13), is situated on the Reykjanes peninsula and consists of several geothermal fields, such as Seltún, where solfataras, fumaroles, mud pots and hot springs have formed and the soil is colored in bright yellow, red, and green hues. Is3-13 (90 °C, pH 3.5–4.0) is surrounded by other hot springs and shows very limited access to organic materials. The second analyzed site is the volcano Grensdalur (Is2-5S), with an air distance from Krísuvík of about 45 km. This hot spring (85 °C and pH 5.0) was on a slope of a hill with flow through from other hot springs higher up and the sediment is dark gray and very fine, almost sandy. The surroundings are filled with various organic materials, such as moss and lichen as well as the edges of the spring were encrusted with cyanobacterial or algal mats (Menzel et al. 2015).

From the sample Is3-13 the mapped reads (about 8 millions) were assigned to 79% bacterial and 19.7% archaeal microorganisms. Proteobacteria was the dominant phylum, comprising Gamma- (57%) and Beta-proteobacteria (13%) responsible for nitrogen fixation. Within this phylum, *Acidithiobacillus* (52%) was the most abundant genus. The archaeal community was largely composed of Thermoproteales (13%) hydrogen-sulfur autotrophs, with *Thermoproteus tenax* (12%) being the most abundant species, and Sulfolobales (3%).

In Is2-5S (about 7 millions of mapped reads), Menzel and co-workers reported that almost 33% were assigned to Archaea while 62.1% to Bacteria. These latter were largely comprised of Aquificae (29%), mostly belonging to the species *Thermocrinis albus* (14%) and *Sulfurihydrogenibium azorense* (7%). Crenarchaeota was the dominant archaeal phylum (25%) primarily populated by *Pyrobaculum* genus (13%). The analysis of 16S rRNA predicted from the assembled contigs revealed that many sequences showed > 99% similarity to SILVA database sequences, most of them matching with sequences from species annotated as uncultured (Menzel et al. 2015).

In conclusion, the work of Menzel and colleagues suggests that the community structure is not affected by geographic distance, but by environmental parameters. Indeed, Is2-5S (85 °C/pH 5.0) is similar to the Chinese (65 °C/pH 7.0) and Uzon Caldera in Kamchatka (61–64 °C/pH 5.8–6.0) hot springs and a high overlap between samples can be observed in both archaeal and bacterial components. The sample Is3-13 (90 °C/pH 4.0) is similar to Solfatara volcano from Italy (76 °C/pH 3.0) and both samples have the lowest diversity.

5.1.2 Kamchatka Peninsula

The Kamchatka peninsula, which is located in the Far East of Russia, comprises an area of approximately 472,300 km^2 and is described as the land of fire by its first explorers due to the high density of volcanoes and associated volcanic phenomena. To examine the prokaryotic diversity of the microbial communities in this area, three different metagenomic analyses were performed (Table 5.1) (Eme et al. 2013; Wemheuer et al. 2013). Two samples were from the Central thermal field of Uzon Caldera. The first one is derived from the hot spring referred to as Kam37, characterized by a small bottom opening of 10 × 15 cm with a steady discharge and very little rim overflow, at 85 °C, pH 5.5. The second sample, referred to as Uzon, derived from the same caldera but is 81 °C, pH 7.2–7.4 while the third sample, referred as Mutnovsky, is from a thermal and acidic spring (70 °C, pH 3.5–4.0) at the Mutnovsky volcano.

Environmental DNA for metagenomic analyses was isolated from collected sediment samples by direct cell lysis, and archaeal and bacterial 16S rRNA genes were amplified by PCR and analyzed to assess the prokaryotic community. In particular, Kam37 has been analyzed by constructing a fosmidic metagenomic library and two SSU rRNA gene libraries (one archaeal- and one prokaryotic-specific library). 108 clones obtained with prokaryotic primers and 149 clones from the archaeal-specific library were analyzed. In addition, six selected clones of the fosmid library were sequenced using the 454-pyrosequencing FLX technology, and reads were assembled and annotated. The retrieved 16S rRNA gene sequences were analyzed using QIIME (Caporaso et al. 2010b) to assess the prokaryotic community structure of Uzon and Mutnovsky samples.

The SSU rRNA gene survey of Kam37 suggested that the community was dominated by uncultivated members of the Aquificales, Euryarchaeota, Crenarchaeota, and MCG (Miscellaneous Crenarchaeotic Group), whereas sixteen sequences were similar to Thaumarchaeota, one of the new major archaeal lineages, including putative phyla such as Korarchaeota and Nanoarchaeota. These metagenomic analyses provided the first genomic data from two novel major (hyper)thermophilic archaeal lineages HTC1 and HTC2 (Hot Thaumarchaeota-related Clade 1 and 2, respectively), which represent either an ancient lineage of high-taxonomic rank within Thaumarchaeota. Interestingly, Thaumarcheota, together with Proteobacteria and Thermotogae, dominated also the Uzon and Mutnovsky sites. A study by Meyer-Dombard et al. (2005) investigated the prokaryotic community in three thermal springs in the YNP (the Sylvan Spring, the Bison Pool, and the Obsidian Pool). They have observed that the Sylvan Spring has a low pH of 5.0, whereas the other pools have a rather neutral pH. However, the prokaryotic community structure of this acidic spring was different to that found in the acidic Mutnovsky spring sample. Meyer-Dombard et al. identified the Crenarchaeota as the most abundant archaeal group, whereas Thaumarchaeota were the most abundant group in the acidic Mutnovsky spring sample. Thaumarchaeota are the dominant archaeal group also in hot springs on the Tibetan Plateau (Huang et al. 2011). By contrast, the analysis of the

Uzon sample revealed a more diverse prokaryotic community than in the Mutnovsky sample, being dominated by uncultured members of the MCG and *Enterobacteriaceae*. Despite the geographical separation, the Obsidian Pool and the Uzon Caldera hot spring share a very similar community structure, as almost the same dominant archaeal and bacterial groups were identified, together with the rare phylum of Korarchaeota (Meyer-Dombard et al. 2005). These results confirm also the presumption proposed for other sites that similar extreme environmental conditions result in similar microbial communities (Simon et al. 2009).

5.1.3 Furnas Valley, Azores

Azores is a group of islands of volcanic origin in the Atlantic Ocean, with the main hydrothermal area being the Furnas Valley on the Island of São Miguel. The largest spring is at the highest elevation and is alkaline, whereas some of the lower springs are smaller and more acidic (Brock and Brock 1967). This is in opposition to the displacement in YNP and in Iceland where the higher springs are small and acidic and the lower ones are large and alkaline (Allen and Day 1935; Barth 1950). Sahm and co-workers have analyzed nine sites in the Valley of Furnas, (AI-AIV near Caldeira Do Esgucho, BI-BIII near Caldeirão and CI and CII near Caldeira Asmodeu) with a wide variety of physico-chemical characteristics with a range of temperature between 51 and 92 °C and pH values between 2.5 and 8.0 (Sahm et al. 2013) (Table 5.1). The study was performed through a mixed approach including the analysis of 16S rRNA, both by PCR amplification and metagenomic, fluorescence in situ hybridization (FISH), and denaturing gradient gel electrophoresis (DGGE) to estimate the prokaryotic diversity. The DGGE-profile on all the sites indicates that the pH showed the prominent effect on the microbial complexity in the different samples. Indeed, the lower diversity was observed in combination of extremes of acidic pH and temperature (pH 2.5–3.0 and 84 °C), while the highest microbial diversity was detected with temperatures between 55 and 85 °C and pH values between 7.0 and 8.0. The DGGE-profiles of archaeal 16S rRNA revealed an overall lower number of bands showing a quite identical pattern in the closely spaced sites with higher temperatures AIII and AIV (85 °C pH 8.0 and 92 °C pH 8.0, respectively). The samples AI (51 °C, pH 3.0) and AIV are connected with the outflow of AIV running into AI and show a wide diversity in temperature and pH. The samples were analyzed by sequencing of the 16S rRNA, by using the amplification of the regions V2/V3 with specific primers for Archaea and Bacteria, and by FISH by using domain-specific probes to quantify the relative abundances of Bacteria and Archaea. Sahm and co-workers reported that, by FISH, AI site was dominated by Bacteria (68% of total cells) and the Archaea could not be detected at all. By contrast, the site AIV showed that the relative contribution of the specific domains was almost 35% Archaea and 40% Bacteria (Sahm et al. 2013). The partial amplification of 16S rRNA genes using specific primers for archaeal and bacterial V2/V3 regions produced 93,576 clean sequences (74% of the total raw sequences)

with an average length of 390 bases. In addition, the sample AIV was successively analyzed by sequencing the overall 16S rRNA from the metagenomic DNA producing 725 partial 16S RNA genes sequences that were analyzed against SILVA database (Pruesse et al. 2007). The overall results indicate that the acidic spring AI was dominated by Proteobacteria (80%), prevalently of the acidophilic genera such as the heterotroph *Acidicaldus* (38%) and the chemolithoautotroph *Acidithiobacillus* (43%), and by the phylum of the Firmicutes (10%) related to *Anoxybacillus*. Instead, bacteria belonging to the phyla of Thermotogae, Firmicutes and Dictyoglomi, with the genera of the Fervidobacterium, Caldicellulosiruptor and Dictyoglomus, respectively, dominated the site AIV. These genera constituted up to 61% and 88%, regarding the metagenomic pyrosequencing and the amplified V2/V3 16s rRNA, respectively, of the bacterial community, indicating a high abundance of heterotrophic microorganisms. The sequencing of the metagenomic 16S rRNA of this sample also revealed high abundance of two genera from the phylum Aquificae, with the chemolithoautotrophic *Sulfurihydrogenibium* being the dominant genus (22%) (Sahm et al. 2013).

The archaeal community of the two sites showed substantial differences. The Archaea present in AI were only Euryarchaeota, mainly belonging to the genus Thermoplasma (89%) while the archaeal population of the site AIV was almost exclusively composed by Crenarchaeota, belonging to the family of Desulfurococcaceae (75%), with the genera *Sulfophobococcus* (55%) and *Desulfurococcus* (19%), and the family of Thermoproteaceae with the genus *Pyrobaculum* (25%).

A more detailed analysis of the 16S rRNA genes indicates that in AIV the partial sequences are more than 99% identical to those from cultivated organisms. The authors reported that for the genera of *Fervidobacterium*, *Dictyoglomus* and *Caldicellulosiruptor* more than 85% of the sequences showed an identity >99% to *F. islandicum*, *D. thermophilum*, and to *C. lactoaceticus* species, respectively, while only few sequences (11%) were 97% identical to cultivated or related to uncultivated species. A different situation was observed for AI where 94% of the *Acidicaldus*-specific sequences were related to uncultivated organisms and 99% of the *Acidithiobacillus*-sequences were 99% identical to *Acidithiobacillus caldus* (Sahm et al. 2013).

This global result is in contrast to the majority of other environmental studies, in which the largest proportion of sequences cannot be assigned to cultivated species. Moreover, the dominant genera *Caldicellulosiruptor*, *Dictyoglomus*, and *Fervidobacterium* in AIV site, have also been detected in in situ enrichment cultures in hot springs from Uzon caldera in Kamchatka (Kublanov et al. 2009). The first two genera are able to grow on polysaccharides (cellulose and chitin) while *Fervidobacterium* grows on proteinaceous substrates, suggesting that the Furnas hot springs could be a reliable source of new polymer-degrading enzymes and organisms.

16S rRNA analyses indicated the dominance of heterotrophic bacterial genera in both springs in contrast to many other studies where Aquificales have been repeatedly found to dominate. To explain this discrepancy, the authors suggest that chemolithotrophic physiology probably based on the oxidation of H_2 or reduced sulfur compounds is the major metabolic pathway in both samples and that,

depending on the chemical characteristics of the spring, different subgroups of Aquificales could be dominating. In particular, it was reported that high-sulfide and sometimes iron-rich habitats were dominated by Aquificales branches J and S which today are attributed to *Sulfurihydrogenibium* (Hugenholtz et al. 1998; Yamamoto et al. 1998; Reysenbach et al. 2000). According to Sahm and colleagues, the high abundance of *Sulfurihydrogenibium* (9 and 22%, by FISH and metagenomic respectively) from site AIV fitted into the picture. The low relative abundance of chemolithoautotrophic organisms at site AIV might be related to the high DOC concentration of 284 mg/L, while data from other hot springs where a clear dominance of Aquificales was observed indicate DOC contents between 0.41 and 10 mg/L (Yamamoto et al. 1998; Hetzer et al. 2007; Hall et al. 2008). Thus, a 20–400-fold higher concentration of DOC in the Furnas spring could be a reason for the abundance of heterotrophic bacteria. About the Archaea in the site AIV, the metagenomic rRNA data suggest that the heterotrophic genera *Sulfophobococcus* spp. and *Desulfurococcus* spp. represent approximately 74% of the sequences while the remaining 26% could be related to chemolithotrophic genera *Pyrobaculum* (25%), *Stetteria* (0.5%) and *Staphylothermus* (0.5%) (Sahm et al. 2013).

The study of Sahm and collaborators represents the state of the art on the characterization of the microbial communities in the Furnas springs which, according the authors, reflect a natural enrichment in this area of heterotrophic and polymer degrading genera that could be promising for the search of new thermostable biocatalysts for biotechnological applications.

5.1.4 Malaysian Sungai Klah

The Sungai Klah (SK) hot spring, the second hottest geothermal spring in Malaysia, is a shallow, 150 m long, fast-flowing stream, with temperatures varying from 50 to 110 °C and a pH range of 7.0–9.0. Hidden within a wooded area, the SK hot spring is continually fed by plant litter, resulting in a relatively high degree of total organic carbon (TOC). In 2015, Chan and coworkers studied the middle of SK stream (75–85 °C, pH 8.0) by performing a metagenomic analysis of a mixture of water and sediment sample (Chan et al. 2015) (Table 5.1). The sample extracted from this site was analyzed by 16S rRNA sequencing and shotgun analyses. For the metagenome sequencing the Illumina HiSeq 2500 sequencer and a dual-indexed 151 (Paired-End sequencing) strategy was used. Paired-end sequencing reads were filtered with the Trimmomatic 0.30 trimming tool (Bolger et al. 2014) for a minimum terminal base quality score of 20 and a length >30 bp. *De novo* assembly was performed using the IDBA-UD assembler and all contigs <300 bp were discarded. This strategy allowed obtaining a total of 278,434 contigs with coverage exceeding 10X. The authors found that 88.44% of the predicted ORFs belonged to Bacteria, and 10.14% and 0.67% were from Archaea and Eukaryota, respectively. A small fraction of the total contigs belonged to viruses and unclassified sequences. The metagenome shotgun sequencing approach allowed the identification of 83 phyla, the top 6 of which are

Firmicutes, Proteobacteria, Chloroflexi, Bacteroidetes, Euryarchaeota and Crenarchaeota. This distribution of the major phyla was similar between both the 16S rRNA and shotgun metagenome approaches. A total of 1,203,458 full-length protein-coding genes identified within the shotgun metagenome dataset were analyzed and, among these, 817,831 ORFs were annotated and classified.

Interestingly, Chan and coworkers used the sequence affiliations to understand the relationship between the geochemical parameters and the population diversity within this hot spring. They showed that the SK hot spring community uses diverse means for growth, as suggested by the analysis of carbon metabolism, since a fraction of the community exhibits a complete metabolic pathway, whereas the others may benefit from syntrophic relationships. In addition, microorganisms in the SK community contain more than one carbon fixation pathway thus allowing the use of different inorganic carbon sources. The community appears to survive using mutualistic or commensalistic symbiotic relationships to thrive under multiple environmental stresses. The authors suggested that the uniqueness of the diversified pathways observed is likely a result of the physical characteristics of the hot spring and of additional factors, such as dissolved gases, minerals, and trace elements. The SK hot spring is richer in aluminum, iron, sulfate, and sulfur in comparison to other Malaysian hot springs. The authors compared SK hot spring to 60 other Malaysian sites, and reported that it is unique due to the natural environment of the site. They identified four key factors; (i) the stream contains multiple spring pools with temperatures exceeding >100 °C; (ii) temperature along the streams fluctuate from 50 to 110 °C; (iii) pH along the streams is not uniform and ranges between 7.0 and 9.0, and (iv) the SK hot spring is fed with plant litters that enhance its carbon contents. The analysis of the microbial diversity present in another hot spring named Little Hot Creek (LHC) (78.7–82.5 °C and pH 6.75–6.97), determined by 16S rRNA analysis, revealed that the dominant phyla are Aquificae, Thermodesulfobacteria, Deinococcus–Thermus, Thermotogae, Chloroflexi, and Dictyoglomi (Vick et al. 2010). This is in contrast with SK, where approximately 56% of the phyla were composed of Firmicutes and Proteobacteria. As suggested by the authors, the biodiversity of the SK hot spring is due to combinations of the three aforementioned factors (i–iii) and to the plant litter enriching the SK microbiome diversity of thermophiles by providing additional carbon sources.

5.1.5 Tengchong, China

One of the most active geothermal areas in the world is Tengchong in China, which is located on the northeastern edge of Tibet–Yunnan geothermal zone between the Indian and Eurasian plates. The Rehai ("Hot Sea") and Ruidian geothermal fields in Tengchong are two regions of intense hydrothermal activity with numerous springs and pools (Table 5.1). Physicochemical conditions span a wide range of temperature (58–97 °C) and pH (1.8–9.3) (Hou et al. 2013). Rehai harbors various types of hot springs: small source, high discharge springs such as Gumingquan and

Jiemeiquan; small, shallow acidic mud pools, such as those in Diretiyanqu, that formed a decreasing temperature gradient; shallow acidic pool Zhenzhuquan; and shallow spring with multiple geothermal sources such as Shuirebaozha. Large pools with neutral pH such as Gongxiaoshe and Jinze are located in the Ruidian geothermal field (Wang et al. 2014). Based on physical characteristics, the hot springs can be divided in 4 groups: (i) high temperature and neutral-alkaline pH (in both Rehai and Ruidian regions), (ii) moderate temperature and neutral pH (Ruidian), (iii) low temperature and low pH (Rehai), (iv) high temperature and low pH (Rehai). In 2013, Hou and co-workers published the first comprehensive census of the microbial community in 16 different hot springs of Tengchong. Previously, few metagenomic studies focused only on Crenarchaeota (Song et al. 2010) or ammonia oxidizing archaea (AOA) (Jiang et al. 2010) have been reported. Hou's work focused on the relationship between thermophilic microbial communities and geochemical conditions. The microbial community was analyzed through PCR amplification of the bacterial and archaeal V4 V8 variable regions and sequencing of the 16S rRNA genes. The sequences were aligned with the PyNAST method (Caporaso et al. 2010a) and operational taxonomic units (OTUs) were identified with Chimera Slayer (Haas et al. 2011). More than 90% of total sequences from Rehai springs were composed of Archaea, mainly Crenarchaeota. Desulfurococcales and Thermoproteales (mainly *Pyrobaculum*) were the dominant orders present in the springs with neutral-alkaline pH (pH 6.7–9.4) and high concentrations of silica, Na^+, K^+ and Cl^+. Instead Sulfolobales, predominantly the genus *Sulfolobus*, were dominant in high temperature, acidic, and sulfur-rich springs (85.1–89.1 °C and pH 2.5–4.8). Within acidic sites, temperature exerted a strong control on community composition. With decreased temperature, a Sulfolobus-dominated community was replaced by the bacterial taxa *Hydrogenobaculum*, with Aquificae as the most abundant bacterial phylum. Some bacterial and archaeal groups, such as *Hydrogenobaculum* and *Sulfolobus*, were found only in Rehai, whereas *Thermaceae* and *Rhodothermaceae* were fairly abundant only in Ruidian. Putative ammonia-oxidizing *Thaumarchaeota* were the dominant archaea in Ruidian.

The correlation between microbial diversity and environmental geochemistry was measured by Chao1 (predicted number of OTU), Shannon and equitability indices based on 16S rRNA gene sequence data. These diversity indices were tested for their correlation with the geochemical data using Mantel test. Higher microbial richness, equitability, and diversity in Ruidian than in Rehai were found. According to Hou et al., this might be due to the neutral pH, moderate temperatures, and high TOC contents as well as the different mineralogy (carbonates and silicates) of the Ruidian springs relative to those from Rehai (Hou et al. 2013).

More recently, the correlation between microbial diversity and geochemistry in Tengchong has been analyzed by considering the seasonal changes (Wang et al. 2014), since the hot springs in Tengchong are located in a subtropical area with heavy temporal monsoon rainfall (Briggs et al. 2014). Wang and co-workers compared their samples collected in the rainy season (June and August) with the analysis of Hou and colleagues obtained by samples collected in the dry season (January). They found that the seasonal effects on the microbial diversity are more

pronounced in sediment relative to water sample. In acidic springs the water communities between January and June were highly similar to each other and both were predominated by *Sulfolobus*. Instead, in August, *Hydrogenobacter* became the most abundant taxon followed by *Sulfolobus*. In the sediments of two acidic springs, named Zhenzhuquan (89.1 °C, pH 4.79) and Diretiyan-1 (85.1 °C, pH 2.58), from January to August the dominant taxa *Sulfolobus* was replaced by *Desulfococcus* and *Ignispaera*, respectively. In the neutral-alkaline spring, the water community remained constant in both dry and rainy seasons, except for the *Shuirebaozha* spring (79.8 °C, pH 8.28) where, among the most abundant taxa, *Fervidobacterium* changed in *Hydrogenobacter* from June to August, while *Persephonella* remained constant over time. The two alkaline springs, characterized by fast-flowing and high discharges, harbored *Hydrogenobacter* in January and June but the community structures showed a notably change in August, where *Persephonella* and the candidate phylum OP1 became the dominant members. Both studies revealed that Ruidian sediments contained more diverse microbial lineages than Rehai sediments thanks to the neutral pH and moderate temperatures. Specifically, the neutral spring contained similar microbial lineages in January and June, but in August a single dominant lineage of *Thermus* emerged.

The exact reasons of the seasonal changes in microbial community structure were difficult to establish. Wang and co-workers suggested that pH was a primary factor influencing the microbial community shifts, followed by temperature and DOC. For example, in Zhenzhuquan, where the pH ranged from 4.8 in January to 6.1 in August, the change of dominant microbial lineages from *Sulfolobus* to microaerophilic *Hydrogenobacter* (in the water) and anaerobic *Desulfococcus* (in the sediments) was observed. Indeed, the pH 6.1 exceeded the growth pH range for *Sulfolobus* (0.9–5.8) (Brock et al. 1972) but fits well with the pH range of *Hydrogenobacter* (near neutral pH) (Takacs-Vesbach et al. 2013) and *Desulfococcus* (pH 6.0–6.5) (Faith 1992). Instead, in the other acidic spring Diretiyanqu-1 the shift of dominant taxa from *Sulfolobus* to *Ignisphaera* was not driven by the increased pH but by the high accumulations of NH_4^+, K^+, and Na^+ ions that could inhibit the growth of *Sulfolobus* species by affecting their RNA polymerase activity (Park and Lee 1999).

Microbial community composition and its correlation with geochemistry in Tengchong could be better understood by a comparison to other geothermal systems in the world. In terms of mechanisms and genesis, the Tengchong hot springs are very similar to those in YNP in that they are both volcanically driven. As a result, many springs in YNP and Tengchong, in particular in Rehai, harbored similar microbial communities at the phylum and family/genus levels such as the bacterial and archaeal phyla Aquificae and Crenarchaeota, respectively. In more detail, four genera of *Aquificales* were present in both Tengchong and YNP springs (Hou et al. 2013) but their relative abundance was highly different. For example, in neutral alkaline springs (>78 °C) *Hydrogenobacter* dominated on *Thermocrinis*, although, the former was more abundant at lower temperatures (60–80 °C) than *Thermocrinis* (75–92 °C) (Eder and Huber 2002). Instead, *Sulfurihydrogenibium* is more scarcely present in Tengchong than YNP springs (Inskeep et al. 2005;

Yang et al. 2011). Hou and coworkers suggested that this may be related to maximum growth temperature of 78 °C of *Sulfurihydrogenibium*, which was below the lowest temperature in the neutral and alkaline samples (78–90 °C), except Ruidian springs (74–82 °C). In the latter springs, however, the relatively high dissolved O_2 content may be incompatible with the microaerophilic nature of *Sulfurihydrogenibium* (Nakagawa et al. 2005). *Deinococcus-Thermus*, the most abundant species in Ruidian spring (73.8 °C, pH 7.29), has a geochemical distribution similar to YNP and the crenarchaeal class Thermoprotei is largely abundant in Rehai and in many sites in YNP (Meyer-Dombard et al. 2005).

In conclusion, the two geothermal fields Rehai and Ruidian were highly diverse in their environmental conditions, in terms of temperature range (55–94 °C) and pH (2.5–9.4). The bacterial phylum Aquificae and the archaeal phylum Crenarchaeota are dominant in all Rehai samples and in the water samples of Ruidian but their specific compositions are highly dependent on geo-physical characteristics of the springs and seasonal physicochemical changes (Hou et al. 2013; Wang et al. 2014). Therefore, these studies suggest that the temperature, the pH and other geochemical conditions play a key role in shaping the microbial community structure in Tengchong hot springs.

5.1.6 Taupo Volcanic Zone, New Zealand

The Taupo Volcanic Zone (TVZ) consists of a complex group of high temperature geothermal systems in the central North Island of New Zealand. One of the major geothermal fields in the TVZ is Waiotapu, which is characterized by a large number of springs (Table 5.1) with elevated arsenic concentrations (Mountain et al. 2003). The largest feature at Waiotapu is Champagne Pool (CP), ~65 m in diameter with an estimated volume of ~50,000 m^3 (Hedenquist and Henley 1985), and an arsenic concentration between 2.9 and 4.2 mg L^{-1}. The inner rim of CP is characterized by subaqueous orange amorphous As-S precipitate (Jones et al. 2001). The narrow outflow channel (~40 cm wide and 5 cm deep), in a sub-aerial sinter dam, drains the spring water out across a shallow siliceous sinter terrace. Convection in CP stabilizes water temperature at 75 °C, while in the surrounding silica terrace ("Artist's Palette"), the temperature decreases to 45 °C. Water-rock interactions beneath the pool lead to silica dissolution and sulfide oxidation (Ellis and Mahon 1967) and provide sources of acidity to CP waters. In 2014, Hug and co-workers studied the microbial contributions to coupled arsenic and sulfur cycling at Champagne Pool, with implications for understanding the evolution of microbial arsenic resistance in sulfidic geothermal systems. In this study, the hot spring was divided in four sampling sites on the basis of distinctive physical and chemical characteristics. These sites were located along a natural hydrologic gradient from the inner pool (CPp) through the inner rim (CPr) and outflow channel (CPc), with pH ranging between 5.5 and 5.8, and on to an outer silica terrace (AP), pH 6.9. Total dissolved arsenic concentrations of 3.0, 2.9, 3.6, and 4.2 mg L^{-1} were measured at

sites CPp, CPr, CPc and AP, respectively. All sampled sites contained total dissolved sulfur concentrations between 91 and 105 mg L^{-1} (Hug et al. 2014). High temperatures and high concentrations of dissolved toxic metal(loid)s determine a strong selective pressure on extant microbial communities. Indeed sulfide, elemental sulfur, thiosulfate, and sulfate are common electron donors or acceptors for microorganisms under hydrothermal conditions (Macur et al. 2013), and sulfide ions are highly reactive with arsenic. Indeed, Stauder and co-workers suggested the transformation of arsenite into thioarsenates via elemental sulfur indicating that, microbially-mediated sulfur cycling could exert a profound, although indirect, influence on arsenic speciation, by thioarsenate species control (Stauder et al. 2005). In comparison to arsenite and arsenate, thioarsenates are considered to be less toxic for microorganisms, as the sulfurarsenic bond leaves no free electron pair to bind with sulfhydryl-groups in amino acids. Despite in the work by Planer-Friedrich et al. (2008) thioarsenate species were identified as potentially toxic to microorganisms over longer exposure times, it has been demonstrated that many microbes employ a range of strategies to detoxify arsenic (Planer-Friedrich et al. 2008). The most ubiquitous arsenic resistance mechanism is the expression of the *ars* operon encoding for proteins that identify and transport arsenic (Páez-Espino et al. 2009).

To determine potential microbial contributions to arsenic speciation in CP and to characterize the microbial diversity, Hug and co-workers extracted total genomic DNA from sediments and the samples were sequenced using Illumina Miseq. Sequence analysis was performed using the rapid annotation subsystems technology for metagenomes (MG-RAST) bioinformatics package. The numbers of clean reads obtained by the sequencing were about 2 million for CPp, CPr and CPc and about 4 million for the AP sample. For taxonomic analysis, 16S rRNA gene sequence data were compared to all accessory databases. These analyses showed that the lowest species abundance was detected at CPc, with species abundances of CPp and CPr closer to CPp than to AP. At CPp, 12% of the sequences belonged to Archaea, consisting almost exclusively of *Thermofilum*, *Sulfolobus* and *Pyrobaculum* (together 8%). Instead, 16S rRNA gene sequences resulted ~21–28% of Archaea at CPr and CPc, respectively, mostly assigned to genera *Sulfolobus, Thermofilum, Pyrobaculum, Desulfurococcus, Thermococcus,* and *Staphylothermus*. In AP only 2% of the sequences belonged to Archaea, with no dominant genus present. Hug reported that, across all sites, most abundant sequences belonged to Bacteria closely related to the genus *Sulfurihydrogenibium,* in particular 19% at CPp and CPr, 13% CPc and 10% AP. Additionally, bacterial genera *Anoxybacillus* and *Persephonella* comprised 38 and 3% of the total sequences at CPp, respectively; whereas at AP, alongside *Sulfurihydrogenibium, Thiomonas* and *Thermus* were the most abundant bacterial genera in the community with 9% and 4% of total sequences.

Hug and colleagues reported that microbial community, including a large group of sulfur-cycling microorganisms, increased its richness with decreasing temperatures and increasing pH. They suggested that this could be an evidence of the indirect biological mediation of arsenic speciation via microbial sulfur cycling (Ullrich et al. 2013; Hug et al. 2014). At CPp, the main proportion of sulfur

metabolizing genes belonged to sulfur oxidation genes, whereas at CPr and CPc belonged to sulfur reduction genes. Instead at AP the proportion of sulfur metabolizing genes changed again to sulfur oxidation genes. According to the authors, the combination of sulfide dehydrogenase and sulfur oxygenase-reductase encoding genes, detected as major sulfur oxidation genes at CPp, suggests a two-step sulfide oxidation process to sulfite and thiosulfate, also producing sulfide. Thiosulfate could be further oxidized via oxygen at the surface of the pool. The annotation of sulfur oxidation genes detected at CPp revealed a close relationship to the genus *Sulfolobus*. This sulfur-oxidizing genus (Brock et al. 1972) enhances the potential for production of thiosulfate and sulfate. Other 16S rRNA gene sequences detected in CPp were closely related to members of the order *Aquificales*, primary producers in high temperature ecosystems (Eder and Huber 2002) and capable of oxidizing H_2 or reduced sulfur species. The presence of sulfur reduction genes at CPp, belonging to the *dsr* and *asr* gene complexes, is consistent with the thiosulfate or elemental sulfur-reducing genus *Pyrobaculum* (Stetter et al. 1990). The resulting biogenic sulfide produced, would then be available to transform arsenite to monothioarsenate and yielding H_2. At CPr the increase in sulfur reduction genes is consistent with the detection of close relatives from the genera, which have the potential to reduce thiosulfate or elemental sulfur to sulfide. Since the sulfide concentration at Cpr did not increase significantly, Hug and collaborators assumed that biogenic sulfide was probably rapidly reoxidized via sulfur oxidation by *Sulfolobus* (Hug et al. 2014). In addition, the authors observed that alongside the indirect impacts on arsenic transformation from microbial sulfur cycling, the metagenomic data for all sites revealed the presence of arsenic resistance genes. The dominance of the *ars* operon supports the high degree of utility and conservation of this arsenic resistance mechanism (Hug et al. 2014).

In conclusion, phylogenetic analysis of 16S rRNA genes from metagenomic sequencing revealed the dominance of *Sulfurihydrogenibium* at all sites and an increased archaeal population at the rim and outflow channel. Several phylotypes were found closely related to known sulfur- and sulfide-oxidizers, as well as sulfur- and sulfate-reducers. Bioinformatic analysis revealed genes underpinning sulfur redox transformations, consistent with sulfur speciation data, and illustrating a microbial role in sulfur-dependent transformation of arsenite to thioarsenate. Metagenomic analysis also revealed genes encoding for arsenate reductase at all sites, reflecting the ubiquity of thioarsenate and a need for microbial arsenate resistance despite anoxic conditions.

5.1.7 Phlegraean Fields, Italy

The Phlegraean Fields is a large volcanic area situated to the west of Naples, Italy. The area of the caldera comprises 24 craters and volcanic features, lying mostly underwater and showing hydrothermal activity at Lucrino, Agnano and in the town of Pozzuoli.

Solfatara volcano is located in the central part of the repeatedly collapsed Phlegraean Fields caldera, and is one of the youngest volcanoes formed within this active volcanic field (Rosi and Santacroce 1984; Orsi et al. 1996; Isaia et al. 2009). It is a tuff cone located at 100 m above sea level, about 2 km off the town of Pozzuoli. The central part of the crater is occupied by the "Fangaia mud pool", at which the water table emerges and a continuous rising of hydrothermal fluids generates diffuse bubbling (Table 5.1) (Petrosino et al. 2012).

Pisciarelli spring is an area with intense hydrothermal activity and currently exhibits one of the most impressive degassing manifestations. A direct relationship between the increase of hydrothermal activity in the Pisciarelli solfataric area and ground uplift in the caldera has been observed. For this reason, its activity is considered a direct indicator of the volcanic dynamics (Troiano et al. 2014). Pisciarelli spring is only about 800 m^2 in size, but contains more than 20 physically and chemically different springs and mud holes (Table 5.1). A typical feature of continental solfataric fields is the existence of two dominating zones in the soil. The upper oxidized zone is an orange-colored Fe-oxid mat. Generally, the thickness depends on the volcanic activity and exhalation of reducing volcanic gases such as H_2S and H_2, and is between a few centimeters and about 50 cm. Under the oxidized layer, there is a black-colored anoxic zone rich in ferrous sulfide. The surface between these zones, with a width of a few millimeters, is slightly yellow-colored and characterized by the presence of elemental sulfur. This element is formed by chemical oxidation of H_2S coming from below by molecular oxygen penetrating from the surface into the soil. In this high-temperature environment, beyond sulfide, arsenic is one of the most prominent heavy metals (Huber et al. 2000). This suggests the existence of hyperthermophiles, in arsenic rich environment, able to use arsenic compounds in their metabolism, as Hug and co-workers found in Champagne Pool (New Zealand) (Hug et al. 2014). Previous phylogenetic analyses of a highly acidic sample from Pisciarelli (original temperature 95–97 °C) showed archaeal 16S rRNA sequences belonging to the *Sulfolobales* and *Thermoplasmatales*, which are known to grow optimally between pH 1.0 and 3.0 (Huber et al. 2000). In particular, *Sulfolobus solfataricus* strain P2 was isolated for the first time from Pisciarelli (Zillig et al. 1980).

In 2015 Menzel and colleagues analyzed Solfatara volcano (It6) and Pisciarelli hot spring (It3) by metagenomic approach aiming to define the biodiversity, genome contents and inferred functions of bacterial and archaeal communities (Menzel et al. 2015). It6 sample (76 °C, pH 3.0, water/sediment) was sequenced by Illumina Hiseq and analyzed for estimating species abundances by MEGAN (Huson and Weber 2013). From this sample 78.6% of the mapped reads were assigned to Bacteria and 17.6% to Archaea. The most abundant phyla were Proteobacteria (72%) and Thermoprotei (15%). The former was mostly comprised of the genus *Acidithiobacillus* (64%) and a smaller number of Firmicutes (4%). Archaea were composed by Crenarchaeota (15%), with *Acidianus hospitalis* being the most abundant species (4%), and Euryarchaeota (3%), with 2% of the reads assigned to *Ferroplasma acidarmanus*. By the assembly of the reads, Menzel et al. reported 8 distinct 16S rRNA archaeal sequences. Among these, two sequences were assigned

to *Acidithiobacillus*, three to *Sulfolobus*, two to *Sulfobacillus* species. One predicted full-length 16S sequence showed 99.8% identity to *Ferroplasma acidarmanus*. According to the authors, the metabolism of these genera reflected the organic and inorganic content of the pool; for example, the aerobic and autotrophic bacterium, *A. thiooxidans* is a sulfur oxidizer, acidophilic, chemoautotrophic whose carbon requirements are fulfilled by CO_2 from the atmosphere (Ko et al. 2013; Menzel et al. 2015). Instead *F. acidarmanus* is iron-oxidizing archaeon.

In addition, Menzel and co-workers analyzed Pisciarelli site (It3) (86 °C, pH 5.5, water/sediment). Total genomic DNA was extracted from sample and sequenced by Roche/454 Titanium FLX. To estimate the species abundance MEGAN was used to analyze the cleaned reads (about 680,000). It3 almost exclusively contains Archaea (96.6%), with 50% of clean reads assigned to *Acidianus hospitalis* and 32% to *Pyrobaculum* species. These data have been confirmed by 16S rRNA sequences analysis. Among the 9 identified sequences, 3 were >98% identical to *Acidianus* species, including one full length with >99% identity to *Acidianus hospitalis W1*. Moreover, 3 sequences showed highest identity to *Pyrobaculum* species (Menzel et al. 2015).

In conclusion, Menzel and colleagues suggested that the community structure was largely determined by a combination of environmental parameters, rather than geographical distance. Indeed, It3 sample (86 °C, pH 5.5) was almost exclusively comprised of Crenarchaeota (30% of *Pyrobaculum* species), which also corresponded to other moderately acidic pools in YNP (79 °C, pH 1.8) (Inskeep et al. 2013a). In contrast, the sample It6 (76 °C, pH 3.0) was largely comprised of species belonged to the bacterial genus *Acidithiobacillus*, which is known to be an acidophilic mesophile. However, members of this genus have recently been observed in a hydrothermal spring (51 °C/pH 3) of the Azores (Sahm et al. 2013), in a highly acidic river metagenome (59 °C/pH 1) from Argentina (Sofía Urbieta et al. 2014) and in a thermoacidophilic hot pool (70 °C/pH 3.5–4) at the Mutnovsky volcano (Wemheuer et al. 2013).

5.1.8 Metagenomics of Hydrothermal Sites to Access to Novel Enzymes for Biotechnology

The sources of new enzymes were technically limited to a minor fraction of total microbial diversity, the culturable microorganisms, which have been estimated as representing less than 1% of the real diversity in most environments (Amann et al. 1995). This drawback is even more prominent with extremophilic microorganisms. Thus, metagenomics of extreme environments play a key role in the discovery of new enzymes with unique feature combinations crucial for industrial development. Indeed, thermostable enzymes from thermophilic microorganisms are important biocatalysts for industrial and biotechnological purposes, given that they can work at high temperatures in which mesophilic enzymes would be denatured. Two types of protein thermostability are of interest for industrial purposes: thermodynamic

stability (when an enzyme is used under denaturing conditions such as high temperature or presence of an organic solvent) and long-term stability (Sharma et al. 2012). Enzymes from thermophiles, often show both types of thermostability and are able to resist also at the combination of different denaturants (temperature and organic solvents, detergents and extreme pH values) (Haki and Rakshit 2003). Due to these unique properties, thermozymes are of tremendous importance for biotechnological applications and, therefore, screening for novel biocatalysts from extremophiles represents a valuable alternative to elaborative engineering procedures for the optimization of available enzymes from mesophiles (Cobucci-Ponzano et al. 2015).

Since the classical example of Taq DNA polymerase from *Thermus aquaticus,* purified and isolated from hot springs (Chien et al. 1976), which made the development of the PCR amplification technique possible, there are now many thermophilic enzymes being used for biotechnological and industrial purposes. Studies of the biodiversity in hot springs revealed the presence of complex communities containing novel microorganisms, which can be potential sources of novel enzymes with unique features of interest in industrial applications. Indeed, thermophiles and hyperthermophiles produce a variety of hydrolytic enzymes such as lipases, glycosidases, peptidases, which are of applicative interest. Thermophilic enzymes were primarily screened in a culture-based manner, but bioprospecting of extreme temperature metagenomes by either homology based DNA sequence data mining or functional screening of metagenomic DNA libraries is currently regularly providing new thermostable biocatalysts with potential in biotechnology applications. Heterotrophic (hyper)thermophilic prokaryotes are capable to utilize various polymeric substrates as carbon sources. A resourceful enzyme repertoire that is stable at specific extreme environmental conditions facilitates the efficient degradation of complex natural polymers including starch, lignocellulose, chitin as well as proteins and fats (Elleuche et al. 2015). In recent years biocatalysts search in metagenomic DNA libraries from hot springs was mainly focused on the discovery of esterases, lipases and glycosidases, instead only one DNA polymerase and one protease were reported. We here describe some recent examples of new thermostable enzymes with high potential in biotechnological applications identified from high temperature metagenomes (for a review see López-López et al. 2013; Lewin et al. 2013).

Viral metagenomes are an unexplored source of sequence diversity for the development of new enzymes. Moser and coworkers constructed a viral metagenomic library from Octopus hot spring in YNP and analyzed 21,198 Sanger sequence reads. BLASTx alignment to the Genbank protein sequence database identified hundreds of potential polymerase genes. Among these, they identified the DNA polymerase 3173 with both high thermostability and innate reverse transcriptase (RT) activity. An easier-to-use exonuclease-deficient derivative was incorporated into a PyroScript RT-PCR master mix and compared to one enzyme (Tth) and two-enzyme (MMLVRT/Taq) RT-PCR systems for quantitative analyses. Specificity and sensitivity of 3173 Pol-based RT-PCR were higher than Tth Pol and comparable to three common two-enzyme systems. The performance and simplified

set-up make this enzyme a potential alternative for research and molecular diagnostics (Moser et al. 2012).

Lipases and esterases are ubiquitous in nature and can be found in animals, plants and microorganisms, but most industrial lipases are of microbial origin. Nowadays, lipases and esterases represent a major portion with high growth potential in the World Industrial Enzymes Market. They have many applications in the food and paper industry, synthesis of biopolymers, biodiesel production, synthesis of fine chemicals, bioremediation and waste treatment. Most industrial processes in which lipases/esterases are used as biocatalysts are carried out at temperatures above 45 °C and in the presence of organic solvents. Lipases from extremophiles can provide special features that make them more suitable for specific applications where a lipolytic biocatalyst is required (López-López et al. 2014). Several thermostable esterases have been isolated in recent years by functional screening of metagenomic libraries from hot spring. EstE1 and Est1 were identified from a functional screening of metagenomic library of samplings in the Sileri region of Indonesia (80–95 °C, pH 4.0–6.0) (Rhee et al. 2005) and of Jae Sawn hot spring in Thailand (70 °C, pH 7.0) (Tirawongsaroj et al. 2008), respectively. These enzymes display typical thermophilic profiles: extremely stable at 80 and 70 °C in the absence of any stabilizer, with high optimal temperatures of 95 and 70 °C, respectively. The activity at lower temperatures is remarkably high in EstE1: 20% and 30% of its optimal activity is retained at 30 and 40 °C, respectively. In addition, from the functional screening of the same Jae Sawn hot spring metagenome, Tirawongsaroj and coworkers identified a novel patatin-like phospholipase (PLP) containing four conserved domains, similar to other patatin-like proteins with lipid acyl hydrolase activity, and exhibiting high *V*max toward *p*-nitrophenyl butyrate. PLP and Est1 enzymes had activity toward both short-chain (C4 and C5) and long chain (C14 and C16) fatty acid esters. Therefore, PLP and Est1 are novel lipolytic enzymes from unculturable microbes, different from other known patatin-like phospholipases and esterases, which usually show no activity for substrates longer than C10 (Tirawongsaroj et al. 2008). More recently, three novel genes encoding lipolytic enzymes (*plpBW1*, *estBW1*, and *estBW2*), including a new patatin-like protein, have been identified by the functional screening of the metagenomic library prepared by Wemheuer and coworkers from the Uzon caldera in Kamchatka peninsula (see above). The closest relatives of all identified protein sequences originated from known thermophiles. They were similar to uncharacterized putative gene products derived from *Sulfurihydrogenibium azorense* (PlpBW1 and EstBW2), and *Thermobaculum terrenum* (EstBW1). The characterized lipolytic enzymes (PlpBW1, EstBW1, and EstBW2) showed features similar to those of other metagenome derived esterases, which were identified in thermophilic sites (Wemheuer et al. 2013).

The functional screening of the metagenomic library from the Uzon caldera allowed the identification of first metagenome-derived peptidase from a thermophilic environment (Wemheuer et al. 2013). The authors identified a gene sequence (*pepBW1*) that is almost identical to that of a putative gene encoding a serine peptidase of *Desulfurococcus kamchatkiensis*, belonging to the

Crenarchaeota, whose 16S rRNA gene sequence was found in the 16S analysis of the Uzon sample. The serine peptidase PepBW1 was affiliated to the subtilisin family (family S8). The recombinant *E. coli* strain containing PepBW1 was tested towards different proteins and showed proteolytic activity with skim milk and elastin-Congo red but not with azoalbumin or azocasein; however, no more enzymatic details are reported (Wemheuer et al. 2013).

Production of biofuels from the renewable lignocellulosic biomass is gradually considered as a promising way to replacement of fossil fuels. However, its bioconversion has been limited by the saccharification step because the main components of the lignocellulosic biomass (cellulose, hemicellulose and lignin) are tightly held together. Enzymatic hydrolysis is the most common process to degrade the cellulose and hemicellulose into fermentable sugars such as glucose and xylose. Many extreme thermophiles are able to utilize a variety of carbohydrates pertinent to the conversion of lignocellulosic biomass to biofuels. Identification and characterization of the glycoside hydrolases from these extremely thermophilic microorganisms is likely to generate new opportunities for the use of renewable resources as biofuels (Blumer-Schuette et al. 2008). Recently, a family 1 β-glucosidase (Bgl1) of archaeal origin was isolated by functional screening of a metagenome from the hot spring Caldeirão on the island São Miguel (Azores, Portugal) (Schröder et al. 2014). The samples collected at Furnas Valley, with temperatures ranging from 60 to 70 °C, pH 6.0–7.0, included water, mud and sediment. The putative protein Bgl1 exhibited 50–53% identity to putative glycoside hydrolases from species belonging to the class *Thermoprotei* of the phylum Crenarchaeota. The recombinant enzyme showed a broad substrate spectrum with activity toward cellobiose, cellotriose and lactose with a Ki value for glucose of 150 mM. Compared to most enzymes, extremely high specific activity with 3195 U/mg was observed at 90 °C and pH 6.5. Bgl1 was completely stable at pH 4.5–9.5 for 48 h at 4 °C and more than 40% of activity was measured at 105 °C. These distinctive characteristics distinguish Bgl1 from other enzymes described so far and make this enzyme suitable for application in numerous biotechnological applications that run at high temperatures (Schröder et al. 2014). To identify novel enzymes to be used in second generation biofuel technology, a very interesting approach based on the enrichment of microbial consortia on a selected biomass has been reported by Graham and collaborators (Graham et al. 2011). The authors assumed that given that no characterized hyperthermophilic archaeal species contain a minimum set of exo-, and endo-hemicellulases required to grow on lignocellulose above 90 °C, isolating a single cellulolytic species could be problematic. Hence, they set out to reconstitute a consortium of hyperthermophiles that could deconstruct lignocellulosic biomass at 90 °C. The sample was collected at Great Boiling Springs, a circumneutral geothermal pool at 94 °C near Gerlach, Nevada. The sediment was inoculated into minimal salts medium with the pulverized *Miscanthus gigas* as the primary carbon and energy source; then, after 3 weeks at 90 °C, a secondary enrichment with microcrystalline cellulose and a tertiary step with strips of Whatman No. 3 filter paper as a sole carbon source were set up. After

this enrichment strategy, since repeated efforts to separate the individual species were unsuccessful, they analyzed the consortium as a whole for potential cellulases of interest. The analysis of the metagenome of this consortium allowed the identification of a multidomain cellulase, the most thermotolerant endoglucanase reported to date, with a unique domain architecture. This approach, a compromise between environmental metagenomics and classical microbial isolation, resulted in the selection of a limited archaeal enrichment capable of growing on crystalline cellulose at 90 °C. The authors found that the consortium enriched on Avicel consisted of three strains but the closest characterized organism to the dominant member is related to *I. aggregans* DSM17230, representing a divergent new species of this genus. Since single isolates from this enrichment were not possible to obtain, the authors suggested that the diverse enzymes needed for lignocellulose utilization (cellulases, cellulose binding domains, xylanases, and cellobiohydrolases) do not allow for the survival of a single isolated hyperthermophilic Archaeon (Graham et al. 2011).

The studies surveyed in this paragraph prove that sequence-based and functional metagenomics are powerful strategies to discovering new hyperthermophilic enzymes with unique combinations of biochemical features and potential use in industrial applications. In the sequence-based metagenomic approach, new enzymes are discovered by exploring metagenomic data for enzymes homologous to known activities. Another common strategy is a PCR-based method with degenerate primers designed according to the conserved regions of already-known classes of enzymes. A disadvantage is that both approaches tend to detect only enzymes related to previously reported families, and might overlook those with completely new sequences. However, it is well documented that single amino acid differences may have drastic influences on enzyme properties and substrate recognition. Alternatively, metagenomic libraries can be subjected to functional screening to detect clones that exhibit the enzymatic activities of interest. The advantage of the function-driven approach is the potential for discovering entirely new classes of biocatalysts, with no similarity to known enzymes. However, the success of such screening relies on the kind of biotransformation process that should produce metabolites that can be easily detected by simple activity tests (such as reaction color) and on compatibility of the cloned genes with the transcription and translation machinery of the heterologous host, usually *Escherichia coli*. To overcome this limitation, a special fosmid vector was developed for the expression of metagenomic libraries from thermophiles. This vector, allowing the library to be constructed in *E. coli* and subsequently transferred to *Thermus thermophilus* (Angelov et al. 2009), could be of great help for the correct expression and folding of enzymes form hyperthermophilic microorganisms and the functional screening of metagenomic libraries from extreme environments.

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Chapter 6
Crenarchaeal Viruses of Hot Springs: Diversity, Ecology and Co-evolution

Alice C. Ortmann

Abstract Hot springs vary in temperature and pH, even within the same geological region. At temperatures above 68 °C, eukaryotes are unable to survive, and the microbial communities become dominated by Bacteria, Archaea and viruses. As temperatures rise and pH levels decrease, Archaea dominate the communities with few Bacteria being present. It is these hot (>80 °C), acidic (pH < 3) springs from which most of our understanding of thermal viruses comes. This chapter will address what we know about the morphological and genetic diversity of the viruses from studies of viral isolates as well as the application of culture independent methods to hot springs. Additionally, it will address areas where we know relatively little, such as the ecological role of these viruses, areas where we are rapidly learning more, such as the functioning and role of the CRISPR/cas system, and finally what future research in thermal virology may be headed.

6.1 Introduction to Hot Spring Environments

Hots springs, where geothermal activity heats underground water, vary in temperature, pH and chemical composition leading to significant variability in their microbial communities. Hot springs can be roughly classified based on their temperature and pH. Once temperatures are greater than 68 °C, eukaryotes are no longer significant contributors to the microbial community. In these high temperature springs, pH is a strong factor selecting for specific microbial communities. Generally, alkaline hot springs tend to be dominated by Bacteria, especially members of the Aquificales (Schoenfeld et al. 2008; Hou et al. 2013). As pH moves to neutral or slightly acidic, the Archaea increase, with members of the uncultured Korarchaeota being present in springs with pH values around 5–7 (Reigstad et al. 2010; Miller-Coleman et al. 2012) along with members of the crenarchaeal

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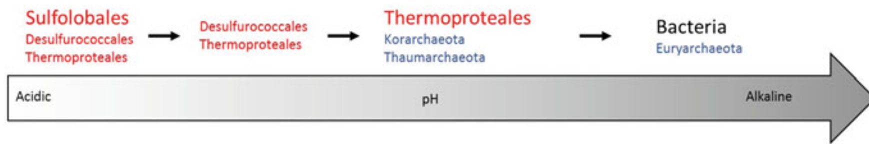

Fig. 6.1 Change in the relative abundance of Crenarchaeota (*red*) families along with other Archaea (*blue*) and Bacteria as the pH of hot springs increase. At temperatures above 68 °C, Eukaryotes are excluded. Large type indicates groups that dominate within pH ranges. While these organisms dominate springs at each pH, they may not be the only organisms present in any given spring

Thermoproteales order (Inskeep et al. 2010; Hedlund et al. 2013a; Hou et al. 2013). More acidic springs tend to be dominated by Crenarchaeota, specifically Sulfolobales (Young et al. 2005; Snyder et al. 2007). Euryarchaeota may be found in more neutral or alkaline hot springs, with higher abundances in lower temperature springs (Schoenfeld et al. 2008; Hedlund et al. 2013a) (Fig. 6.1).

The majority of studies looking at viruses in hot springs have focused on the hot (>80 °C), acidic (pH < 3.0) springs dominated by the Sulfolobales. Generally, these springs have microbial communities with low diversity, with ~10 microbial species detected using DNA sequencing techniques (Young et al. 2005; Snyder et al. 2007; Bolduc et al. 2015). *Sulfolobus* spp. are one of the most common organisms in these springs and have become model organisms for Archaea due to the ease in isolating and growing them using heterotrophic conditions. However, *Sulfolobus* spp. can also grow autotrophically, oxidizing reduced sulfur compounds and H_2 (Young et al. 2005). The majority of thermal viruses that have been studied have been isolated using *S. solfataricus* or *S. islandicus* (e.g. Martin et al. 1984; Rice et al. 2001; Xiang et al. 2003). In *Sulfolobus*-virus interaction studies, experimental conditions usually use a heterotrophically growing host. How these interactions may differ if the cells were growing autotrophically is unknown, but may be of importance in understanding the ecology of these viruses under natural conditions. A closely related genus, *Acidianus*, an obligatory autotroph common to acidic hot springs, has also been used as a host for isolating thermal viruses (e.g. Bettstetter et al. 2003; Häring et al. 2005a, c). Other common microbes detected in acidic hot springs include other genera of the Sulfolobales (*Stygiolobus*, *Metallosphaera*), members of the Desulfurococcales (*Aeropyrum*, *Caldococcus*, *Desulfurococcus*, *Pyrodictium*) and Thermoproteales (*Caldivirga*, *Thermocladium*, *Vulcanisaeta*) (Snyder et al. 2007; Inskeep et al. 2010). These organisms include both anaerobic and aerobic species, with some capable of autotrophy while others are heterotrophic. This mixture may be due to the presence of micro-niches in these springs caused by the mixing of subsurface and surface water and formation of mineral particles. Alternatively, because these studies have used DNA for analysis, it is possible that some of these organisms were introduced into the spring from the subsurface, but were not active in the spring itself. Understanding the ecology of viruses in the hot acidic springs will require refining our knowledge of the ecology

of the microbial hosts under environmental conditions. However, through laboratory cultures and experiments and the application of sequencing techniques to hot springs, we have begun to elucidate the complex and diverse world of crenarchaeal viruses.

6.2 Crenarchaeal Virus Isolates

Previous reviews have highlighted the wide range of morphologies associated with archaeal viruses (e.g. Peng et al. 2012; Uldahl and Peng 2013; Dellas et al. 2014; Snyder et al. 2015). While many viruses infecting Euryarchaeota have head and tail morphologies found in bacterial viruses, other viruses, including all of those infecting members of the Crenarchaeaota, have unusual morphologies. Based on these morphotypes, viruses isolated from Archaea have been assigned to 15 families, of which 12 are newly recognized or have been recently proposed. Ten of these new families include viruses infecting Crenarchaeota isolated from hot springs or hydrothermal vents. This remarkable diversity in morphology detected in theses hot springs is even more remarkable when the diversity of the host Archaea are considered. Crenarchaeal viruses have been isolated for only 8 archaeal genera, with the majority of the viruses infecting *Sulfolobus*, *Acidianus* and *Aeropyrum*. Below is a brief description of the morphology, genetics and life cycles of the virus families infecting crenarchaeal hosts (Table 6.1).

6.2.1 Ampullaviridae

Perhaps the most unusual shaped virus isolated to date, the *Acidianus* bottle-shaped virus (ABV) has been classified in the family Apullaviridae (Häring et al. 2005a). This virus was isolated from an Italian hot spring with a pH of 1.5 and temperatures ranging from 87 to 93 °C. ABV has been found to infect a single strain of *Acidianus* (*A. convivator*). Like many of the crenarchaeal viruses, cells are not lysed by ABV, however growth rates are severely reduced in the presence of the virus (Häring et al. 2005a). The ABV particle is bottle-shaped; with a length of 230 nm, a bottom width of 75 nm and a neck width of 4 nm. Short filaments are arranged in a circle around the bottom end of the bottle and are likely involved in cellular attachment (Peng et al. 2007). The bottle-shaped structure is surrounded by an outer lipid envelope. ABV has a 23.9 kb linear dsDNA genome with a 590 bp terminal repeat and 57 predicted open reading frames (ORFs) (Peng et al. 2007). One of these ORFs may encode a protein primed DNA polymerase with similarity to eukaryotic and bacterial viruses. ABV is the only crenarchaeal virus in which a DNA polymerase has been identified.

Table 6.1 Families and characteristics of viruses isolated which infect thermophilic Crenarchaea. Characteristics are general to the family or genus with details provided in the text

Family	Genus	Shape	Isolates	Hosts	Characteristics
Ampullaviridae	*Ampullavirus*	Bottle-shaped	ABV	*Acidianus*	• Linear dsDNA • DNA polymerase
Bicaudaviridae	*Alphabicaudavirus*	Two tails, spindle-shaped	ATV, ATV2	*Acidianus*	• Circular dsDNA • Tails grow outside of host • Integrase • ATV is lytic
	Betabicaudavirus	One tail, spindle-shaped	STSV1, STSV2, SMV1	*Sulfolobus*	• Circular dsDNA • Integrase
			ATSV	*Acidianus*	
Fuselloviriadae	*Alphafusellovirus*	Spindle-shaped	SSV1, SSV2, SSVRH, SSVK1, SSV4, SSV5, SSV6, SSV7	*Sulfolobus*	• Circular dsDNA • Integrase • SSV1, SSV2 and SSV7 UV inducible
	Betafusellovirus		ASV1	*Acidianus*	• Circular dsDNA • Integrase • Gene duplication
	Gammafusellovirus		APSV1	*Aeropyrum pernix*	• Circular dsDNA • Integrase • Induced through sub-optimal growth
	Deltafusellovirus		PAV1, TPV1, A3	Euryarchaeaota	• Circular dsDNA • Integrase
	Epsilonfusellovirus		His1, His2	Euryarchaeaota	• Linear dsDNA • DNA polymerase
Clavaviridae	*Clavavirus*	Bacilliform	APBV1	*Aeropyrum pernix*	• Circular dsDNA • Smallest genome to date

(continued)

Table 6.1 (continued)

Family	Genus	Shape	Isolates	Hosts	Characteristics
Globulaviridae	*Globulovirus*	Spherical	PSV	*Pyrobaculum, Thermoproteus tenax*	• Linear dsDNA • Stable carrier state
			TTSV1	*Thermoproteus tenax*	
Guttaviridae	*Alphaguttavirus*	Droplet-shaped	SNDV	*Sulfolobus*	• Circular dsDNA • Highly methylates
	Betaguttavirus		APOV1, APOV2	*Aeropyrum pernix*	• Circular dsDNA • Integrase
Lipothrixviridae[a]	*Alphalipothrixvirus*	Rod-shaped	TTV1	*Thermoproteus tenax*	• dsDNA • Multiple variants • Rapid mutation
	Betalipothrixvirus	Filaments	SIFV, SIFV2	*Sulfolobus*	• Linear dsDNA • Inverted terminal repeats • Low complexity AT-rich regions
			AFV3, AFV6, AFV7, AFV8, AFV99	*Acidianus*	
			TTV2, TTV3	*Thermoproteus tenax*	
	Gammalipothrixvirus	Filaments, claw-like ends	AFV1	*Acidianus*	• Linear dsDNA • Stable carrier state
	Deltalipothrixvirus	Filaments, bottle-brush ends	AFV2	*Acidianus*	• Linear dsDNA • tRNA gene • Stable carrier state

(continued)

Table 6.1 (continued)

Family	Genus	Shape	Isolates	Hosts	Characteristics
Rudiviridae[a]	*Rudivirus*	Rod-shaped, short terminal fibers	SIRV1, SIRV2, SIRV4	*Sulfolobus*	• Linear dsDNA • Inverted terminal repeats • Covalently closed • Holliday junction helicase and resolvase involved in replication • SIRV2 is lytic
			ARV1	*Acidianus*	
			TTV4	*Thermoproteus tenax*	
			SRV	*Stygiolobus*	
Spiraviridae	*Alphaspiravirus*	Cylindrical, coil-shaped	ACV	*Aeropyrum pernix*	• Circular ssDNA • Stable carrier state
Turriviridae	*Alphaturrivirus*	Icosahedral, turrents at vertices	STIV, STIV2	*Sulfolobus*	• Circular dsDNA • STIV is lytic

[a]These two families have been assigned to the order Ligamnevirales

6.2.2 Spindle-Shaped Viruses

One of the most commonly observed morphologies for viruses in hot springs and hydrothermal vents, as well as hypersaline environments, is the spindle-shaped virus particle (Rice et al. 2001; Rachel et al. 2002; Geslin et al. 2003a). The actual shape may vary from more elongated to lemon-shaped, and may or may not include tails. Recent analysis has suggested that the spindle-shaped viruses can be assigned to two large virus families based on the analysis of their structural proteins (Krupovic et al. 2014). The Fuselloviridae includes all non-tailed spindle-shaped viruses, representing at least five genera and including viruses infecting Crenarchaea, thermophilic Euryarchaea and hypersaline Euryarchaea. The tailed spindle-shaped viruses fall into two genera in the Bicaudoviridae, but all isolated viruses infect members of the Sulfolobales.

6.2.2.1 Bicaudaviridae

The tailed spindle-shaped viruses tend to be larger than the non-tailed spindle-shaped viruses, however, the size of the virions often vary in size (Hochstein et al. 2015). Of the five isolated, two infect *Acidianus* spp. and the other three infect *Sulfolobus* spp. Isolates with two tails, the *Acidianus* two-tailed virus (ATV), represents the *Alphabicaudavirus*. The ATV virus is unique in that the two tails actually grow after the virus particles are released from the cell (Häring et al. 2005b). When stored at low temperatures, the tailless particles remain stable, but at 75 °C, the volume of the particles decrease and the tails elongate, likely through the polymerization of virus proteins (Häring et al. 2005; Prangishvili et al. 2006b). The particle contains a 62.7 kb circular dsDNA genome with 72 predicted ORFs. Eleven of the ORFs encode structural proteins associated with the virion. Several of the ORFs have some sequence similarity to transposases from *Sulfolobus* plasmids, while others show similarity to unknown ORFs from other crenarchaeal viruses (Prangishvili et al. 2006b). Like ABV, ATV was only successfully amplified in an *A. convivator* strain. However, unlike ABV, ATV lysed the cells around four days post-infection when grown at 75 °C. When incubated at 85 °C, no lysis occurred, and instead the cells were lysogenized (Prangishvili et al. 2006b). This suggests that environmental conditions control the life cycle of the virus. A second isolate in this family, ATV2, has been reported, with a slightly smaller genome of 57.9 kb, but details of its biology and sequence have yet to be published (Garrett et al. 2015).

The other four tailed spindle-shaped viruses have been assigned to the *Betabicaudavirus* (Krupovic et al. 2014). These virions are released with a single tail already present. Three of these viruses, *Sulfolobus tenchongesis* spindle-shaped virus 1 (STSV1) (Xiang et al. 2005), *Sulfolobus tenchongesis* spindle-shaped virus 2 (STSV2) (Erdmann et al. 2014b) and *Sulfolobus* monocuadavirus 1 (SMV1) (Erdmann and Garrett 2012) have been isolated using *Sulfolobus* spp. as the host, while the fourth, *Acidianus* tailed spindle virus (ATSV) infects *Acidianus*

(Hochstein et al. 2015). Like ATV, these viruses all have circular dsDNA genomes, which range from 48 to 76 kb and have as many as 96 putative ORFs (Hochstein et al. 2015). All *Betabicaudavirus* contain a tyrosine recombinase family integrase, suggesting that the viral genomes can integrate into their host genomes (Hochstein et al. 2015). While temperature appears to be important in the induction of the lytic cycle in ATV, no factors leading to induction of the *Betabicaudavirus* have been identified (Erdmann et al. 2014b). Additionally, the *Betabicaudavirus* do not appear to lyse their hosts, suggesting the particles are released through an uncharacterized mechanism common to other crenarchaeal viruses (reviewed in Dellas et al. 2014).

6.2.2.2 Fuselloviridae

The non-tailed spindle-shaped viruses have been assigned to the family Fuselloviridae. This family contains the largest number of virus isolates, including viruses infecting halophilic Euryarchaeota, thermophilic Euryarchaeota from deep-sea hydrothermal vents and Crenarchaea. The first fusellovirus, SSV1 (Sav-1), was reported in 1984 to be a temperate, UV inducible virus (Martin et al. 1984). Since the first virus was isolated, thirteen other spindle-shaped viruses have been isolated, which have recently been assigned to five different genera based on analysis of the major capsid proteins and the number of shared genes (Krupovic et al. 2014). The *Alphafusellovirus*, *Betafusellovirus* and *Gammafusellovirus* include viruses infecting thermophilic Crenarchaeaota, while the *Deltafusellovirus* and *Epsilonfusellovirus* infect Euryarchaeaota. All of the fuselloviruses have a spindle-shape, however some are more elongated or club-shaped, rather than round and lemon-like. Additionally, the abundance, shape and stickiness of the end filaments vary between virus isolates (Redder et al. 2009).

The *Alphafusellovirus* includes the eight *Sulfolobus* viruses (SSV1, SSV2, SSVRH, SSVK1, SSV4, SSV5, SSV6 and SSV7) (Stedman et al. 2003; Wiedenheft et al. 2004; Redder et al. 2009). The genomes from members of this genus are circular dsDNA, and much smaller than the Bicaudaviridae, ranging from 14.7 to 17.6 kb. The genomes all include a tyrosine recombinase family integrase (Palm et al. 1991; Muskhelishvili et al. 1993) and twelve other core genes. SSV1 is the best studied *Alphafusellovirus*. It infects the cell, produces a few particles with limited impact on growth and integrates into the host genome. Usually three to four circular SSV1 genomes in plasmid form remain in the cell (Schleper et al. 1992). After UV exposure, multiple particles are released, but no cell lysis has been observed (Martin et al. 1984; Schleper et al. 1992). To characterize the infection cycle, virus transcription after UV induction of the virus has been described (Reiter et al. 1987; Frols et al. 2007). While some viral genes are constitutively expressed even without induction (Reiter et al. 1987), a short mRNA transcript is produced immediately after UV exposure. This is followed by three early transcripts and five late transcripts. Each transcript includes multiple genes, with DNA binding proteins transcribed early and structural genes on the late transcripts (Frols et al. 2007). Recently, a small transcript encoding a DNA binding protein was identified in

non-induced cells (Fusco et al. 2013), which may regulate lysogenic expression of SSV1. While SSV2 and SSV7 can also be induced by UV irradiation (Stedman et al. 2003; Redder et al. 2009), induction has not been reported for the other members of the *Alphafusellovirus*.

The single tailless spindle shaped *Acidianus* spp. virus (ASV1) (Redder et al. 2009) has been classified as a *Betafusellovirus*. While ASV1 shares genomic similarity to *Alphafusellovirus*, its genome is larger (24.2 kb) and its major capsid protein (MCP) is sufficiently different to consider it as a new genus (Krupovic et al. 2014). The virus was identified as an integrated form and as an extrachromosomal element in the genome sequence of *Acidianus brierleyi* DSM 1651 (Redder et al. 2009). Production of virus particles in the culture was sufficient to characterize and sequence ASV1. The larger genome of ASV1 is partly due to gene duplication and partly due to genes not present in the *Alphafusellovirus* genomes. Most of the ASV1 genes were unique and did not match publically available sequences (Redder et al. 2009).

The *Gammafusellovirus* include a single virus, *Aeropyrum pernix* spindle-shaped virus 1 (APSV1) (Mochizuki et al. 2011), infecting *Aeropyrum pernix*, a crenarchaeal hyperthermophile originally isolated from a coastal sulfidic vent (Sako et al. 1996). Related organisms have also been detected in terrestrial hot springs using DNA sequencing techniques (Young et al. 2005; Snyder et al. 2007). While initially, APSV1 was thought to be more similar to the Bicaudaviridae, subsequent analysis has determined it is more similar to the Fuselloviridae (Krupovic et al. 2014). The particles are similar to those observed for *Alphafusellovirus* and *Betafusellovirus*, but sufficient differences exist in the sequence of the MCP to assign APSV1 to a new genus (Krupovic et al. 2014). Additionally, the genome of APSV1, at 38 kb, is much larger than the SSV1-like or ASV1 viruses, although it is also circular dsDNA. APSV1 was not produced when *A. pernix* was grown under ideal conditions, but when shaking of the culture was reduced and growth rates decreased, virus production appeared to be induced, again indicating that environmental conditions affect virus-host interactions. APSV1 genome was integrated into the *A. pernix* genome when the virus was identified and sequenced, indicating the virus lysogenizes the host cells, but no extrachromosomal plasmid form was observed (Mochizuki et al. 2011).

The remaining two genera of fuselloviruses contain isolates that infect members of the Euryarchaeota. These viruses include the thermophilic viruses, *Pyroccous abyssi* virus (PAV1) and *Thermococcus prieurii* virus (TPV1), both isolated from hydrothermal vents (Geslin et al. 2003b; Gorlas et al. 2012). These two viruses have been assigned to the *Deltafusellovirus* based on the MCP sequence and genome content (Krupovic et al. 2014). A virus isolated from *Methanococcus voltae* A3, a mesophilic methanogen, has also been assigned to this group (Wood et al. 1989; Krupovic et al. 2014). The *Epsilonfusellovirus* includes those viruses previously identified as the Salterprovirus. This genus includes His1 and His2, which infect the halophilic Euryarchaea *Haloarcula hispanica* (Bath and Dyall-Smith 1998; Bath et al. 2006). While the virions of the *Deltafusellovirus* have circular dsDNA genomes and have been shown to integrate into the host genome, the

Epsilonfusellovirus have linear dsDNA genomes with inverted terminal repeats and no reports of integration. The His1 and His2 genomes share little similarity, however both appear to include a DNA polymerase, unlike the other fuselloviruses (Bath et al. 2006).

6.2.3 Clavaviridae

The single isolated virus belonging to the proposed family Clavaviridae is the *Aeropyrum pernix* bacilliform virus (APBV1) (Mochizuki et al. 2010). This virus has a ridged rod shape, with one rounded end and one pointed end. The genome is circular and dsDNA, but at 5.3 kb, is the smallest crenarchaeal virus genome. Only 14 ORFs have been identified, none of which have any similarity to known virus sequences. This highlights the challenge of identifying novel crenarchaeal viruses in environmental sequences. With little to no similarity to known viruses, alternative approaches to identifying novel viruses have been developed (see metagenomics below). APBV1 does not lyse infected cells, nor does it appear to impact growth rates of the cells (Mochizuki et al. 2010), even though the genome does not integrate into the host genome. A stable relationship between the host and virus may benefit APBV1 in the extreme conditions of the hot springs, but whether there are costs or benefits to the host and how that may impact the ecology of these organisms is unknown.

6.2.4 Globulaviridae

This family of archaeal viruses has two isolates, *Pyrobaculum* spherical virus (PSV) (Häring et al. 2004) and *Thermoproteus tenax* spherical virus (TTSV1) (Ahn et al. 2006), infecting members of the Thermoproteales. PSV is reported to infect both the *Pyrobaculum* sp. D11, from which it was isolated, and *Thermproteus tenax*. This is the only archaeal virus reported to infect multiple genera, although other viruses have successfully infected different species of the same genus (e.g. SSV1, STIV). The Globulaviridae are spherical particles between 70 and 100 nm, with linear dsDNA genomes surrounded by a lipid envelope. PSV has a 28.3 kb genome, while TTSV1 has a smaller 21.6 kb genome. While the ORFs encoding the structural proteins could be identified, the remainder of the ORFs did not match any sequences in public databases. These viruses appear to exist in a stable carrier state in the hosts and do not lyse the cells to release virions. PSV was originally isolated from cells collected from an 85 °C, pH 6.0 hot spring in Yellowstone National Park, USA (Häring et al. 2004). Viral metagenomes from two other neutral to slightly alkaline hot springs were found to have sequences that could be matched to the PSV genome, indicating that these viruses occur in multiple springs throughout the park (Schoenfeld et al. 2008).

6.2.5 *Guttaviridae*

The Guttaviridae includes two isolates, but only one has a sequenced genome. The *Sulfolobus neozealandicus* droplet-shaped virus (SNDV) was originally described as having a droplet-shape, ribbed surface and a methylated, circular dsDNA genome (Arnold et al. 2000a). The pointed end of the particle had several fibers. Unfortunately, this virus was not sequenced and is no longer in culture, but this virus has been classified in the *Alphaguttavirus*. Based on a similar droplet-shaped morphology, although without the tail fibers, the recently isolated *Aeropyrum pernix* ovoid virus 1 (APOV1) was classified with the Guttaviridae, but in a new genus, *Betaguttavirus* (Mochizuki et al. 2011). APOV1also has a circular dsDNA genome, slightly smaller at 13.8 kb compared to the 20 kb one described for SNDV. It was confirmed that APOV1 integrates into the host genome, and an integrase was one of the few ORFs on the virus genome with any similarity to known sequences (Mochizuki et al. 2011). A second virus APOV2 has also been identified, but not fully characterized, and infects a different strain of *A. pernix*.

6.2.6 *Ligamnevirales*

There are two families of linear crenarchaeal viruses that have been described, the Lipothrixviridae and the Rudiviridae. Comparative genomic analysis and structural studies suggest that these two families should be classified together (Prangishvili et al. 2006a; Prangishvili and Krupovic 2012). The strong similarity in the MCP sequences of these virus and the presence of shared genes between families, supports a shared evolutionary history, but may also indicate the potential for recombination to drive evolution of these viruses in hot springs today.

6.2.6.1 Lipothrixviridae

One of the largest groups of virus isolates infecting the Crenarchaea is the Lipothrixviridae. The first members of this family were isolated from cultures of *Thermoproteus tenax* (TTV1, TTV2 and TTV3) (Janekovic et al. 1983). Under heterotrophic growth, lysis occurred after sulfur was depleted. Three different particles were characterized: TTV1 described as rods, TTV2 described as thin filaments and TTV3 described as long filaments with pointed tips (Janekovic et al. 1983). All of the eleven isolated lipothrixviruses are flexible filaments, varying in length from 900 to 2000 nm with linear dsDNA genomes (reviewed in Pina et al. 2011). The ends of the virions vary, with different numbers of filaments and structures. The genomes show some similarity to each other, but with sufficient differences to classify them into four genera.

TTV1 is the only member of the *Alphalipothrixivirus*. The virion is composed of four major proteins with an envelope surrounding a core composed of a 15.9 kb dsDNA genome (Neumann et al. 1989). Sequencing of the genome resulted in multiple variants, with associated variations in structure suggesting rapid mutations of this virus within the host (Neumann and Zillig 1990).

The largest group of isolates belongs to the *Betalipothrixvirus*. This genus includes viruses infecting *Sulfolobus* (SIFV and SIFV2) (Arnold et al. 2000b; Garrett et al. 2015), *Acidianus* (AFV3, AFV6, AFV7, AFV8, AFV9) (Bize et al. 2008; Vestergaard et al. 2008b) and *Thermoproteus* (TTV2, TTV3). These isolates share similar morphologies and genomic content. Studies have revealed that the ends of the linear dsDNA genomes are not covalently bound, but that proteins are bound to the ends of the DNA, which also have inverted terminal repeats of varying lengths (Bize et al. 2008). Additionally, all *Betalipothirixvirus* have low complexity AT rich regions with few or no ORFs within their genomes. The protein coat of these viruses is composed of a helix of proteins surrounding the genome with an outer lipid envelope. The *Betalipothrixvirus* have larger genomes than the *Alphalipothrixvirus*, with sizes ranging from 37 to 41 kb compared to the 15.9 kb of TTV1. None of the *Betalipothrixvirus* isolates have been reported to lyse their hosts.

The *Gammalipothrixvirus* and *Deltalipothrixvirus* are each represented by one isolate that infects the genus *Acidianus*. AFV1, in the *Gammalipothrix*, has a 20.8 kb linear dsDNA genome and is 900 nm long with claw-like structures at the end of the filaments (Bettstetter et al. 2003). AFV1 is reported to exist in a carrier state and does not appear to lyse the host cells. The *Deltalipothrixvirus*, AFV2, differs from other lipothrixviruses in its morphology, showing no regular repeating structure on its surface. The particle contains seven proteins which form structures at the ends that resemble bottle brushes with round caps (Häring et al. 2005c). Comparison of the 51 putative ORFs on the 31.8 kb genome showed similarities to other lipothrixviruses, but some unique characteristics. A tRNA was identified in the genome, which has not been reported for other crenarchaeal viruses. AFV2 also exists in a carrier state and particles are released without cell lysis (Häring et al. 2005c).

6.2.6.2 Rudiviridae

The second family of linear viruses is the Rudiviridae. These viruses are non-enveloped stiff rods ranging in size from 610 to 900 nm, depending on the length of their linear dsDNA genomes (reviewed in Pina et al. 2011). There are six isolated rudiviruses infecting four genera of Crenarchaea. *Sulfolobus islandicus* rod shaped virus 1 (SIRV1), SIRV2 and SIRV4 infect *Sulfolobus* (Prangishvili et al. 1999; Garrett et al. 2015), while ARV1 infects *Acidianus* (Vestergaard et al. 2005) and SRV infects *Stygiolobus* (Vestergaard et al. 2008a). TTV4 infects *Thermoproteus tenax* (Zillig et al. 1998), but few details about the morphology or genome content of this isolate exist. The glycosylated capsid proteins of the

rudiviruses form a superhelix around the DNA and the stiff rod shaped particle end with short terminal fibers (Prangishvili et al. 1999). The Rudiviridae have genomes ranging from 24.6 to 35.4 kb. The ends of the linear genome have inverted terminal repeats and form covalently closed hairpin structures similar to those seen in some eukaryote viruses (Blum et al. 2001). The four well characterized isolates share seventeen genes, including structural genes, a dUTPase (SIRV1, SIRV2, SRV) or a thymidylate synthase (ARV1, SRV), glycosyl transferases, a methytransferase and a Holliday junction helicase and resolvase. Replication of rudiviruses occurs through a site-specific single-strand nicking within the inverted terminal repeats, generating concatemers of the genome. The genomes are split into monomers using a virus-encoded Holliday junction resolvase (Peng et al. 2001).

Transcriptional analysis of SIRV1 and SIRV2 indicates little temporal regulation of transcription (Kessler et al. 2004). Thirty minutes after infection, transcripts for all virus genes except one could be detected, mostly as polycistronic message. After two to three hours, transcription of the MCP increased and particle assembly soon followed. A single gene was then transcribed prior to release. While initial reports indicated that SIRV1 and SIRV2 existed in a stable carrier state and were not lytic, more recent work has indicated that SIRV2 does lyse the cells (Bize et al. 2009). Prior to virion release, pyramids form on the surface of the cells due to the production of large amounts of one protein (Quax et al. 2010). These pyramids break at the apex, releasing viruses. Interestingly, this mechanism of cell lysis has also been identified for STIV (Turriviridae, see below) (Ortmann et al. 2008; Brumfield et al. 2009). The gene associated with the pyramid structures is homologous in SIRV2 and STIV has been identified in the genomes of SIRV1 and SRV, but not in ARV1 or STIV2 (Quax et al. 2010). This suggests that this mechanism for lysis, occurring across different families of viruses infecting different genera of crenarchaeal hosts, may be transferred between viruses via horizontal gene transfer.

6.2.7 Spiraviridae

The *Aeropyrum* coil-shaped virus (ACV) represents the only crenarchaeal virus isolated that does not have a dsDNA genome (Mochizuki et al. 2012). Instead, this virus has a positive sense single-stranded DNA (ssDNA) genome. The viral particle is non-enveloped, cylindrical and hollow, with appendages at the ends of the particles. The genome is circular and 24.8 kb, making it one of the largest ssDNA genomes isolated to date. The genome is predicted to encode 57 ORFs, only four of which have any similarity to public databases. Modeling of predicted protein structure enabled putative functions to be assigned to fourteen ORFs. These include thioredoxins, carbohydrate metabolism, DNA binding proteins, a protease and a recombinase. ACV does not appear to lyse its host, an *Aeropyrum pernix* strain isolated from the Yamgawa Hot Spring in Japan, and was unable to infect any other strain of *Aeropyrum*, indicating a narrow host range where it exists in a stable carrier state. The isolation of a ssDNA virus was surprising as the harsh conditions

of hot, acidic pools was thought to preclude these types of viruses and select for the more stable dsDNA viruses. Other ssDNA archaeal viruses had been previously isolated from hypersaline conditions, indicating that extreme environments do not preclude ssDNA viruses (Maija et al. 2009). Additionally, previously conclusions that crenarchaeal viruses were not lytic due to these conditions were also shown to be incorrect, suggesting that the study of hot spring viruses may yield more surprises in the future.

6.2.8 Turriviridae

The proposed family, Turriviridae, includes two closely related virus isolates, *Sulfolobus* turreted icosahedral virus (STIV) (Rice et al. 2001) and STIV2 (Happonen et al. 2010). These viruses have a clear icosahedral shape with turrets associated with the vertices, although the turrets vary significantly between the two isolates. The structure of the MCP has been shown to be related to that of PRD1, a bacteriophage, and adenoviruses infecting eukaryotes (Rice et al. 2004; Happonen et al. 2010). Additionally, the virus SH1, infecting a halophilic Euryarchaea appears to be related to this family (Porter et al. 2005; Jalasvuori et al. 2009). The turrivirus particles have internal lipid membranes derived from the host cell surrounding the genome (Maaty et al. 2006; Khayat et al. 2011; Fu and Johnson 2012). STIV and STIV2 have circular dsDNA genomes, and share significant similarity in ORFs and genomic organization. Some genome rearrangement can be observed between the two viruses (Happonen et al. 2010). One gene that is not present in STIV2 is the one that is associated with pyramid formation and cell lysis (Quax et al. 2010). While initial reports suggested that STIV2 may lyse the cells, this has not been confirmed and would not be predicted based on the lack of this gene.

Infection of a susceptible isolate of *Sulfolobus solfataricus* by STIV has been well characterized using transcriptomics and proteomics. The viral genes are all transcribed by eight hours post infection, with peak expression around 24 h (Ortmann et al. 2008). By 32 h, cells begin to lyse. Similar to SIRV1 and SIRV2 (Kessler et al. 2004), little temporal regulation of transcription was observed for SITV. Analysis of infected cells with TEM showed step-wise assembly of virus particles starting around 24 h after infection (Brumfield et al. 2009). Initially, cellular membranes increase, then crystalline structures are observed. Immature, spherical particles of lipids with protein shells are then observed, followed by insertion of the turret structure into the vertices and then insertion of the genome. This assembly is similar to that of PRD1 (Karhu et al. 2007), which has a similar morphology supporting a shared evolutionary history (Ortmann et al. 2006). Proteomic analysis of infected *S. solfataricus* cells detected thirteen STIV proteins, with four appearing to have undergone post-translational modification (Maaty et al. 2012). The majority of the detected proteins were part of the virion, which should be produced at high levels. Additionally, high levels of C92, the protein associated

with pyramid formation was also detected, although later in the infection cycle, just prior to lysis (Snyder et al. 2013).

6.3 From Isolates to the Environment

Studies of crenarchaeal virus isolates have provided the opportunity to develop understanding of the evolution and cell biology of Crenarchaea. The development of genetic systems (Aucelli et al. 2006; Wirth et al. 2011) and shuttle vectors has enabled manipulation of host-virus systems and host genomes for studying fundamental cell biology. As almost all of the isolates have fully sequenced genomes, multiple comparative genomics studies have provided insights into the evolutionary relationships between viruses (Wiedenheft et al. 2004; Prangishvili and Krupovic 2012; Krupovic et al. 2014), and in some cases plasmids (She et al. 1998, 2004; Stedman et al. 2000; Berkner and Lipps 2007). However, what these isolates cannot provide is a clear understanding of the diversity, abundance and ecological role of crenarchaeal viruses in the environment. To obtain this information, multiple approaches have been applied to environmental samples including incubations, using DNA signatures of known viruses, exploiting the CRISPR/Cas system and application of metagenomics.

6.3.1 Virus Dynamics in Hot Springs

One way to characterize the contribution of viruses to the ecology of a habitat is by quantifying the rates of virus production and loss or turnover. In marine systems, multiple studies have used several different approaches to measure the impact of viruses on microbial communities (e.g. Heldal and Bratbak 1991; Wilcox and Fuhrman 1994; Fuhrman and Noble 1995; Bongiorni et al. 2005; Danovaro et al. 2009). These studies have determined that viruses remove an average of 25% of the prokaryote production in ocean waters, although the estimates range from almost none to over 100% (reviewed in Wilhelm and Suttle 1999). Temperate viruses can be induced using mitomycin C, resulting in estimates that ~25% of the prokaryote community is lysogenized (Ortmann et al. 2002; Weinbauer et al. 2003; Long et al. 2008). There are only two published studies that measure the abundance and production rate of viruses in hot springs (Breitbart et al. 2004a; Lee et al. 2007).

The first study was carried out in hot springs around the Long Valley caldera in California, USA, (Breitbart et al. 2004a). The sampled springs ranged in temperature from 73 to 84 °C with pH values around 7.6. The other study took place at the hot springs in the Alvord Desert in Oregon, USA, where temperatures ranged from 61 to 96 °C and the pH was approximately neutral (Lee et al. 2007). Both studies measured similar abundances of prokaryote cells and viruses, with cells on the order of 10^5 ml^{-1} and viruses ranging from 10^4 to 10^6 ml^{-1}. The estimated virus to

prokaryote ratios averaged 5.2 in the Long Valley caldera, but was higher at 10.52 in the Alvord Desert. These values are similar to estimates made for marine systems (reviewed in Wommack and Colwell 2000), although much higher than the average of 0.3 measured for eight acidic and two neutral hot springs in Yellowstone National Park, USA (Schoenfeld et al. 2008; Ortmann, unpublished data).

In the neutral hot springs, rates of virus production ranged from 3.8×10^4 to 1.5×10^6 viruses ml^{-1} d^{-1} (Breitbart et al. 2004a; Lee et al. 2007). These particles may be the result of cell lysis, but may also be due to release of virus particles from cells without lysis. Assuming lysis and a burst size of 20 virions per cell, these studies estimated that between 9 and 32% of the prokaryote cells were lysed daily. Mitomycin C was used to induce temperate phages, with varying results. In the Alvord Desert springs, 18–21% of the cells were determined to be lysogenized (Lee et al. 2007), compared to only 1–9% of cells in the Long Valley caldera (Breitbart et al. 2004a). While variable, these values are similar to estimates from marine systems (reviewed in Wommack and Colwell 2000).

In both of these studies, the microbial communities in the hot springs likely included both Bacteria and Archaea. In some of the springs with lower temperatures, eukaryotes may also have been present. Thus, these studies do not directly reflect what may be occurring in springs dominated by Crenarchaeaota. The variability in the number of viruses induced by mitomycin C agrees with the variability in the response of isolates to the treatment. While SSV1, ATV and the euryarchael virus PAV1 have been reported to be induced by mitomycin C (Liu and Huang 2002; Geslin et al. 2003b; Prangishvili et al. 2006b), none of the other isolates have been induced in this manner. Mining of metagenomics data for homologs to the C92/P98 proteins associated with pyramid structures in the lytic viruses STIV and SIRV2 (Quax et al. 2010; Snyder et al. 2013) did detect multiple groups of this protein present in two hot springs in Yellowstone National Park, USA, but at relatively low abundance suggesting that lysis is not common in these habitats (Snyder et al. 2011). The production of viruses with minimal impact on growth rates and few infections resulting in lysis would produce very different biogeochemical cycling than an ecosystem dominated by lytic viruses. Rather than releasing organic matter into the environment where it becomes available to heterotrophic species, chronic virus release without lysis could result in more competition for fewer resources or a dependence on autotrophic metabolism. Variation in the abundance of C92-like proteins over time in a single hot spring suggests that lysis rates may vary as well (Snyder et al. 2011). Potentially, when high rates of lysis do occur, organisms like *Sulfolobus* spp. may be able to exploit a rare resource by turning to heterotrophic growth.

6.3.2 Dynamics of Specific Viruses in Hot Springs

One technique that has provided information on the abundances, dynamics and diversity of viruses in lakes and oceans is the targeting of conserved genes within a

group of known viruses. This has enabled studies of Myoviridae, with specific studies targeting Cyanomyoviridiae (Short and Suttle 2005), as well as monitoring the abundance and diversity of the Phycodnaviridae, a group of large algal viruses (Short and Suttle 2002; Rozon and Short 2013). In hot springs, only one study has been carried out which monitored the diversity of specific viruses using conserved genes. In a study of three hot springs over a two-year period, the diversity of *Alphafusellovirus* and Rudiviridae was monitored (Snyder et al. 2007). Using PCR primers designed to detect the largest ORF in SSV-like viruses and the MCP of SIRV-like viruses, the diversity of these viruses were monitored. Within each individual spring, multiple clades of each virus were detected, with different clades detected over time. The virus clades were detected in all three springs, but not at the same time. Models indicated that movement of the viruses among hot springs was responsible for maintaining the observed patterns in virus diversity rather than mutation within a single hot spring. This study suggests that hot springs have diverse virus populations that can easily be dispersed between pools, potentially through the air (Snyder et al. 2007). In contrast to the viruses, the host cells have been shown to have limited dispersal ability (Whitaker et al. 2003; Held and Whitaker 2009), potentially due to the importance of the spring chemistry in selecting the microbial community (Macur et al. 2004; Inskeep et al. 2010).

While a PCR-based approach can provide details regarding individual virus groups in the environment, it is limited in the need for prior sequence information. The two virus groups chosen for this study were both represented by multiple, closely-related isolates (four for SSV-like and three for SIRV-like) (Snyder et al. 2007), enabling PCR primers to be designed to detect multiple known viruses, something that cannot be done when only one isolate exists. With many of the crenarchaeal viruses representing novel families, there are few opportunities to generate consensus sequences and broadly targeted primers. With the increase in the number of virus isolates, this approach may be applied to such genera as the *Betabicaudavirus*, the *Betalipothrixvirus*, however, determining the diversity of viruses not represented by isolates requires a different approach.

6.3.3 Metagenomic Analysis of Hot Springs

One approach to understanding virus diversity that has worked well in other environments is metagenomics, specifically targeting the virus fraction. In this approach, samples are processed to remove the cellular fraction, viruses are concentrated and the DNA or RNA is extracted and sequenced. Because of the low concentrations of viral nucleic acids, whole genome amplification is often used. This approach has demonstrated the high diversity of viruses in oceans, sediments and other environmental samples (Breitbart et al. 2003; Daniel 2005; Angly et al. 2006; Culley et al. 2006; Bench et al. 2007; McDaniel et al. 2008). Remarkably, few metagenomics studies have been carried out in hot spring environments.

One study reported on the sequencing of the virus fraction of two different neutral hot springs (Schoenfeld et al. 2008). These springs were inhabited by both Crenarchaea and Bacteria, however, the majority of matches between the viral metagenomes and known viral isolates were to archaeal viruses. Significantly, sequences matching almost all of the ORFs of PAV were detected in these two springs. This matches with the temperature and pH of the springs, which would predict more Thermoproteales under neutral conditions compared to Sulfolobales (Inskeep et al. 2010). Sequences matching SRV, ARV, STSV1, SIFV and ATV were also detected. Multiple lysin-like genes were detected in the metagenomes, suggesting lytic viruses were common in the hot springs. The presence of integrases also suggests that some viruses were temperate. This would support the findings that virus production can be increased by mitomycin C induction in neutral hot springs (Breitbart et al. 2004a; Lee et al. 2007). The PHACCS tool was used to estimate the number of virus genomes present in these two hot springs (Angly et al. 2005). While the estimates of 1400 and 1310 was lower than estimates for marine systems (e.g. Breitbart et al. 2004b; Angly et al. 2006), it was higher than the estimates from metagenomes from an acidic hot spring (Bolduc et al. 2015).

Nine viral metagenomes were generated from sampling an acidic (pH 2.0–4.5) hot spring over a two-year period, with a tenth sample collected three years later. These metagenomes were analyzed using network analysis rather than simply generating contigs and matching them to known sequences (Bolduc et al. 2015). Using the PHACCS tool, the nine metagenomes collected over the two years were predicted to contain between 192 and 1342 virus genomes. Using network analysis, 110 virus groups were identified, which likely represent family/sub-family clusters. Thus, while grouping individual strains together, which could then be targeted using a PCR-sequencing approach (Snyder et al. 2007), network analysis also gives an overall view of the virus community within a spring. Of the 110 virus groups, 76 were always present in the hot spring, and 109 were present in at least half of the time points. Additionally, several of the viral sequences were detected in a second hot spring more than 30 km away with similar geochemistry (Bolduc et al. 2015).

The network analysis was also able to identify that only seven of the 110 virus groups shared sequence similarity with known virus isolates. These groups included most of the Fusseloviridae, SIRV1/SIRV2, *Alphabicaudavirus*, *Gammalipothrixvirus*, *Betalipothrixvirus*, Turriviridae and a group that included ARV, SRV, and STSV1 and STSV2. While the relative abundances of sequences associated with each group did vary over time, it is difficult to interpret reads based on amplified DNA. However, there appears to be a relatively persistent, stable virus community in acidic hot springs (Bolduc et al. 2015). This would agree with the PCR-based analysis of SSV-like and SIRV-like viruses over a two year period (Snyder et al. 2007). Perhaps more interesting than detecting known viruses in this hot spring, is the detection of 93 novel virus groups within a spring characterized by low cellular diversity and dominated by Crenarchaea (Bolduc et al. 2012). This suggests that the diversity of the isolated crenarchaeal viruses underestimates the true diversity of viruses in hot springs. Thus, extending our understanding of host-virus interactions between cultured hosts and viruses to environmental samples is potentially more

limited than previously expected. The large number of uncharacterized viruses cannot be assigned to a particular lifestyle (e.g. lytic, temperate, stable carrier state), and their potential impacts on their hosts are completely unknown.

The previous viral metagenomes were targeted to detecting DNA viruses. As all of the isolated crenarchaeal viruses have DNA genomes, it was first believed that RNA viruses may not exist in the extreme conditions of the hot springs. However, using the same acidic hot spring as for the network analysis, viral RNA was targeted and successfully sequenced (Bolduc et al. 2012). Assembly of the reads resulted in the identification of two contigs containing RNA-dependent RNA polymerases (RdRp), indicative of positive-strand RNA viruses. A reanalysis of this dataset identified nine more contigs in three lineages from the same hot spring representing a diverse community of positive-strand RNA viruses (Wang et al. 2015). Although multiple other RNA-based metagenomes were analyzed from many different environments, the only one which contained the hot spring associated RdRp were from the original spring. Most of the sequences from the RNA-based viral metagenome did not match known sequences, suggesting they may represent novel crenarchaeal RNA viruses (Bolduc et al. 2012). Together, this indicates that a single acidic hot spring in Yellowstone National Park has a low diversity microbial community composed of eight archaea, one bacteria, 110 DNA viruses and at least eleven RNA viruses, which vary in abundance and strain diversity over time (Snyder et al. 2015). This series of studies gives the best picture of virus dynamics in hot springs to date, yet lacks estimates of rates or an understanding of the impacts of the viruses on biogeochemical cycling or host growth and diversity.

6.3.4 Crenarchaeal CRISPR/Cas Systems

Since first demonstrated to be a prokaryotic immune system in 2007 (Barrangou et al. 2007), studies of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR associated) system have increased rapidly. The defining characteristics of a CRISPR loci is the presence of direct repeats (DR) separated by spacer regions. Usually, a leader sequence can be found at one end of the loci along with the Cas genes. Analysis of CRISPR loci led to the observation that many of the spacer sequences were homologous to viruses, plasmids and other extrachromosomal elements (Pourcel et al. 2005; Shah et al. 2009). This, in turn, led to experiments showing that CRISPR/Cas systems were a type of prokaryotic adaptive immune system, with co-evolution between the host spacer sequences and the homologous regions of the virus genomes (Barrangou et al. 2007; Deveau et al. 2008; Horvath et al. 2008). Although specifics will vary depending on the type of CRISPR/Cas systems present, a general mechanism has been descripted for spacer acquisition and immunity. In response to invasion by viral or plasmid nucleic acid, a complex of Cas proteins recognizes the nucleic acid as foreign and creates a new spacer at the leader end of a CRISPR loci. A secondary

exposure to foreign nucleic acids results in transcription of the CRISPR loci and processing of the RNA into individual spacers, which binds to a Cas protein complex. Spacer/Cas complexes attach to oreign nucleic acid with a 100% match to the spacer, targeting it for degradation (reviewed in Horvath and Barrangou 2010).

The CRISPR/Cas system is present in about 40% of bacterial genomes (Kunin et al. 2007). The mechanism of CRISPR immunity in Bacteria is well characterized (e.g. Brouns et al. 2008; Deveau et al. 2008; Marraffini and Sontheimer 2010), and the bacterial Cas9 endonuclease has been successfully used to develop a powerful tool for genome editing and engineering for biotechnology and medical applications (reviewed in Hsu et al. 2014). CRISPR/Cas systems also exist in most archaeal genomes, with the largest number in Crenarchaea, followed by the Korarchaea and the Euryarchaea (Brodt et al. 2011). The fewest have been identified in the Thaumarchea. Generally, there are multiple CRISPR loci present in the Sulfolobales strains for which multiple virus isolates exist (Garrett et al. 2015), providing good lab systems for studying the function and role of CRISPR/Cas under controlled conditions. Surprisingly, the archaeal CRISPR/Cas systems especially those found in the Sulfolobales, appear to be more complex than bacterial systems and may have additional functions beyond viral immunity (reviewed in Garrett et al. 2015).

Most members of the Sulfolobales have two to six different CRISPR loci. The CRISPR systems are classified into families based on the DR, the leader sequences and the associated Cas genes (Lillestol et al. 2009). In *S. solfataricus* P2, one of the most commonly used laboratory strains, there are six different CRISPR loci (Zhang and White 2013). These loci can be divided into two different repeat families and seven different interference complexes representing three different CRISPR types. While five of the CRISPR loci are complete, the sixth loci is missing a leader sequence and appears to be incapable of adding new spacer sequences. Comparisons of the spacer sequences in *S. solfataricus* P1, P2 and P3, multiple differences were observed in the spacers in the A, B, C, D and E loci, but the spacers were identical in the F locus (Garrett et al. 2015). As P3 was isolated several decades after P1 and P2, this highlights the importance of the leader sequence in the addition, and loss, of new spacers.

Laboratory studies have shown that the leader, along with specific Cas genes, is also necessary for transcription and processing of the CRISPR loci (Lillestol et al. 2009). Both strands of the locus are transcribed (Lillestol et al. 2006), but they undergo different processing depending on the strand to produce the short RNA that interacts with specific Cas proteins to form the functional part of the CRISPR/Cas system. Like the bacterial system, the archaeal CRISPR/Cas system gains new spacer sequences at the leader end (Erdmann and Garrett 2012; Erdmann et al. 2013). There is loss of spacers over time, although new spacers appear to be less stable than older spacers (Erdmann et al. 2014a), explaining why the tail end of CRISPRs seems to be more conserved across strains compared to the leader end (Held and Whitaker 2009; Held et al. 2010; Garrett et al. 2015). From the multiple lab studies with crenarchaeal hosts, a complicated picture of CRISPR/Cas systems

is emerging that suggests that immunity from viral or plasmid infection is not their only role.

Exposure to viruses with 100% matches to CRISPR spacers should prevent infection. For some crenarchaeal systems, this appears to occur, but in other instances it has not been observed (Ortmann et al. 2008; Brodt et al. 2011; Garrett et al. 2015). Additionally, exposure to multiple viruses at once can affect the response of the CRISPR/Cas system to infections. A transcriptomic study of infection by SSV1, SSV2 or both viruses together highlights these different responses (Fusco et al. 2015). When stably infected with SSV1, there is little response in genome expression compared to uninfected controls. Significantly, there is no change in the expression levels of the CRISPR loci or Cas genes. In contrast, infection with SSV2, which has increased virus production in late growth phase, results in a large response in the host gene expression as was previously observed in *S. solfataricus* P2 in response to STIV and SIRV2 (Ortmann et al. 2008; Okutan et al. 2013). Additionally, SSV2 infection results in the upregulation of CRISPR loci A, B, C, D and F, which have spacers matching fuselloviruses. CRISPR loci E lacks a spacer match to fuselloviruses and is not upregulated. Following this upregulation of CRISPR loci and Cas genes, SSV2 is integrated into the host genome, extrachromosomal copies are reduced to a small number and a stable carrier state appears to be achieved. This suggests that interactions between viruses and the archaeal CRISPR/Cas system may be important in stabilizing the carrier state observed for many of the crenarchaeal viruses isolated to date. Interestingly, co-infection of cells with SSV1 and SSV2 resulted in no induction of the CRISPR/Cas genes and expression of virus genes similar to that observed in cells infected with only one of the viruses. This observation indicates interactions among the two viruses and the CRISPR/Cas system of the infected cell.

A series of studies using SMV1 have also detected interaction between viruses and the CRISPR/Cas system of *Sulfolobus*. After co-infection of a host with SMV1 and either a conjugative plasmid (pMGB1) or STSV2, CRISPR loci in *Sulfolobus* were found to have acquired spacers from pMGB1 or STSV2, and the cells became resistant to the plasmid or STSV2 (Erdmann and Garrett 2012; Erdmann et al. 2013, 2014a). The cells continued to be susceptible to SMV1. The complexity arises when cells were infected with only pMGB1 or STSV2 without SMV1. Under those conditions, no spacers were acquired and a stable existence between the plasmid/virus and the host formed. Additionally, co-infection with STSV2 and SIFV2 or by STSV2, SIRV3 and SSRV resulted in stable infections of all viruses. When infected with just SMV1, growth was reduced and cells appeared to die (Erdmann et al. 2014a). A similar observation was made when *S. islandicus* was infected with SSV9 (Bautista et al. 2015). Following infection with SSV9, the cells entered a period of dormancy. When the CRISPR/Cas system is active in the cell, dormancy is reversed and the cell recovers, having been cured of the virus. If the system is not active, the cells eventually die, although they do not appear to be lysed. Analysis of the viruses involved in these studies suggest that in some cases,

viruses may be able to evade the CRISPR/Cas system. STSV2 has a modified DNA genome, while SMV1 carries an ORF that produces a DNA-binding protein that may protect the genome (Erdmann et al. 2014a), however, these hypotheses have yet to be tested.

Analysis of the seven *Thermoproteus tenax* CRISPR loci revealed that some of the spacer sequences were exact matches to the *T. tenax* genome (Plagens et al. 2012). Matches were also identified to other archaeal genomes, several crenarchaeal viruses, halophilic euryarchaeal viruses and some unclassified viruses previously detected in an enrichment culture from a hot spring (Garrett et al. 2010). Analysis of spacers across all the sequenced archaeal genomes indicate that most of the spacer match plasmids, viruses and other mobile DNA, however, multiple spacers were found to match cellular organisms (Brodt et al. 2011). This included self matches and matches to other Archaea, but also matches to insects, mammals and plants. How or why these Archaea would acquire spacer sequences to these other organisms remains a mystery. However, it is known that cells do not appear to respond to self matches with an autoimmune-type response, supporting an additional role of CRISPR/Cas beyond immunity (Brodt et al. 2011).

In the *T. tenax* study, five CRISPR loci were found to be expressed constitutively (Plagens et al. 2012). The two loci that were not expressed were not associated with Cas proteins, suggesting they may be inactive. An important observation in the study was the change in transcription in response to UV exposure or osmotic stress. Abiotic stress was also found to impact the acquisition of spacers in cells exposed to SMV1 (Erdmann et al. 2013) where a freeze-thaw cycle induced spacer acquisition. Thus, another potential role of CRISPR/Cas systems in crenarchaea may be to respond to environmental stresses. Stress has been associated with induction of temperate viruses and following infection with STIV, *S. solfataricus* upregulated multiple genes associated with physiological stress (Ortmann et al. 2008). Evidence also exists that suggests CRISPR/Cas systems are associated with toxin/antitoxin systems, which are also associated with physiological stress (He et al. 2014; Fusco et al. 2015), further supporting a connection between viral immunity and a stress response system.

The complexity of the archaeal CRISPR/Cas system and the results from the laboratory studies strongly suggest multiple functions for these systems in Archaea (Garrett et al. 2015). Viruses appear to be able to circumvent immunity from the system, possibly through genome modification or DNA binding proteins (Erdmann et al. 2014a). Additionally, viruses may be able to undergo mutations to avoid triggering a response. Analysis of Rudiviridae genomes detected 12 bp indels across the genomes which have been proposed to be in response to CRISPR/Cas system, illustrating co-evolution between hosts and viruses (Vestergaard et al. 2008b). This connection between viruses and CRISPR spacers, regardless of the functioning of the CRISPR/Cas system in cells, can be exploited to explore the ecology and diversity of viruses in the environment.

6.3.5 *CRISPR/Cas Systems in Environmental Studies*

A small number of studies in hot springs, as well as acid mine drainage and hydrothermal vent systems, have used CRISPR spacer sequences to better understand the diversity and ecology of viruses in extreme systems. These approaches have led to the analysis of cellular populations at a fine scale (Tyson and Banfield 2008; Held et al. 2010), characterization of the distribution of viruses (Held and Whitaker 2009), identification of new viruses (Andersson and Banfield 2008; Bolduc et al. 2012) and monitoring of unknown viruses over time (Snyder et al. 2010).

In hot springs, 39 strains of *S. islandicus* were isolated from a single hot spring (Held et al. 2010). Isolates were analyzed using multiple loci sequence analysis and by sequencing CRISPR loci. Based on the twelve sequences loci, one dominant population was detected in the spring, with multiple genotypes existing at very low abundances. Analysis of the three CRISPR loci present in these isolates revealed a more complex pattern. Although some loci could not be sequenced, analysis of CRISPR loci indicate no single dominant population, but much higher diversity, with almost all individuals having a different complement of spacers. This was different than in a lower-diversity, bacterial-dominated, acid mine drainage system where evidence of selective sweeps in response to viral infection was observed (Tyson and Banfield 2008). This may be due to differences in the functioning of the archaeal CRISPR/Cas system compared to the bacterial system. The pattern of high CRISPR spacer diversity across multiple individuals has been described as distributed immunity (Held et al. 2013; Childs et al. 2014). With distributed immunity, individuals share equally fit immune alleles (spacers) across the population. Based on models, this would lead to a diverse, stable host population and low virus population abundances, which has been observed in multiple hot springs sampled over time (Snyder 2005).

Another application of CRISPR/Cas systems to environmental studies was to characterize the biogeography of viruses infecting a single host. Previous work has shown that the same virus types can occur in habitats separated by large distances, but that work has generally focused on conserved virus sequences (Breitbart and Rohwer 2005; Short and Suttle 2005; Angly et al. 2006). Analysis of CRISPR spacers in eight strains of *S. islandicus* isolated from four different regions along with sequences of nine SSV-like (*Alphafusellovirus*) viruses was carried out to determine the distribution of viruses across the planet (Held and Whitaker 2009). The viruses appeared to be more similar to those isolated from geographically close locations than to viruses isolated from larger distances. Additionally, viruses tended to match more CRISPR spacers in host strains isolated from the same region than from host strains isolated from other areas. This pattern would suggest stronger biogeographical structuring hot spring viruses compared to oceanic viruses, which could be due to the fragmented nature of the hot spring habitat.

Because most of the sequences generated from a viral metagenome do not match any sequences in the database and cellular nucleic acid can sometimes contaminate the sequences, CRISPR spacer sequences may be one way to identify contigs in

metagenomes that belong to viruses. In the acid mine drainage, this approach was used successfully to construct whole virus genomes and connect the viruses to their hosts using CRISPR spacer matches (Andersson and Banfield 2008). In acidic hot spring, although low diversity compared to soil and marine systems, the diversity of viruses and hosts is too high to fully assemble whole genomes. CRISPR spacers were used to provide additional support to analysis of an RNA-based virus metagenome (Bolduc et al. 2012; Wang et al. 2015). Matches between the putative RNA viral contigs and CRISPR spacers in crenarchaeal species, suggests that these sequences likely represent RNA viruses infecting members of the Sulfolobales.

One final study which used CRISPR/Cas systems to carry out environmental studies developed microarrays using CRISPR spacer sequences from cellular metagenomes (Snyder et al. 2010). A single hot spring was sampled over time and virus enriched DNA was hybridized to the arrays. Over the four time points, between 224 and 650 spacers were detected, with 187 spacers always detected in the hot spring. The matches to the other spacers changed over time, although samples collected closer together in time were not always more similar to each other. This study also identified two communities of viruses in the hot springs, one that is persistent over time and one that is more transient, which agrees with studies using a targeted PCR-based approach as well as viral metagenomics (Snyder et al. 2007; Bolduc et al. 2015).

6.4 The Future of Crenarchaeal Virology

It seems that every study of viruses infecting the crenarchaea raises new questions about the diversity, ecology and co-evolution of these organisms. Although isolating new viruses is challenging, new families are still being added to the virus family tree, suggesting this avenue should still be pursued. A shift of the focus away from hosts from the Sulfolobales to other orders could provide new insights into the diversity of known families and, as seen for *A. pernix*, likely the addition of more new virus families. While studies of isolates in the lab continue to provide new understanding of the molecular biology of Archaea and the interactions between hosts and their viruses, it is perhaps the role of viruses in the hot springs where the biggest questions lie. How fast are viruses produced in hot springs? What percentages of viruses lyse their hosts? How does the stable carrier state impact growth, nutrient acquisition and competitiveness of the host cells? How does autotrophic growth impact host-virus interactions compared with heterotrophic growth? What role does the CRISPR/Cas system play in diversity of crenarchaeal viruses and hosts? All of these questions will require the application of field-based studies along with molecular biology-based techniques to hot springs representing different geochemical conditions and different geographical locations. By applying diverse techniques, a more complete view of crenarchaeal viruses in hot springs will emerge, although likely with a few more surprises and interesting discoveries along the way.

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Chapter 7
New Insights into the Microbial Diversity of Polar Desert Soils: A Biotechnological Perspective

Josie van Dorst, Nicole Benaud and Belinda Ferrari

Abstract Microorganisms represent the most abundant cold-adapted life-forms on earth. Far from just surviving, microorganisms appear to be thriving in cold climates, with microbial richness present in polar soils often in line with temperate soils. Recent advances in molecular techniques have allowed the true extent of global microbial diversity to be revealed. Antarctica in particular, has been found to harbour diverse and unique microbial populations comprise of high proportions of Chloroflexi, Actinobacteria, and unknown, previously uncultured taxa. Microorganisms have been the targets for bioprospecting for many years but efforts have thus far largely focused on easily obtainable temperate organisms, readily cultured within the laboratory. The extreme conditions that push the limits of life within cold environments leads to the evolution of unique physiologies and functional capabilities. Actinobacteria are well known to be prolific producers of useful natural products. Their high relative abundance along with the plethora of rare and previously unknown organisms highlights the potential for new biotechnological discoveries within cold adapted microorganisms. With limited to no higher organisms, Polar soils also provide ideal model ecosystems to examine the mechanisms driving microbial patterns of distribution. Thus far microbial communities have been found to be largely endemic and exhibit spatial patterns over meter, kilometre, regional and continental scales. While the mechanisms driving the patterns are not completely understood, a number of key biotic and abiotic factors, in particular pH, C/N ratio, NH_4 and N concentrations, phosphorus and plant cover, have been identified as influencing polar microbial communities and their survival in these extreme environments. Identifying and understanding key environmental drivers of microbial populations through biogeochemical analysis, structural equation models, microbial co-occurrence models, or space for time substitution studies, are providing the first step towards identifying the distribution of populations with desirable genetic or functional capacity and likewise polar regions that may contain unique communities for protection. At the same time this research is improving our capacity to predict microbial responses to disturbance due to both a changing climate and anthropogenic contamination.

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7.1 Polar Soil Microbial Diversity

Cold environments span more than 70% of the Earth and represent one of the largest biospheres. Microorganisms dominate these cold environments, and as such they represent the most abundant cold-adapted life forms on Earth. The polar soils of Antarctica represent an under-explored, extreme environment comprised of microorganisms with great potential for the field of biotechnology (Lee et al. 2012; Babalola et al. 2009). Of its 13,661,000 km^2, only 0.36% of the Antarctic continent is ice-free, and the ice-free areas consist primarily of permafrost; that is ground which retains a temperature at or below 0 °C for a minimum of two years consecutively (Guglielmin 2012; Stewart et al. 2012; Dobinski 2011). The Antarctic continent has also been described as the driest, windiest and coldest place on earth, with temperatures ranging from a record minimum of −89 °C, to a summer maximum of +10 °C, and yearly averages of around −10 °C (AAD 2002). In comparison to Antarctica, the Arctic region climate is less extreme, supporting more plant and animal life. For example, the Norwegian archipelago of Svalbard, which boasts the northernmost populated township in the world, Longyearbyen, experiences an annual average temperature of −6.7 °C, with a record low of −46 °C, and maximum of +21 °C (Førland and Hanssen-Bauer 2001; Brage et al. 2014). Climate change is impacting the Arctic at a greater rate than the world average, with Svalbard experiencing an increase in annual mean temperature of 4 °C over the last 90 years (Madeleine et al. 2009). In both the Arctic and Antarctica, extended periods of darkness occur during winter months, while high UV radiation is experienced during summer due to months of 24 h daylight (Blanc et al. 2012; (AAD), A.A.D 2012). Together, these factors mean that life within the polar soils of Antarctica and the Arctic are examples of survival at the limits of life. In order to survive the extremes conditions, soil organisms need to develop unique physiologies and or functional capabilities, many of which are yet to be explored.

Thanks to the application of next generation sequencing technologies, we now know that cold desert environments harbor microbial life much more diverse than once thought, with microbes also constituting the majority of biomass in many polar ecosystems. (Yergeau et al. 2007; Cary et al. 2010; Ganzert et al. 2011; Ganzert et al. 2014; de la Torre et al. 2003; Ferrari et al. 2015). In terms of microbial community diversity, both richness and phylogenetic diversity within Arctic soils appear to align well with temperate soils, however, the diversity observed in the more extreme Antarctic environment appear to be lower (Ferrari et al. 2015; Chu et al. 2010). Generally, bacterial phyla that are most abundant in temperate soils; Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes, are also dominant in polar soils (Pearce et al. 2012; Van Horn et al. 2014; Chong et al. 2011; Roesch et al. 2012). In contrast, permafrosts and arid, polar desert soils have consistently been shown to contain a higher proportion of Actinobacteria, which are prolific producers of useful natural products (Babalola et al. 2009; Cary et al. 2010; Ferrari et al. 2015; Berdy 2005; Lee et al. 2012). These phyla are ubiquitous,

well adapted to cold environments (Moyer and Morita 2001; Hinsa-Leasure and Bakermans 2013), and offer the potential for new biotechnological discoveries (Fig. 7.1).

Fig. 7.1 The Antarctic environment is more extreme than the High Arctic region. (*Top*) Svalbard Island in Norway contains both vegetation and animal life. (*Bottom*) The barren patterned ground landscape of Browning Peninsula, Antarctica. *Source* Ian Snape AAD

7.2 Microbial Ecology of Antarctic Soils Today

A wealth of information exists on the diversity of two major areas of terrestrial Antarctica, the Dry Valleys and the Antarctic Peninsula. On the other hand, eastern Antarctica is an under-explored region, and its location and relatively undisturbed landscape are providing a unique opportunity to undertake research that cannot be replicated anywhere else in the world. A large microbial biodiversity study recently discovered these extremely barren soils were dominated not by Proteobacteria, Actinobacteria and Firmicutes (as with most terrestrial and polar soils), but Actinobacteria, Chloroflexi and suprisingly, Candidate Division bacteria WPS-2 and AD3 (Ferrari et al. 2015; Ji et al. 2015). Moreover, even within the well know phyla recovered, a high proportion of bacterial and fungal taxa found to be present in relatively high abundances, were unclassified (Ferrari et al. 2015; Ji et al. 2015). Such a community structure has not been reported elsewhere and consequently, it was proposed that the locations containing this unique community structure, Mitchell Peninsula and Robinson Ridge, represent a potential "biodiversity hot-spot", which should be protected in the future.

7.3 Drivers of Polar Soil Microbial Diversity

Polar soils, with limited to no higher order organisms, are ideal model ecosystems to examine the mechanisms driving microbial patterns of distribution. Over the last two decades, the rapid progression and reduction in costs of molecular genetic techniques has encouraged efforts to disentangle the effects of climate, geography, latitude, edaphic factors and niche verses neutral community assembly processors, responsible for polar soil microbial diversity. Microbial molecular genetic tools have progressed from sanger sequencing, to community fingerprinting techniques, and now most commonly, pyrosequencing and metagenomics. However, it should be noted that fingerprinting methods have been reported to remain comparable to 454 tag pyrosequencing in their capacity to identify major diversity patterns (van Dorst et al. 2014; Gobet et al. 2014). The functional potential of polar soil microbial communities has also been targeted with Geochip functional gene array (Chan et al. 2013), qPCR and microarrays (Yergeau et al. 2010). Despite the variable approaches, the integration of molecular genetic techniques with biogeochemical analysis has provided evidence that instead of cosmopolitan distribution (Finlay 2002), microbial populations are largely endemic and exhibit spatial patterns over meter, kilometre, regional and continental scales (Yergeau et al. 2007; Ferrari et al. 2015; Fierer and Jackson 2006; Siciliano et al. 2014; Zeglin et al. 2009) Furthermore, while the mechanisms driving the patterns are not completely understood, a number of key biotic and abiotic factors have been identified as influencing microbial polar populations and their survival in these extreme environments.

In 2007, Yergeau et al. conducted a survey of soil-borne bacteria from polar soils spanning 27° of latitude, along a ~3200 km Southern polar transect, including both vegetated and bare, fell-field or frost boil sites. The bacterial diversity calculated from extensive 16S rRNA gene clone libraries was observed to decline with increased latitude, consistent with established biodiversity patterns of flora and fauna (Willig et al. 2003; Peat et al. 2007). In contrast, both the bacterial diversity and density at vegetated sites was consistent across geographical locations, suggesting opposing influences of vegetation and climate on bacterial communities and highlighting the need to consider specific habitat details when comparing biodiversity indicators across geographical locations (Yergeau et al. 2007).

Low microbial diversity and the latitude-diversity relationship was assumed to also hold true for Northern polar soils (Heal 1999; Hodkinson and Wookey 1999; Staddon et al. 1998). However, more recent studies have observed that microbial diversity throughout the Arctic is more likely determined by location specific biotic and abiotic drivers, and furthermore, that bacterial structure, diversity and gene abundances' can be equivalent, or higher, to other biomes including tropical and temperate forests, boreal, grasslands, desert and prairie (Chu et al. 2010; Fierer and Jackson 2006; Neufeld and Mohn 2005; Lauber et al. 2009). A high level of variation exists between Arctic microbial populations, which is consistent with the considerable variation in temperature, precipitation and soil characteristics, both spatially and temporally of terrestrial Arctic ecosystems. For bacterial, archaeal (Hoj et al. 2005) and fungal populations (Gittel et al. 2014), community diversity and structure has been observed to vary not only between ecosystems, but also with depth/horizon, and between permafrost and active layers (Gittel et al. 2014; Chu et al. 2011; Høj et al. 2006; Rooney-Varga et al. 2007; Kim et al. 2014; Shi et al. 2015; Lipson et al. 2013; Frank-Fahle et al. 2014). A number of abiotic and biotic factors have been linked to the variable distribution and response of Arctic microbial populations including C/N ratio, NH_4 and N concentrations, phosphorus and plant cover (Chu et al. 2011; Rooney-Varga et al. 2007; Kim et al. 2014; Shi et al. 2015; Geyer et al. 2014; Blaud et al. 2015). However, the most consistent factor identified as influencing terrestrial Arctic microbial populations is soil pH (Ganzert et al. 2014; Chu et al. 2010; Kim et al. 2014; Shi et al. 2015; Männistö et al. 2007).

An extensive microbial survey integrating sites from both poles also found that pH remains a dominant microbial driver at a global scale. In Siciliano et al. (2014) structural equation models were developed to interrogate the relationship between bacterial and fungal population dynamics from 223 soils across 8 polar locations and 41 biogeochemical factors, ultimately linking edaphic properties to measures of α and β-diversity. It was concluded that pH is specifically associated with community composition, whereas soil fertility (defined as organic matter, nitrogen and chloride content) is associated with fungal and bacterial richness. The SEM developed by Siciliano et al. was further applied to an additional set of 33 Arctic heath soil samples with broader soil parameters, yet still successfully predicted the microbial richness based on the biogeochemical factors present. While structural equation models have been used widely to examine the links between

environmental drivers and plant productivity and plant diversity, their current use in microbial ecology is limited (Siciliano et al. 2014; Grayston et al. 2004).

Utilising the same polar soil archive dataset (Siciliano et al. 2014), Ferrari et al. (2015), developed microbial co-occurrence networks to further disentangle the biotic influences such as organismal interactions, and landform connectivity on community assembly that were not explicitly examined within the SEMs (Ferrari et al. 2015). It was concluded that bacterial and fungal communities inhabiting well connected polar landscapes responded consistently to regional scale gradients in biotic and edaphic factors as described above. Conversely, the repeated freeze thaw cycles that characterise fragmented or frost boil landscapes create barriers within the landscape leading to a breakdown in microbial richness and co-occurrence networks. Despite the high variability between sites and the significant geographical and ecological differences between Arctic and Antarctic regions the significant effect of geological connectivity on microbial connectivity remains, suggesting the patterns observed were robust.

The environmental extremes characteristic of polar soils supports novel microorganisms with unique adaptations and metabolisms. The microorganisms that inhabit Polar Regions, are also critically involved in regulating the earth's climate and carbon balance. Identifying and understanding key environmental drivers of microbial populations through biogeochemical analysis (Lee et al. 2012; Chan et al. 2013; Zeglin et al. 2009; Geyer et al. 2013, 2014), SEMs (Siciliano et al. 2014), microbial co-occurrence models (Ferrari et al. 2015), or space for time substitution studies (Van Horn et al. 2014), are providing the first step towards identifying polar regions that may contain unique communities for protection. At the same time this research is improving our capacity to predict microbial responses to disturbance due to both a changing climate and anthropogenic contamination (van Dorst et al. 2014; Bissett et al. 2013; Arbel et al. 2015).

7.4 What is the Potential of Natural Products Discovery in Polar Soils?

There is an urgent need to develop new therapies, particularly novel antibiotics to combat emerging diseases and the growing numbers of antibiotic resistant pathogens such as gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) in the community (De Castro et al. 2014; Butler et al. 2013). Historically, most natural product discovery efforts have focused upon readily accessible biota such as plants, marine invetebrates, temperate bacteria and fungi as sources of new compounds. But, the growing need for novel agents to treat disease warrants further investigation of extreme sources of biodiversity, for the discovery of new natural products, in particular antibiotics (Woodhouse et al. 2013). Other applications of natural products with commercial value include pesticides, dyes, fragrances and sunscreens. Based on their potential for untapped biological chemical diversity, natural product discovery

programs are now targeting bacteria and fungi from extreme environments, such as deep ocean, thermal vents and harsh arid lands as potential sources of new compounds. Permanently cold dessert environments are exposed to a variety of extreme environmental stressors, including but not limited to; low nutrients, limited availability to liquid water. High UV exposure and of course low to below freezing temperatures. As unicellular microorganisms lack the physiology to regulate their temperature, cold-adapted microorganisms have evolved both structural and functional differences to sustain life in the cold, including; modified proteins, amino acids, cell wall and membrane components and bioactive chemical compounds, such as siderophores and antibiotics. (Amato 2013; Casanueva et al. 2010; Corsaro et al. 2008; Rice et al. 2015). Such adaptations are worth pursuing in the search for novel compounds.

Studies on polar soil bacteria in terms of antimicrobial production, and antimicrobial associated genes have been modest in both number and scale (Lee et al. 2012; Gesheva 2010; Zhao et al. 2008, 2011; Encheva-Malinova et al. 2014). Moreover, the majority of antimicrobial investigations carried out within Polar Regions have focused on marine sources, including sponges and sediments. For example, in 2001, Shekh et al. (2011) cultured 218 psychrotolerant bacterial isolates from sediment cores, and samples of soil, faeces and feathers from penguin rookery areas in Antarctica; as well as Arctic permafrost soils and glacial water. The isolates were screened for anti-fungal activity and seven produced good activity against the *Candida* strains tested, including multi-drug resistant strains. Based on 16S rRNA gene analysis, those taxa exhibiting activity were found to belong to Proteobacteria genera *Psychrobacter* and *Yersinia*, and Firmicutes genera *Enterococcus*, *Bacillus*, and *Carnobacterium*. One of the isolates with good activity against seven different *Candida* strains, an *Enterococcus* sp., was further characterised with the authors concluding that the anti-fungal activity was most likely attributable to a small bacteriocin-like peptide of around 30 kDa (Shekh et al. 2010). In Gesheva (2010) isolates were cultured from four eastern Antarctic soils and 5 of the recovered Actinomycete strains were tested for production of antimicrobial compounds; all five strains showed activity against several gram positive and fungal pathogens, but no activity against the gram negative pathogen, *E. coli* (Gesheva 2010). Finally, Lee et al. (2012) cultured soil Actinobacteria from Barrientos Island, Antarctica, and screened the isolates for antimicrobial activity. Of 39 Actinobacteria cultivated, 15 exhibited antimicrobial activity, predominantly against *C. albicans* and *S. aureus*, including four which were active against a methicillin resistant *S. aureus* strain. Based on 16S rRNA sequence homology, their isolate's identity to known species ranged from 96.9 to 99.9% (Lee et al. 2012).

In microorganisms, secondary metabolites, which are a major source of natural products, are often produced by type-I or II polyketide synthases (PKS-I or PKS-II) and/or non-ribosomal peptide synthetase (NRPS) genes. Increasingly, molecular studies targeting these genes are being used as a screening process for natural products potential. For example, Zhao et al. (2008 and 2011) analysed Antarctic coastal sediments for type-I polyketide synthases (PKS) and non-ribosomal peptide synthetase (NRPS) genes. They PCR amplified the genes from environmental DNA

and based on the analysis of amino acid sequences, found genes with closest homology to members of Cyanobacteria, Firmicutes and Proteobacteria, although the primers used do show some bias toward these phyla. The sequences exhibited low sequence similarity (~50–80%) to PKSs and NRPSs of known organisms (Zhao et al. 2008, 2011) thus, they may potentially produce novel compounds. Yi Pan et al. (2013) cultured 95 Actinomycetes from 8 soils from Signy Island Antarctica, and evaluated antibiotic activity against 3 test pathogens and screened the isolates for the presence of NRPS genes. Their strains exhibited 96–100% sequence similarity with known organisms based on 16S rRNA sequences. Of 16 representative strains, 9 were positive for NRPS genes, although these sequences were not subsequently analysed for identification (Yi Pan et al. 2013). In 2014, Encheva et al. screened 23 *Streptomyces* strains from Livingston Island Antarctica for antibiotic-associated genes including Type-II PKS and NRPS, and antimicrobial activity. Eleven of the isolates were positive for NRPS genes, and all were positive for Type-II PKS. Eight of the strains displayed antimicrobial activity against selected test pathogens (Encheva-Malinova et al. 2014).

Most recently, Benaud (2014) carried out a pilot study of predominantly Actinobacterial strains, isolated from soils from the Windmill Island region in eastern Antarctica and sub-Antarctic Macquarie Island. Nineteen isolates were tested for antimicrobial activity against a selection of gram negative, gram positive and yeast strains, using the cross-streak agar method (Carvajal 1947; Hopwood 2007). Seven of the 19 strains, which were all *Streptomyces* spp., exhibited measurable activity against at least one of the test strains (Table 7.1), and screening for the presences of NRPS, Type I and Type II PKS (Ayuso-Sacido and Genilloud 2004; Metsä-Ketelä et al. 2002; Metsä-Ketelä et al. 1999) revealed the majority of isolates tested positive to multiple secondary metabolite genes. The *Streptomyces* spp. examined here shared high homology (99–100%) with known strains based on their 16S rRNA gene, yet sequencing of the PKS and NRPS genes for 2 selected isolates revealed a 72–98% homology with known gene clusters. From this preliminary study it was concluded that these Polar soils warrant a more extensive examination in terms of secondary metabolite genes and antimicrobial producers.

Other recent investigations by Charlop-Powers et al. (2014, 2015) used high throughput 454 pyrosequencing to conduct diversity and richness assessments of PKS and NRPS genes in geographically and chemically diverse soils throughout the USA, and later on a global scale, including sites from Asia, Africa, Hawaii, Australia and Dominican Republic. Soil types included temperate and alpine forests, rainforests, deserts and coastal sediments and their results suggested arid soils presented the greatest biosynthetic potential. Desert soils also contain a population bias toward natural products-rich phyla such as Actinobacteria and Proteobacteria, and this is likely to be a driving force behind PKS and NRPS richness in these soils types (Charlop-Powers et al. 2014, 2015). While some research in the area has been carried out, one major question that remains is whether extreme polar soils represent a better target for biomining of novel natural products than temperate soils? It is unclear if antimicrobial production may provide a competitive advantage for survival in these extreme cold desert soils. Currently, there are efforts towards

Table 1 Antimicrobial activity and presence of secondary metabolite genes in 19 bacterial isolates from polar soils

Polar isolate		Test pathogen distance of growth from isolate streak (mm)					Secondary metabolite encoding genes		
No.	Genus	*M. luteus* *ATCC 10240*	*B. subtilis* *ATCC 11774*	*E. coli* *ATCC 25922*	*S. aureus* *ATCC 25923*	*C. albicans* *ATCC 10231*	PKS I	PKS II	NRPS
1	*Rhodococcus*	0	0	0	0	0	-	-	+
2	*Streptomyces*	0	†	2	0	11	+	+	+
3	*Streptomyces*	0	†	1	0	12	+	+	+
4	*Streptomyces*	10	†	0	0	0	+	+	+
5	*Streptomyces*	0	0	0	0	0	-	+	+
6	*Paenisporosarcina*	0	0	0	0	0	-	-	-
7	*Streptomyces*	7	13	0	5	13	+	-	+
8	*Streptomyces*	11	†	0	0	8	-	+	-
9	*Mycobacterium*	0	0	0	0	0	+	-	+
10	*Streptomyces*	†	†	†	†	†	+	+	-
11	*Paenisporosarcina*	0	†	0	0	0	-	-	-
12	*Arthrobacter*	0	0	0	†	†	-	-	+
13	*Kribella*	0	0	0	0	0	-	-	+
14	*Hymenobacter*	0	0	0	0	0	-	-	-
15	*Streptomyces*	11	†	0	†	†	+	+	+
16	*Mycobacterium*	0	0	0	0	0	+	-	+
17	*Streptomyces*	0	†	0	†	0	-	+	-
18	*Streptomyces*	7	9	0	†	†	+	+	-
19	*Streptomyces*	0	†	0	0	0	-	+	+

†—Inhibited growth; test organisms grew up to isolate streak but less robustly

adapting high throughput sequencing technologies to screen polar soils for the presence of PKS and NRPS genes. Such studies hope to link the biogeochemical drivers within the environment to the relative biosynthetic potential of cold deserts in an attempt to determine the ecological relevance of these genes in extreme environments.

7.5 Microbial Dark Matter and the Potential for New Biotechnological Discoveries

It is well established that less than 1% of total bacterial diversity (Ferrari et al. 2008; Handelsman 2004) and 5% of fungal taxa are able to grow under traditional culture conditions (Ferrari et al. 2011). Recently, this yet-to-be described diversity has been termed microbial dark matter (MDM), and deep sequencing studies are suggesting the diversity of MDM may be even greater than once thought (Nobu et al. 2015;

Rinke et al. 2013). Polar soils are no exception with metagenomic studies continuing to highlight that the majority of bacteria identified are yet-to-be cultured, including species within the well-known Actinobacteria phylum (Lee et al. 2012; Babalola et al. 2009; Ferrari et al. 2015; Ji et al. 2015). In environments that are understudied, the percentage of uncultured or poorly cultured species is potentially much greater. For example, it was recently proposed Mitchell Peninsula and Robinson Ridge within eastern Antarctica to be a biodiversity hotspot, due to the unique community structure in its soil, with an average relative abundance of Actinobacteria (12%), Chloroflexi (15%) and remarkably, Candidate Division bacteria WPS-2 (9.3%) and AD3 (8.7%) most dominant (Ji et al. 2015). Areas that harbor such unique organisms not only hold promise for the discovery of novel compounds, but hold intrinsic value as unique ecosystems. Although microorganisms are not commonly considered in conservation efforts, it was suggested in (Ji et al. 2015) and (Hughes et al. 2015) that this microbial diversity hotspot, and other unique polar soil ecosystems warrant protection on the basis of biological significance.

State-of-the-art technologies continue to uncover a wealth of information on microbial diversity from a plethora of environments, with MDM now challenging the phylogenetic boundaries of life (Tytgat et al. 2014). These capabilities are also accelerating the rate of gene discovery (Handelsman 2004; Jansson and Tas 2014). Metagenomics investigations aimed at the recovery of rare genomes, while limited in scope for soil due to its inherent complexity, are now applicable to extreme Antarctic environments. Indeed, the first genomes of WPS-2 and AD3 have been recovered from polar soils within the Windmill Islands, by combining shotgun sequencing with differential coverage binning (Ji 2016). Ultimately such studies will lead to not only the discovery of novel microbes with unique functional capabilities, but the hope is for new biotechnological discoveries.

7.6 Future Risks for Polar Desert Soils

Despite the relative isolation and extreme conditions, polar soils across the Arctic and the Antarctic are at risk from increasing anthropogenic activities. Effects from climate change are more pronounced throughout the Arctic and maritime Antarctica than anywhere else on earth (AMAP 2011; Turner et al. 2005; ACIA 2005). In the Arctic, the rapid warming and decreasing sea ice (AMAP 2011) increases the risk of severe consequences for global carbon feedback systems (Schuur et al. 2015; Schuur et al. 2008), and also opens up new travel, exploration and resource extraction opportunities, increasing the likelihood of additional nutrient and pollutant inputs (Peters et al. 2011). In the Antarctic, changing conditions are expected to alter microbial diversity and productivity (Van Horn et al. 2014; Newsham et al. 2016). At the same time, the limited ice free areas are increasingly impacted from human activities such as the construction of research stations, airstrips, roads and other infrastructure. Human activities not only contribute to pollution, habitat

disruption and destruction (Tin et al. 2009; Braun et al. 2014; Aislabie et al. 2004; Bargagli 2008), but also increase the risk of contamination from non-native microorganisms and genetic material (Hughes et al. 2015; Cowan et al. 2011; Hughes and Nobbs 2004). As a result, it is increasingly difficult to identify pristine areas that are known to be categorically free from earlier human activity (Hughes et al. 2011).

The Antarctic treaty was designed with the overreaching aim of protecting Antarctica as “a natural reserve devoted to peace and science”. One of the key features of the protocol is the prohibition of collecting mineral resources for purposes other than scientific investigation. There is an increasing realization that these unique environments harbor microorganisms that are a potentially valuable resource for bioprospecting activities (Hughes et al. 2015; Hemmings 2010). Although the treaty aimed at preventing mineral mining, the current research into bio-mining may be considered at odds with the benefit sharing ideals of the treaty. The existing identification and management process of the Antarctic Special Protection areas (ASPs) is considered by many inadequate to protect Antarctica’s macroscopic fauna and unique ecosystems (Hughes et al. 2015; Shaw et al. 2014; Terauds et al. 2012). Without taking microbial inhabitants into consideration, the current system is also inadequate to protect ecosystems that harbour unique microorganisms. In the future, even the smallest life forms on earth should be viewed as worthy of protection, and protected on the basis of biological significance. As highlighted by Hughes et al. (2015); microbiologists throughout the Polar Regions are in the unique position of being both conservation champions and biotechnological exploiters. A balance needs to be found in bio-discovery and microbial ecology research between (1) furthering our understanding of the biological and chemical diversity present in polar soils, (2) increasing the accuracy with which we can identify unique, vulnerable and/or pristine ecosystems, (3) predict the consequences of a changing climate and contamination events and (4) protect pristine areas for the generations and the scientific tools of tomorrow.

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Chapter 8
Exploring the Viral Ecology of High Latitude Aquatic Systems

Caroline Chénard and Federico M. Lauro

Abstract Viruses are abundant and ubiquitous in aquatic systems. While most of our knowledge in the field of aquatic viral ecology comes from the study of temperate environments, new insights are starting to emerge from polar systems. It is becoming increasingly evident that viruses play a pivotal role in structuring high latitude aquatic systems where microbes are important players and major drivers of biogeochemical cycles. In this chapter, we summarize the latest findings about the abundance, distribution and production of viruses from polar regions. We also review viral-mediated bacterial mortality and phage-host dynamics in freshwater and marine polar waters. For example, we summarize temporal studies performed in polar freshwater systems, showing a seasonal trend with a high percent of lysogenic bacteria in the winter and an undetectable rate in the summer. These findings suggest that lysogeny represents an important life strategy in polar regions. We conclude with the latest analysis of large scale meta-omics datasets, which suggest that polar viral assemblages might be a reservoir of new lineages of viruses and unknown viral diversity.

8.1 Introduction

The presence of viruses in aquatic systems has been known for many decades, however it was only twenty-five years ago, with the advent of transmission electron microscopy (TEM), that they were revealed to be the most abundant biological entity in aquatic environments (Bergh and Borsheim 1989; Proctor and Fuhrman 1990). Viruses occur in both freshwater and marine systems, from tropical to polar

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regions, and from surface waters to sediments. Viruses have been found in cryoconite sediments from an Arctic glacier (Säwström et al. 2002; Anesio et al. 2007), in the deep water of the Mediterranean Sea (Winter et al. 2014) and in tropical African lakes (Bettarel et al. 2006). In general, viral numerical abundance ranges from 10^6–10^8 mL^{-1} in marine and fresh waters (Maranger and Bird 1995). Their numbers are often correlated with numbers of bacteria and archaea and are generally one order of magnitude higher.

Viruses are not only abundant in aquatic systems, they are also important players in biogeochemical and ecological processes (see reviews by Fuhrman 1999; Suttle 2005, 2007). Viruses cause the lysis of a large proportion of both autotrophic and heterotrophic prokaryotes, shunting nutrients between particulate and dissolved phases (Wilhelm and Suttle 1999; Weitz and Wilhelm 2012) (Fig. 8.1). For

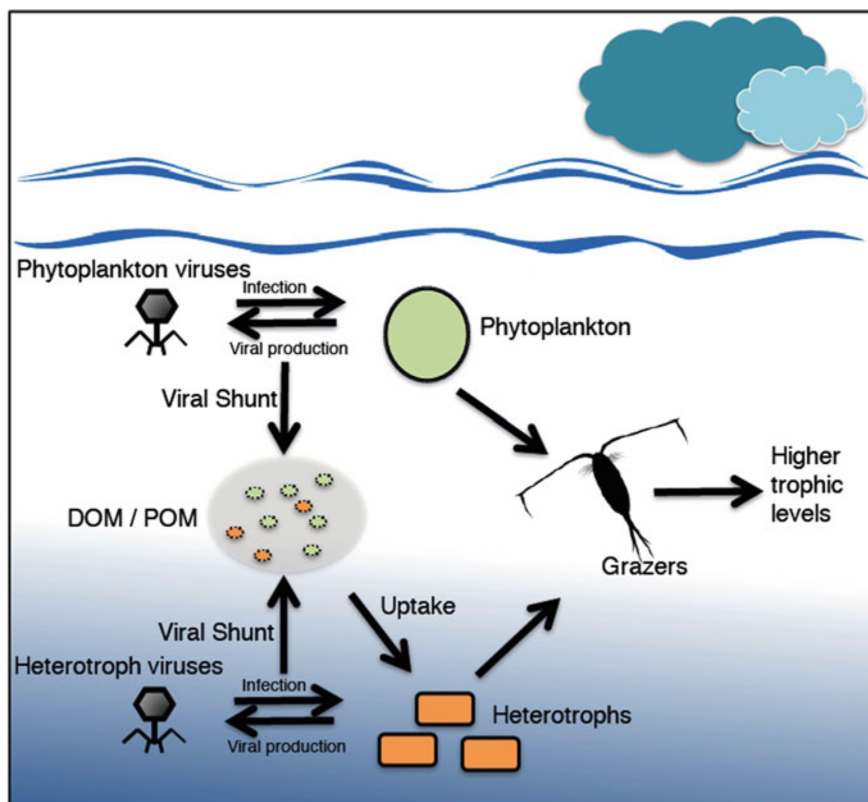

Fig. 8.1 Schematic of the viral shunt. Viruses cause the lysis of a large proportion of both autotrophic and heterotrophic prokaryotes, shunting nutrients between particulate and dissolved phases. Almost half of the bacterial production and about 26% of the total organic carbon fixed by photosynthesis in the ocean are lost daily through viral lysis. As a result of this "viral shunt", viruses redirect matter and energy away from higher trophic levels and generate substrates for heterotrophic prokaryotic producers (adapted from Weitz and Wilhelm 2012)

instance, studies suggest that almost half of the bacterial production and about 26% of the total organic carbon fixed by photosynthesis in the ocean are lost daily through viral lysis (Wilhelm and Suttle 1999; Fuhrman 1999). As a result of this "viral shunt", viruses redirect matter and energy away from higher trophic levels and generate substrates for heterotrophic prokaryotic secondary production (Middelboe et al. 1996; Suttle 2005).

Viruses can also control the community composition by killing specific host strains. The "kill-the-winner" hypothesis states that while one host strain becomes dominant, a virus would infect this strain and decrease its abundance leaving an open niche for a related strain resistant to the virus (Thingstad 2000).

Viruses can confer immunity against infection and influence the lateral gene transfer among hosts (Jiang and Paul 1998). Viruses can also contain metabolic auxiliary genes involved in photosynthesis, carbon and phosphate metabolisms, which may influence primary production and biogeochemical processes through the expression of these host-like genes during infection (Lindell et al. 2007).

While most of our knowledge on aquatic viral ecology has come from temperate environments, recent studies have emerged from higher latitude systems, which highlight that the role of viruses may be somewhat greater than in temperate regions. Polar lakes, especially Antarctic lakes are microbially dominated systems but support fewer grazers, which implies that viruses might be responsible for a high proportion of the bacterial mortality and consequently influence the microbial loop and drive the microbial evolution (Kepner et al. 1998; Anesio and Bellas 2011; Wilkins et al. 2013).

8.2 Viral Abundance and Distribution in High Latitude Systems

In polar freshwater systems, viral abundance range from 2.0×10^4 mL^{-1} to 1.0×10^8 mL^{-1} (Säwström et al. 2008a). While low numbers of viruses are commonly measured in ultra-oligotrophic polar lakes and glacial ecosystems, higher numbers are found in polar saline lakes. For example, a high viral abundance was measured in saline lakes in the Vestfold Hills (Antarctica) such as Highway Lake ($1.24–9.66 \times 10^7$ mL^{-1}), Ace Lake ($0.8–6.13 \times 10^7$ mL^{-1}) and Pendant Lake ($1.15–12.0 \times 10^7$ mL^{-1}) (Madan et al. 2005). A significant negative correlation between viral concentration and temperature was observed in the saline lakes in the Vestfold Hills (Madan et al. 2005). These findings suggest that in winter the viral decay is low because of the lack of solar radiation and lower temperature. Temporal studies on ultra-oligotrophic freshwater lakes demonstrate that viral abundance exhibits a seasonal variation with greatest abundance in summer (Lake Dryzhby, 1.6×10^6 mL^{-1}) or late autumn (Crooked Lake, 9.2×10^5 mL^{-1}) (Säwström et al. 2007b) (Table 8.1).

As for polar freshwater systems, the viral abundance in the Arctic and Southern Ocean is usually an order of magnitude lower than in temperate regions except for the sea ice. Viral abundance collected during a spring ice algal bloom near

Table 8.1 Example of viral abundance and distribution in high latitudes aquatic systems

Location	Viral abundance (mL^{-1})	VMR[a]	Date	Reference
Arctic Ocean				
Sea Ice	9.0×10^6–1.3×10^8	15–72	Apr 22–May 22 1992	1
Cornwallis Island	1.6×10^6	10	Apr 22–May 22 1992	1
Arctic winter sea-ice brines	1.6×10^6–8.2×10^7	3–18	Feb 29–Mar 21 2004	2
Southern Beaufort Sea Shelf (Amundsen Gulf, Mackenzie Shelf)	1.3×10^5–2.3×10^7	4.3–40.2	Nov 2003–Aug 2004	3
Southern Ocean				
Antarctic sea brines (Ross Sea)	1.0×10^9–1.5×10^9	NA	May 9–Jun 11 1998	5
Antarctic sea brines (Ross Sea)	1.8×10^9	NA	Jan–Feb 1999	6
Sub-Antarctic and Polar Frontal Zone of the Autralian Southern Ocean	6.6×10^6–2.1×10^7	5–14	Jan–Feb 2007	7
Antarctic Peninsula (Bellingshausen Sea, Bransfield Strait, Gerlache Strait)	7×10^6–2×10^7	14–81	Dec 1995–Feb 1996	8
Kerguelen Islands	1×10^7–19×10^7	9–37	Jan–Feb 2005	9
Arctic freshwater systems				
Beringia area	9.6×10^5–1.19×10^7	1.6–18	Jul–Aug 2005	10
Sub-Arctic lakes (Sweden)	6.7×10^5–2.8×10^6	3–10	Jul–Sept 2006	10
Svalbard lakes	4.3×10^6–2.8×10^7	11–25	Aug 2003	11
Antarctic freshwater systems				
Saline lakes in the Vestfold Hills				
Highway Lake	1.2×10^7–9.6×10^7	18–126	Dec 2002–Jan 2004	12
Ace Lake	8×10^6–6.1×10^7	30–80	Dec 2002–Jan 2004	12
Pendant Lake	1.1×10^7–1.2×10^8	30–96	Dec 2002–Jan 2004	12
Lake Dryzhby	3×10^5–1.5×10^6	1–8	Dec 2003–Nov 2004	13

(continued)

Table 8.1 (continued)

Location	Viral abundance (mL^{-1})	VMR[a]	Date	Reference
Crooked Lake	1.6×10^5–9.2×10^5	1–8	Dec 2003–Nov 2004	13

[a]Virus to microbial cells ratio

1 Maranger et al. (1994), *2* Wells and Deming (2006b), *3* Payet and Suttle (2008), *5* Gowing et al. (2002), *6* Gowing (2003), *7* Evans et al (2009), *8* Guixa-Boixereu et al. (2002), *9* Brussaard et al. (2008), *10* Säwström et al. (2008a), *11* Säwström et al. (2007a), *12* Madan et al. (2005), *13* Säwström et al. (2007b)

Cornwallis Island in the Canadian High Arctic showed that viruses in sea ice samples could be as high as 9.0×10^6 to 1.3×10^8 ml^{-1} while the adjacent seawater was about 1.1×10^6 ml^{-1} (Maranger et al 1994). Arctic sea-ice brines also showed high viral abundance with values ranging from 1.6×10^6 to 8.2×10^7 ml^{-1} (Wells and Deming 2006b) while Antarctic sea-brines demonstrated higher concentrations with values ranging from 1.0 to 1.5×10^9 ml^{-1} (Gowing et al. 2002; Gowing 2003). The number of viral particles in sea ice is generally 10–100 times greater than in the water columns.

Few studies in the Southern Ocean have addressed viral abundance dynamics (Evans et al. 2009; Guixa-Boixereu et al. 2002). Evans et al (2009) reported viral numbers ranging from 6.6×10^6 ml^{-1} to 2.1×10^7 ml^{-1} in sub-Antarctic and Polar frontal zone of the Australian Southern Ocean. Viral concentration measured within three areas in the Antarctic Peninsula (Bellingshausen Sea, Bransfield Strait and Gerlache Strait) during the austral summer also showed little variation at the surface waters ranging from 7×10^6 mL^{-1} to 2×10^7 mL^{-1} (Guixa-Boixereu et al. 2002).

A temporal study carried out in the shelf area of the Mackenzie River and Amundsen Gulf in the south Eastern Beaufort Sea showed dynamic in the viral abundance with values ranging from 1.3×10^5 mL^{-1} to 2.3×10^7 mL^{-1} (Payet and Suttle 2008). Surface waters showed the greatest variation with concentration two times higher during the spring bloom (May, June) and 1.5× higher during mid-summer (July and August) relative to winter abundance. Interestingly, two different subgroups of viruses could be identified based on their fluorescence signature using flow cytometry. First, a low SYBR green fluorescence group including 70% of the total viral abundance was correlated to the number of bacteria. The second group with a high SYBR green fluorescence signal was correlated to high chlorophyll a concentration. A study in the waters adjacent to Kerguelen Islands, Southern Ocean also reveals a similar pattern (Brussaard et al. 2008). The low SYBR green fluorescence group represented between 60 and 85% of the total virus abundance, while the high SYBR green fluorescence group included about 13–35% of the total virus concentration. Interestingly, the viral abundance in this study was relatively high (1–19×10^7 mL^{-1}) as compared to other studies conducted in the Southern Ocean, probably as a result of the higher system productivity.

In aquatic systems, a positive correlation between the numbers of virus and bacteria is generally observed suggesting that bacteria are probably the host for

most viruses. Indeed the virus-to-bacterium ratio (VBR) or virus-to-microbial cell ratio (VMR) is the statistical proxy used by microbial ecologists to compare different aquatic systems. In polar freshwater lakes, the ratio was found to range between 1 and 34 (Säwström et al. 2008a). This ratio is lower than other freshwater systems from lower latitudes which Säwström et al. (2008a) explain by the lack of allochthonous inputs in high latitudes lakes. An exception to this low VMR in polar regions was found in the saline lakes of Vestfold Hills where the calculated VMR can be as high as 120 (Madan et al. 2005).

In general, viral abundance is indirectly correlated to nutrient concentration given that viruses require the replication machinery of their host to reproduce. Consequently, in polar freshwater lakes where phosphorus is a limiting factor for bacterial production (Dore and Priscu 2001; Granéli et al 2004; Säwström et al. 2007a), viral numbers were also correlated with phosphorus concentration (Lisle and Priscu 2004; Madan et al 2005; Säwström et al. 2008b).

8.2.1 Viral Production and Viral-Mediated Mortality

Various studies have also focused on measuring the viral production rate both in freshwater and marine polar waters. Few studies estimated the rate of viral production in diverse Antarctic lakes and reporting rates varying between 2.0×10^6 and 2.0×10^9 virus L^{-1} h^{-1} (Kepner et al 1998; Säwström et al. 2007b; Laybourn-Parry et al. 2007; Säwström et al. 2008a). Viral production was generally lower in ultra-oligotrophic lake such as Crooked Lake and Lake Druzhby (2.0–29.9 $\times 10^6$ virus l^{-1} h^{-1}) than in productive saline lakes such as Ace Lake and Pendant Lake (0.2–0.8 $\times 10^8$ viruses l^{-1} h^{-1}) (Kepner et al 1998; Säwström et al. 2007b; Laybourn-Parry et al. 2007). Viral production in the polar oceans also showed similar rates. In the Canadian Arctic, the rate of viral production ranged from 7.2×10^4 to 1.8×10^6 viruses ml^{-1} day^{-1} (Wells and Deming 2006b), values which are lower than those measure in the Australian Southern Ocean which ranged from 3.0 $\times 10^6$ to 2.6×10^8 viruses ml^{-1} day^{-1} (Evans et al 2009).

Although the viral production in polar regions falls within the range of reported values calculated in marine and freshwater system of lower latitudes (Wommack and Colwell 2000), high infection rates were observed in Antarctic and Arctic ultra-oligotrophic freshwater environments (Säwström et al. 2007c). To calculate the infection rates, Säwström et al. (2007c) used the frequency of visible phage-infected bacteria, which represents the number of bacteria containing visible viral particles. This number generally represent a small fraction of the total fraction of infected bacteria ($\sim$1/7) given that viral particles are only visible within the cells at the last part of the latent period. In temperate aquatic systems the frequency of visible infected cells ranged between 0.6 and 5% with a burst size of about 26 phages (Hennes and Simon 1995; Wilhelm and Smith 2000; Hofer and Sommaruga 2001; Fischer and Velimirov 2002; Bettarel et al. 2004; Weinbauer et al. 2002). Interestingly, samples from two Antarctic freshwater lakes and cryoconite holes

from a glacier in the Arctic displayed a higher frequency of visible infected cells, ranging between 5.1 and 66.7% (average 26.15%) but a smaller burst size between 2 and 15 (Säwström et al. 2007c).

The viral production can also be used to calculate viral-mediated mortality and to estimate the amount of nutrients released by viral lysis (Wilhelm et al 2002). In Crooked Lake and Lake Druzhby, viral-mediated bacterial mortality ranged from 38 to 251% which caused the release of 0.8–69% of the total dissolved organic matter (Säwström et al. 2007b). In a different study in the Australian Southern Ocean between 17 and 65% of bacterial mortality was attributed to viruses, contributing to the release of a significantly amount of dissolved organic matter (i.e. between 0.8 and 34 μgC l^{-1} day^{-1}) (Evans et al. 2009) In the Bering and Chukcki Seas (Arctic Ocean), it was estimated that virus-mediated bacterial mortality was 9–23% in comparison to 3–25% of the bacterial mortality due to grazing (Steward et al 1996). Boras et al (2010) investigated the effect of ice melting on bacterial mortality caused by viruses and protists in the summer in North Greenland Sea and the Arctic Ocean. While bacterial mortality caused by protist was generally higher than viral-mediated mortality in their study, significantly higher protozoan-mediated mortality and lower viral-mediated mortality were detected in waters affected by ice melting in comparison with unaffected waters. They measured that viruses contribute to the release of 2.63 ± 2.45 μgC l^{-1} day^{-1} in affected water and 4.27 ± 5.45 μgC l^{-1} day^{-1} in unaffected waters. This suggests that sea melting could affect the carbon flow within the Arctic microbial food web.

8.3 Phage-Host Dynamics

8.3.1 Lysogeny in Low Temperature

Various studies in fresh and marine systems suggest that lysogeny play an important role in polar regions (Säwström et al. 2007b; Payet and Suttle 2013; Brum et al. 2016; Evans and Brussaard 2012). Lysogeny is a strategy employed by some phages that have the capacity to selectively integrate of their genomes into the genome of a host cell under difficult environmental conditions. The integrated phage, known as a prophage, remains latent until favorable environmental conditions trigger the activation of the lytic genes and cause phage production, host lysis and subsequent infection of active host cells (Paul 2008). In general, the dynamic balance between lytic and lysogenic infection, is finely tuned to rapidly respond environmental changes.

Temporal studies performed in polar freshwater systems demonstrate a seasonal trend with a high percent of lysogenic bacteria in the winter and an undetectable rate in the summer. Indeed, a temporal study on Crooked Lake (Antarctica) showed that up to 72% of bacteria were lysogenic in the austral winter, conversely no lysogenic bacteria were detected in the summer (Säwström et al. 2007b). A temporal study in the Beaufort Sea also demonstrated a seasonal pattern with

almost no lysogenic bacteria detectable in the winter and up to 38% of lysogenic bacteria in the summer (Payet and Suttle 2013). Similar pattern were also observed in the Southern Ocean (Brum et al. 2016). In addition, a spatial study suggests that lysogeny might be more prevalent in regions with lower nutrients and low bacterial abundance (Evans and Brussaard 2012).

The important role of lysogeny as a life strategy in polar regions is also supported by genomic and metagenomic analysis. Prophage-like sequences were found to be more prevalent in Arctic marine bacteria than in low latitude bacteria (Cottrell and Kirchman 2012). Angly et al. (2006) reported a higher abundance of prophage-like sequences in the viral assemblage from the Arctic Ocean than in the Sargasso Sea, Coast of British Columbia and the Gulf of Mexico. A metagenomic study on the Western Antarctica Peninsula also suggested that temperate phages dominate the region switching from lysogeny to lytic replication as bacterial production increases (Brum et al. 2016). Indeed, the prevalence of temperate phages in the viral assemblage might be one of the major factors differentiating polar viral assemblage from lower-latitudes assemblages in the oceans (Angly et al. 2006; Brum et al. 2016).

Although most of these studies follow the general trend of low host abundance as the main factor for switching between lytic and lysogenic phase, a recent study using data from coral reefs and meta-analysis from different environments suggest an opposite trend. Indeed, Knowles et al. (2016) suggested that temperate dynamic becomes increasingly important in ecosystems with high microbial density, thus "more microbes, fewer viruses". It will be interesting to investigate if such a trend holds true also in high latitude aquatic systems where constant environmental stress factors such as UV and temperature changes are expected to cause prophage induction.

8.3.2 Influence of Virophage on Algal Host-Virus Dynamics

A metagenomic analysis of an Antarctic hypersaline meromictic lake revealed the presence of virophages that likely influence the algal host-virus dynamics in those lakes (Yau et al. 2011). Virophages are circular dsDNA viruses which require the presence of a "host virus" for its replication (La Scola et al. 2008; Desnues et al. 2012; Fischer and Suttle 2011). Indeed, virophages have been shown to use the replicative cytoplasmic virion factory of giants viruses (Megaviridea) for their own replication (Fischer and Suttle 2011; Slimani et al. 2013). A virophage (virophage OLV) was identified using *de novo* assembly of metagenomic sequences of the 0.1 μm size fraction from Organic Lake (Yau et al. 2011). Large fractions of the genomes of the putative host-viruses were also retrieved from the metagenomic data. The "host viruses" (OLPV1 and OLPV2) of OLV were first believed to have a genome of ~300 kb and related to Phycodnaviruses isolates infecting the Prymnesiophytes *Chrysochromulina ercina* (CeV1), *Phaeocystis pouchetii* (PpV) and the Prasinophyte *Pyramimonas orientalis* (PoV) (Yau et al 2011).

However, the sequencing of a new *Phaeocystis globosa* virus (PgV-16T) revealed that OLPVs might belong to the clade of the Megaviridae which also includes viruses infecting *Acanthamoeba* (Megavirus and Mimivirus) and a virus infecting the marine microflagellate grazer *Cafeteria roenbergensis* (CroV) (Santini et al. 2013). Given the close relatedness of OLPVs with PgV-16T, the actual genome size of OLPV1 and OLPV2 can be better estimated. It is now suggested that the genome of OLPV1 was only 86% complete (125/145 genes) while OLPV1 was only 83.4% complete (121/145 genes). Consequently, the genomes of OLPV1 and OLPV2 are likely larger than 300 kb (Santini et al. 2013).

Sequences related to the virophage OLV were also detectable in samples collected two years later in Organic Lake and in an adjacent lake suggesting the prevalence of virophages in polar regions (Yau et al. 2011). This prevalence highlights the potential role of virophage in the dynamic of the algal host (e.g. host cell) and its virus (e.g. "host virus"). In a laboratory setting, virophage was found to reduce its "host virus" production which subsequently lead to a decrease of the host cells lysis (Fischer and Suttle 2011; Slimani et al. 2013). Virophage interfere with their "host virus" propagation and will then increase the survival of the host-cell population. Using a predator-prey dynamic model, Yau et al. (2011) investigated how virophage OLV influence OLPV and it host cells. They suggested that the virophage OLV decrease the host mortality and consequently, increase the bloom frequency and carbon flux through the microbial loop during the summer. This might be an important community adaptive response in those habitats where primary productivity is limited to just a few short months in the austral summer.

8.3.3 Viral Dynamics in a Pristine Meromictic Lake

An integrative study of a pristine meromictic lake ecosystem in Antarctica identified differential viral abundances and viral-like genes signatures in its depth profile (Lauro et al. 2011). The meromictic lake, Ace Lake is located in the Vestfold Hills and has an upper oxic mixolimnion separated by a distinct oxycline on top of a very stable anoxic monimolimnion. First, the viral abundance quantified with epifluorescence microscopy was estimated at $\sim 10^7$, $\sim 10^4$ and $\sim 10^8$ mL^{-1} in the mixolimnion layer (i.e. 5 and 11.5 m), oxicline (12.7 m) and anoxic monimolimnion (18 and 23 m) respectively. Although the sampling procotol, which used sequential filtration on 3.0, 0.8 and 0.1 μm filters, did not specifically target the Ace Lake viral assemblages, the viral-like genes signatures changed along the water column. The mixolimnion was dominated by Phycodnaviridae. These Phycodnaviridae sequences were believed to belong to viruses infecting *Mantoniella*, given that most 18sRNA genes sequences retrieved from the mixolimnion layer were related to the phylum Chlorophyta. At the oxicline, known viral sequenced were completety absent from the metagenomic data, while in the monimolimnion layer, the viral-like sequences were dominated by bacteriophages (Siphoviridae, Myoviviridae, Podoviridae). These findings suggest that there was

little exchange between viral assemblages among the different layers in the water column of the meromictic lake and that the viral community played different roles in structuring the ecosystem depending on the composition of the host-community (i.e. there was a dynamic coupling between top-down and bottom-up factors).

8.4 Reservoir of Unknown Diversity

The analysis of genomes from polar viral assemblages highlighted the presence of a high diversity of unknown viral types. A cyanophage (cyanophage S-EIV1) recently isolated from freshwater systems on Ellesmere Island (Nuvanut, Canada) using the polar *Synechococcus* strain PCCC-A2c was identified as the first representative of a new lineage phage (Chénard et al. 2015). The morphological and genomic characterization of cyanophage S-EIV1 shows little similarity to any characterized lower latitudes cyanophages. First, two distinct morphologies were observed for intact and empty capsids (devoid of nucleic acids) of cyanophage S-EIV1. The intact capsids did not display a tail-like structure, while the empty capsids possess a long and delicate tail-like structure. It was hypothesized that the tail-like structure was ejected during the release of nucleic acids from the capsid. In addition, most predicted open reading frames in the genome of S-EIV1 did not share similarity with genes of known function. The absence of recognizable genes that encode structural proteins in S-EIV1 genome suggest that the genes encoding its structural proteins are distinct from other viruses. Although S-EIV1 was isolated from polar waters, sequence recruitments using metagenomic datasets from different locations indicate that S-EIV1-like phages are cosmopolitan and abundant in a wide range of aquatic systems.

The genome of two marine polar bacteriophages have also been sequenced (Borriss et al. 2007; Colangelo-Lillis and Deming 2013). The first one, bacteriophage 11b infects a marine psychrophilic Flavobacterium and was isolated from Arctic sea ice (Borriss et al. 2007). Its genomic content and organization reveals similarity to other lower latitude non marine siphoviruses similar to lambda. The second, bacteriophage 9A infecting a marine psychrophilic gamma proteobacterium *Colwellia psychrerythraea* was recently isolated from a nepheloid layer at 128 m depth in Franklin Bay (Canadian Arctic) (Wells and Deming 2006a). Based on its morphology and genomic content, bacteriophage 9A also belongs to the *Siphoviridae* but had similarity to T5-like viruses (Colangelo-Lillis and Deming 2013). Although bacteriophage 9A can replicate at low temperature (between −13 and 8 °C), the temperature influence its latent period and burst size (Wells and Deming 2006a). Indeed, its latent period time increases as temperature decreases while burst size was lower at −10 and −8 °C that at −1 °C. Although the influence of temperature on the replication were only observed in a laboratory-setting, these findings might have an ecological implication. An increase in temperature in polar systems due to climate change might influence the viral-host dynamics.

The analysis of polar metagenomes uncovered further reservoirs of unknown viral diversity (Angly et al. 2006; López-Bueno et al. 2009). For example, the first viral metagenomic analysis targeting four major oceanic regions including the Arctic Ocean demonstrated that the 688,590 reads generated from a viral composite of 56 samples from 16 sites across the Arctic Ocean, over 86% did not have any significant similarity with known sequences (Angly et al. 2006). Among the four oceanic regions (Gulf of Mexico, Sargasso Sea, Coast of British Columbia and Arctic Ocean), the Arctic Ocean was the least genotypic-rich (532 predicted genotypes) and diverse correlating with the latitudinal diversity gradient (Hillebrand 2004; Fuhrman et al. 2008; Paez-Espino et al. 2016).

Viral metagenomic analysis was also performed on polar lakes from both Antarctic and Arctic environments, showing that they largely contain unknown and single stranded DNA viruses (ssDNA viruses) (López-Bueno et al. 2009; Aguirre de Cárcer et al 2015). First, López-Bueno et al. (2009) profiled the seasonal variations of the viral assemblage of Lake Limnopolar located in Byers Peninsula (Livingston Island, Antarctica). Over 87% of the 89,347 sequences obtained by high throughput sequencing had no significantly similarity to known sequences. The remaining sequences contained a large fraction of sequences related to ssDNA viruses and Phycodnaviruses. While most of the Phycodnaviruses hits (~87%) were related to a virus infecting the Prasinophyte *Ostreococcus tauri* (OtV5) (Derelle et al. 2008), the ssDNA viral hits belong mostly to the Circoviridae and Microviridae. Interestingly, samples collected before and after the ice cover melted suggested that the community undergoes a transition from mainly ssDNA viruses before the ice cover melted to dominance by double stranded viruses (e.g. Phycodnaviruses) after the melt, suggesting a seasonal change within the host population (López-Bueno et al. 2009). The viral metagenomes of 6 lakes in Spitsbergen (78 N, Svalbard, Norway) were also dominated by unknown sequences (Aguirre de Cárcer et al. 2015). Indeed, over 90% of the sequences had no significant similarity to known sequences in the databases. The sequences with homology were also mostly related to ssDNA viruses (86%) with a large proportion being Circoviridae. Different arctic freshwater viral assemblages shared a high proportion of sequences, but had few homologs in viral assemblages from other environments including those of the Arctic Ocean (Angly et al. 2006) and Lake Limnopolar (Antarctic) (López-Bueno et al. 2009). A notable exception was the presence of a number of viral ssDNA circular contigs, which were shared between Arctic and Antarctic freshwater with percent similarity between 90.8 and 93.8%. This might suggest that ssDNA viruses dominate the polar freshwater lakes. However, both metagenomic studies conducted in the Arctic and Antarctic lakes relied on multiple displacement amplification using Φ29 polymerase amplification, which is known to produce a bias toward ssDNA (Yilmaz et al. 2010).

A second metagenomic study, this time focusing on RNA viruses, was performed on Lake Limnopolar in Byers Peninsula (Lõpez-Bueno et al. 2015). Positive single-strand RNA viruses from the order Picornavirales dominated all three water samples collected during a three year interval. In addition, nearly four full-length genomes related to picorna-like viruses were assembled [Antarctic Picorna-like

virus (APLV) 1–4]. Based on the RNA-dependent RNA polymerase (RdRp) phylogeny, APLV1 clusters with viruses known to infect insects (e.g. Dicistrovididae). The other assembled genomes (APLV2–APLV4) cluster with viruses infecting diatoms (Bacillarnavirus) and algal (Marnaviridae). Given the deep sequencing coverage for the APLV viruses, López-Bueno et al. (2015) assessed the quasispecies structure of the RNA viruses in Lake Limnopolar using single nucleotide variant calling. Although APLV1 was the most abundant assembled genome in the lake water sample, its genome show little genetic variation from 2006 to 2010. On the contrary, APLV2 and APLV3 which were only present in the summer samples demonstrated a high complexity in their quasispecies structures. As APLV2 and APLV3 were also detected in cyanobacterial mats, it was suggested that these findings might reflect the convergence of different viral quasispecies from the run off or a replication in a more diverse host community.

A metaproteome analysis on a hypersaline lake in the Vestfold Hills region of East Antarctica reveals that archaeal viruses are also parts of the viral assemblage in some polar lakes (Tschitschko et al. 2015). Deep Lake which is a hypersaline lake that remains liquid at temperature below −20 °C is dominated by haloarchaea (DeMaere et al. 2013). Indeed, the analysis revealed the presence of at least eight groups of haloarchaeovirus based on the presence of eight distinct major capsid proteins in the dataset (Tschitschko et al. 2015). Five of those groups were similar to the haloarchaeal siphovirus isolates (HCTV-1 like subgroup), one matched the haloarchaeal siphovirus HCTC-2, one matched the haloarchaeal siphovirus HHTV-1 and one matched Halorubrum virus BJ1 (for more info on Archeal viruses see Chap. 7). The presence of prohead proteases and scaffold proteins in the metaproteomic dataset further suggests that the viral assemblage was active in Deep Lake at the time of sampling (Tschitschko et al. 2015).

As in temperate regions, there seems to be a clear distinction between viral assemblages from marine and freshwater systems in polar regions (Short and Suttle 2005; Chénard and Suttle 2008). This is consistent with the assumption that salinity might be the most important driver of the viral community structure (Sullivan et al. 2008; Logares et al. 2009). For example, water samples collected from Milne Fiord an epishelf lake located on the Northern coast of Ellesmere Island demonstrated a clear distinction when analyzing the community fingerprint of the T4-like bacteriophages with Denaturing Gradient Gel Electrophoresis (DGGE) between its freshwater layer (5 m) and its seawater layer (35 m) (Veillette et al. 2011). Similarly, there is little overlap between the metagenomic dataset from the Arctic Ocean and those from freshwater systems in the Arctic and Antarctic (Aguirre de Cárcer et al. 2015). This is consistent with the global viral biogeography which suggest that viruses are predominantly found in similar habitats, regardless of the geographic proximity (Paez-Espino et al. 2016).

8.5 Conclusion

As summarized in this chapter, new insights on viral ecology of high latitude aquatic systems are starting to emerge. It is becoming progressively clear that viruses play a pivotal role in structuring high latitude aquatic systems where microbes are important players and major drivers of biogeochemical cycles. Research conducted in the high latitude systems is still relatively uncommon as a result of the logistical challenges associated with performing fieldwork in remote polar habitats. More sampling opportunities using "field-friendly" technologies and low cost sampling strategies are needed to capture the big picture of viruses in polar waters. Recent advances in sequencing technologies resulting in lower sequencing cost and the development of portable DNA sequencers with low DNA input requirement are a few examples of these technologies (Jain et al. 2015; Rinke et al. 2016). Nevertheless due to the renewed interest in understanding the ecosystem ecology of low temperature ecosystems, we can foresee that the next few of years are going to be exciting for the field of polar viral ecology.

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Chapter 9
The Nature and Relevance of Solvent Stress in Microbes and Mechanisms of Tolerance

Mike Manefield, Matthew Lee and Joanna Koenig

Abstract Solvent stress in microbiology refers to exposure of microorganisms to chemical compounds with relatively low polarity. Environments in which solvent stress is intense are traditionally grouped with other extreme environments with hazardous temperatures, pressures, salinity, acidity and radiation. Extreme Environments with respect to solvents include natural oil or organohalide contaminated environments and industrial settings in which microbes are used to produce solvents or other compounds in dual phase reactor systems. Stress is typically thought to be exerted by interference with membrane function but the ability of solvents to interfere with protein structure is perhaps an underestimated target for solvent stress. It is a significant concern that selection for efflux pumps through exposure to solvents is likely to select for resistance to antimicrobials. Other solvent tolerance mechanisms include membrane adaptation and solvent biodegradation along with more generic strategies such as biofilm formation, motility and endospore formation. Whilst mechanisms of tolerance in aerobic bacteria have been extensively studied, less work has been done on anaerobic bacteria and archaea. An understanding of the nature of solvent stress and microbial strategies to adapt has relevance in natural and biotechnology settings.

9.1 Introduction

Habitats with pH, temperature, pressure or salinity outside the norm are the usual suspects when considering extreme environments, however microbial communities living in the presence of high dissolved or free phase organic solvents also fit the remit. Such environments can be naturally occurring or contrived by humankind either by accident or in biotechnological settings. Much is now known about

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microbial responses to solvent stress, with a variety of model systems having generated a wealth of information. In this book chapter the nature of solvent stress is described along with the relevance and molecular mechanisms of tolerance. This knowledge is routinely exploited in the bioproduction of organic solvents and the bioremediation of solvent polluted environments.

9.2 The Nature of Solvent Stress

Organic solvents encompass natural and anthropogenic hydrocarbons with varying degrees of non-polar character. Examples include oil or petroleum hydrocarbons, which are mostly of natural origin though processed and redistributed on mass by humankind, and halogenated hydrocarbons, which are widespread in nature but mass produced and distributed anthropogenically. Chemical classes include alcoholic, aromatic or aliphatic hydrocarbons.

Organic solvents impose stress on biological systems principally as a consequence of the polarity of the offending molecule and interactions with cell membranes and proteins (Isken and de Bont 1998). There are other more specific mechanisms by which organic solvents impact on biological systems, for example through competitive inhibition of protein-ligand binding sites affecting respiration, interference with gene expression or through transformative activation in mammalian systems to toxic or mutagenic compounds. Competitive inhibition has been reported where VC competes with cis-DCE for reducing equivalents (Kitanidis and McCarty 2012). Chloroform is known to affect gene transcription in *Desulfitobacterium* species (Futagami et al. 2013). In pure cultures of *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* strains grown on 3-chloro-4hydroxyphenylacetate as electron acceptor, chloroform inhibited transcription of the reductive dehalogenase *cprA*. Other specific inhibitory mechanisms were proposed with chlorophenol and chloroethene respiring *Desulfitobacterium* lineages displaying different sensitivities (Futagami et al. 2013). It is also well known that solvents can be highly mutagenic, but generally only after some form of metabolic activation. For example, perchloroethene is not mutagenic in the standard Ames test, however preincubation with glutathione-*S*-transferases results in formation of TCVG and a striking mutagenic response in the Ames test (Vamvakas et al. 1989).

Whilst vinyl chloride, chloroform and perchloroethene are all solvents and in these examples cause growth inhibition or mutation in specific bacterial strains, the described phenomena are not a unique consequence of their polarity. These phenomena are often strain specific and not related to solvent toxicity where polarity is the central determinant.

The impacts of organic solvents increase with concentration. Many organic solvents have a limit to their aqueous solubility and can reach concentrations where a separate phase develops, analogous to a salt falling out of solution. In dual phase systems, solvent stress is related to concentration by virtue of the surface area of the phase divide, rather than the aqueous concentration, which remains constant as

more solvent is applied. Organic solvents in aqueous solutions can also form isolated droplets and emulsions on agitation and interact readily with hydrophobic surfaces. Organic solvents are typically separated from hydrophilic surfaces by a layer of water.

Polarity dictates biological availability via aqueous solubility as quantified with the octanol: water partition coefficient (log P_{OW}). Solvents with a log P_{OW} between 1 and 5 inhibit the growth and metabolic activity of microorganisms in a concentration dependent manner because they are soluble enough in water to interact with microbes but have low enough polarity to partition into cellular membranes. Polarity of the solvent dictates the location in lipid membranes and ultimately how membrane structure is deformed. Note that the relationship between log P_{ow} and membrane solubility is heavily impacted by the specific lipid composition of a lipid bilayer (Sikkema et al. 1995). Partitioning into triacylglyceride lipid bilayers results in disruption of membrane structure and ultimately function (Sikkema et al. 1995). Organic solvent accumulation in the area of a lipid bilayer containing acyl chains or in the area between opposing monolayers of lipid bilayers results in increases in the area occupied by each phospholipid molecule and membrane thickness (Sikkema et al. 1995) (Fig. 9.1). Solvent impacted membranes become permeable to ions (e.g. protons) and larger biomolecules (e.g. ATP) thereby antagonising generation of proton motive force and storage of energy in metabolic nucleotides (Heipieper et al. 1991). Typically, bacterial cells with dual membrane structure (Gram negative) can tolerate higher solvent concentrations compared to those with a cell structure involving a single lipid bilayer membrane (Gram positive) because outer membranes and the intervening periplasm offer some degree of protection to the inner cytoplasmic membrane (Torres et al. 2011; Segura et al. 2012).

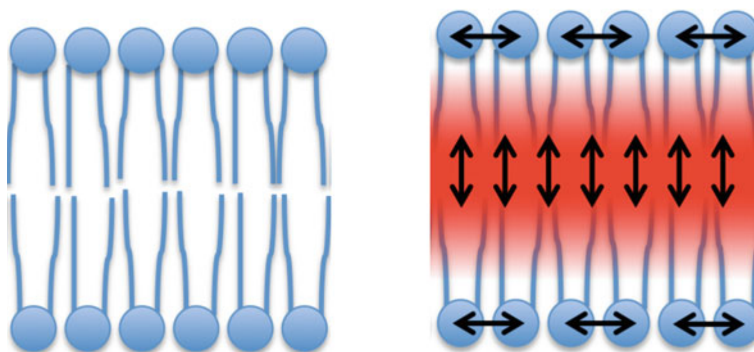

Fig. 9.1 Non-polar solvent impacts on lipid bilayers. Lipid head groups and acyl chains presented in *blue*. *Red* represents accumulated non-polar solvent. *Arrows* indicate forces acting on the bilayer in the presence of solvent. Depending on polarity solvents will penetrate membranes and acculumulate amongst the acyl chains creating forces that separate the phospholipid heads (e.g. hexane) and between the opposing monolayers of acyl chains forcing the membrane to increase in width (e.g. dodecane to hexadecane). Ultimately the natural order is disrupted leading to greater permeability and loss of function

Additionally, organic solvents disrupt protein structure and function. This can occur indirectly through interference with membrane lipids, which constitute the environment in which many important membrane associated proteins fold and function, including the respiratory machinery of microbes (White and Wimley 1999). Solvents additionally disrupt protein function directly owing to displacement of water molecules implicated in protein architecture and catalysis and in the thermodynamics, cooperativity and specificity of ligand binding (Mattos and Ringe 2001). Whilst the impact of aqueous organic mixtures are known to disrupt protein structure and function, it is important to note that many proteins can maintain structure and function in pure organic solvents (Griebenow and Klibanov 1996). Examples of intensively studied proteins in biocatalysis research showing activity in the presence of organic solvents or in neat organic solvents include elastase, thermolysin and lysozyme (Griebenow and Klibanov 1996; Mattos and Ringe 2001). The polarity of the organic solvent as well as the ability to compete for hydrogen bonding dictate the impact of a solvent on protein structure.

Aside from cell integrity maintained by single or double lipid bilayer membranes and anabolic and catabolic catalysis carried out by proteins there is little evidence that solvents impact negatively on other cellular structures or functions. Whilst several organic solvents are known to cause damage in DNA in higher organisms it is understood that this damage occurs as a consequence of processing by mammalian enzymes not present in microorganisms (Irving and Elfarra 2013). To date there is no evidence that organic solvents interfere with the integrity or function of DNA or RNA directly.

9.3 The Relevance of Solvent Stress

Solvent stress and tolerance in microorganisms are relevant in natural and human derived settings. It is not clear when organic solvents were first present on Earth in an abundance that could impart stress and have selected for solvent tolerance in microbes. Organic molecules such as light alkanes (e.g. propane, butane) can be formed abiotically under heat and pressure and are liquid or dissolved under pressure. Such chemistry has been stored 2–3 km into the subsurface in billion year old groundwater environments inhabited by microbes, predominantly sulfate reducing bacteria (Lollar and Ballentine 2009). These may be the earliest environments in which microbes were exposed to solvent stress.

The oil deposits of the Earth, which contain organic solvents, were mostly laid down during the Mesozoic era and are in the order of 100 million years old (Moldowan and Dahl 1994). This is likely the first time microorganisms were widely exposed to solvent stress, with mechanisms of tolerance co-adapted from other forms of stress. Meckenstock and coworkers recently described microbial communities living in small water droplets of deep subsurface origin embedded in a natural asphalt lake (Meckenstock et al. 2014). The communities were dominated by members of the orders Burkholderiales and Enterobacteriales, which contain

many of the extensively studied solvent tolerant bacterial lineages. In environments such as this and others including traditional oil deposits, oil sands, oil shale and marine and terrestrial oil seeps solvent tolerance is a requisite for microbial activity and replication. The characteristics of these hydrocarbon deposits have been influenced by microbes over geological time (Head et al. 2003), which could not have occurred without the ability to tolerate organic solvent stress. Indeed, if microorganisms were unable to tolerate and degrade the 1.3 million tons of oil that enters the environment each year mostly through natural seeps our coastlines would be awash with oil (Head et al. 2006).

Microbes in the environment are also exposed to solvent stress in marine and subsurface environments as a consequence of anthropogenic pollution. The microbiology of infamous marine oil spills such as in the Prince William Sound and the Gulf of Mexico have been studied extensively (Atlas and Hazen 2011), with the ability of microbial communities and the higher trophic levels dependent on microorganisms to rebound in part owing to solvent tolerance. Petroleum leakage from underground storage tanks (BTEX) from omnipresent domestic fuel distribution infrastructure is another anthropogenic phenomenon requiring solvent tolerance in groundwater microorganisms for environmental restoration.

In addition to oil derived solvents, chlorinated aliphatic hydrocarbons are widely distributed in the environment as a consequence of human industry. Organochlorine solvents used extensively as industrial solvents for dry cleaning of fabrics, degreasing engines and in the production of plastics and other chemicals have entered subsurface soil and groundwater environments. Bioremediation of organochlorine contaminated sites has emerged as the most cost effective means of ameliorating the human and environmental health risks of such contamination. This requires aerobic and anaerobic subsurface microorganisms to tolerate high dissolved concentrations of organic solvents and non-aqueous phase pollutant mass.

Solvent tolerance in microorganisms also has relevance in industrial and biomedical chemical production including biofuel production. There are several advantages to performing biocatalytical reactions in biphasic or nonaqueous systems relating to the solubility of reactants and products, removal of toxic products and prevention of contamination (Sardessai and Bhosle 2004). The commercial significance of such bioproduction systems have been a major driver in the isolation and genetic manipulation of solvent tolerant bacteria and research into the mechanisms of solvent tolerance.

Solvent tolerance also has relevance to other tolerances displayed by microorganisms. It has been shown that there is a correlation between solvent and temperature tolerance (Ramos et al. 2002). This suggests that adaptation to high temperature also results in an increase in tolerance to organic solvent stress and is likely related to membrane composition adaptation. A correlation between organic solvent tolerance and resistance to antibiotics has also been observed (Asako et al. 1997). This is important because exposure of microorganisms to organic solvents may increase antibiotic resistance, with implications to infectious disease control and human health. This correlation is related not to membrane structure but to the efficacy and activity of efflux pumps (Fernandes et al. 2003).

9.4 Organic Solvent Tolerant Microorganisms and Mechanisms of Tolerance

There are broad differences in the ability of different microorganisms to tolerate solvent stress that are not in any way related to microbial phylogeny. Indeed solvent tolerant microbes along with solvent sensitivity are well distributed throughout the phylogenetic tree, although detailed information on the mechanisms of solvent tolerance tend to focus on easy to handle laboratory stalwarts such as *Escherichia* and *Pseudomonas* species. Tolerance mechanisms can be broadly divided into two categories. The first encompasses generic mechanisms microbes use to defend themselves against environmental insults generally. The second encompasses mechanisms that are specific to solvent stress Fig. 9.2 summarises generic and specific defence mechanisms against solvent stress.

In environmental settings, microbes protect themselves from environmental stress through aggregation and biofilm formation. Aggregation is mediated by cell surface properties (e.g. hydrophobicity), electrostatic interactions with cations, extracellular DNA (Das et al. 2014) and specific ligand-receptor interactions (sugar-lectin binding) (Rickard et al. 2002). Aggregated cells represent a diffusion barrier to most chemicals, so cells central to the aggregate will be protected from

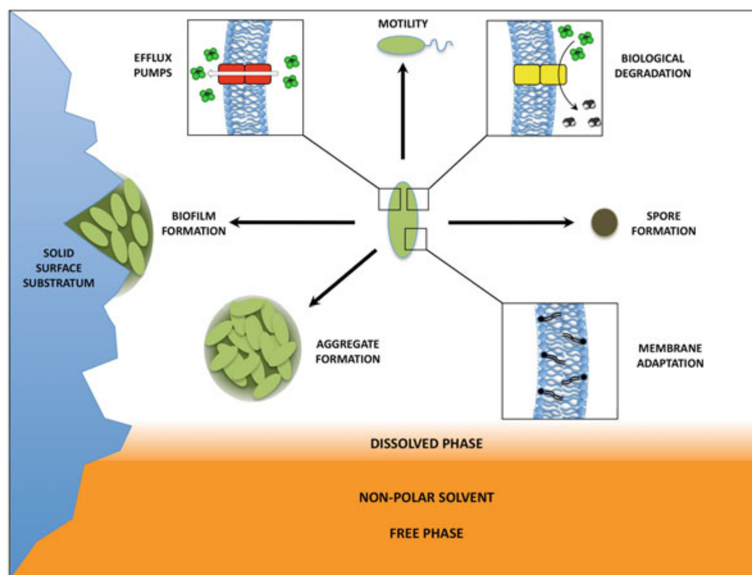

Fig. 9.2 Mechanisms of solvent tolerance dictating the effect of solvent stress on the behaviour and composition of microbial communities in a natural setting (sediment or subsurface). Mechanisms fall into two broad categories. Generic mechanisms include biofilm and aggregate formation, spore formation and motility. Specific mechanisms include increased membrane rigidity, efflux pumps and biological degradation

increases in solvent concentration in the surrounding bulk aqueous phase for a period of time. Surface associated biofilms also consist of aggregated cells with the additional protection of a solid surface, representing a spatial dimension from which solvents cannot access cells, like protecting your back by standing against a wall (Hall-Stoodley et al. 2004). Aggregated cells in flocculates and in biofilms attached to surfaces also generate an extracellular matrix that limits diffusion. These matrices, composed principally of polysaccharides, nucleic acids and peptides protect cells from negative impacts of the surrounding environment (Das et al. 2013). If cells in aggregates or biofilms can degrade the solvent, protection may last indefinitely. Whilst challenging to exhaustively demonstrate, there are no bacteria specifically known for the inability to produce biofilms or aggregates. Cells in biofilms can display low metabolic activity as a consequence of limited access to nutrients and energy but are generally considered to be metabolically active (Hall-Stoodley et al. 2004).

Endospore formation represents another general mechanism by which microbes protect themselves from stress including solvent stress, however this is a dormant, metabolically inactive state (Nicholson et al. 2000). Endospore formation is only recognized within the Firmicutes phylum, though members of this phylum are diverse and widely distributed in the environment, including the *Bacillus* and *Clostridium* genera (Maczulak 2011; Martin and Travers 1989). Endospore formation likely evolved before solvent stress was a major selective advantage, but certainly spore formation will be selected for in complex environmental communities exposed to natural or anthropogenic solvent stress. Simplistically spores consist of an outer coat, a cortex and a core, all of which presumably serve to limit access of solvents to the inner membrane within the cortex and proteins stored in the core, though exactly what imparts solvent tolerance to spores is not known. Bacteria outside the Firmicutes phylum display stress responses analogous to spore formation, whereby cell size reduces, metabolic activity is reduced to a minimum and cell structure becomes more tolerant to insults. This genetic program and resting state mediated principally through ppGpp accumulation and sigma factors (RpoS) has been most intensively studied as a response to starvation, but offers cross protection against solvent exposure (Shimizu 2015).

If one approach to surviving solvent stress is to batten down the hatches (aggregate, biofilm, endospore formation) another is to flee. Many microbes possess sophisticated sensory systems controlling motility (Bren and Esienbach 2000). Motility is most commonly associated with flagella production and action and examples exist whereby solvent challenges to cultures upregulate genes encoding motility (Tomas et al. 2003). Motility is a general response to stress where unicellular organisms attempt to move away from the stressful condition. This is not much help in homogenous culture media bioreactor systems, but potentially useful in escaping down solvent concentration gradients in contaminated saturated subsurface environments (Fig. 9.3).

One of the most extensively studied areas of solvent tolerance is the adaptation of cell membrane composition (Liu 2011). The bulk of this work has concentrated on *Pseudomonas* species, relevant to solvent bioproduction and petroleum product

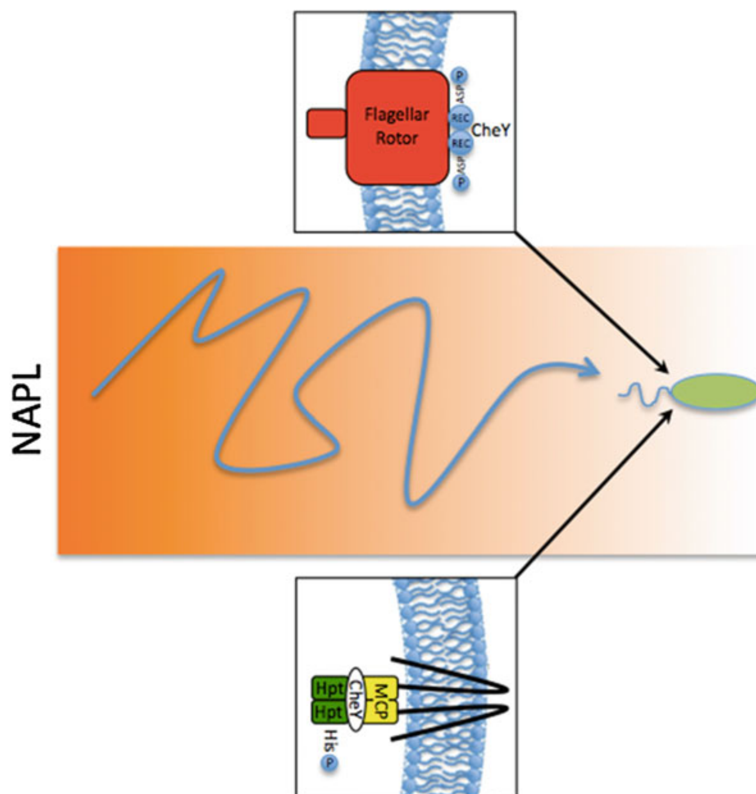

Fig. 9.3 Illustration of a motile bacterial cell with a polar flagellum controlling movement down an organic solvent gradient away from non-aqueous phase liquid (DNAPL or LNAPL). The lower inset shows membrane associated sensory proteins based on the well studied Che system in *Escherichia coli*. When activated by the presence of solvent the sensory system phosphorylates enzymes controlling flagella rotor rotation (upper inset). The bacterial cell tumbles to change direction

bioremediation alike. The toluene tolerant model organism *Pseudomonas putida* and others have been shown to alter outer membrane lipid content to reduce permeability and increase rigidity and hydrophobicity (Ramos et al. 2002; Segura et al. 2004). These changes to membrane properties are achieved through changing the proportions of proteins versus lipids in the membrane and converting lipids with cis (kinked) conformation to the trans (linear) conformation (Pinkart and White 1997). Such adaptations enable survival of short-term exposure to solvents or mild solvent concentrations and require active replication to manifest. The genetic response for membrane and protein adaptation is related to the heat shock response which also requires a decrease in membrane rigidity. The response is orchestrated by the sigma factor RpoH.

The other intensively studied mechanism of solvent tolerance is the use of efflux pumps (Torres et al. 2011). Efflux pumps are enzyme transporter complexes embedded in the cytoplasmic membrane that use energy to transfer out of the cytoplasm molecules that interfere with cellular function. Consequently, solvent stress can confer cross protection and resistance to antimicrobials, including disinfectants and antibiotics. Solvent efflux pumps have been extensively studied in *Pseudomonas putida* (Kieboom et al. 1998) *Pseudomonas aeruginosa* (Li et al. 1998), *Escherichia coli* (Asako et al. 1997) and *Bacillus cereus* (Matsumoto et al. 2002). As long as cells remain metabolically active, efflux pumps offer tolerance to solvents indefinitely and this is one of the reasons why efflux pumps represent possibly the most important mechanism of solvent tolerance, especially in the field of bioproduction. The importance of efflux pumps to solvent tolerance raises an interesting question about the mechanism of solvent toxicity. It is widely believed that solvents principally exert their effects through interactions with membranes (Isken and de Bont 1998), but if this were the case then removal of solvents from the cytoplasm would not be a particularly effective defense strategy. This remains one of the last general paradoxes remaining in the research area of solvent tolerance.

Another widely recognized mechanism of solvent tolerance relates to the ability of microbes to alter or degrade the offending solvent. In the context of bioproduction this is generally undesirable but in the context of bioremediation it is very much the end game. Selection for solvent tolerance through biodegradation to harmless end products is a mean of developing bacterial strains for bioaugmentation of contaminated sites. Again, *Pseudomonas* species have featured heavily in this research area in the context of oxidative oil degradation but perhaps the most interesting examples lie in low redox conditions where members of the Firmicutes and Chloroflexi exploit chlorinated organic solvents as electron acceptors in a process known as reductive dechlorination (Koenig et al. 2014). Because chlorinated solvents have a higher density than water, they are predominantly found deep in groundwater setting where oxygen does not permeate.

So called organochlorine respiring bacteria (ORB) have been shown to tolerate, and be metabolically active in, the presence of some of the highest priority pollutants known (Lee et al. 2012). For example, chloroform is a widespread groundwater contaminant globally and is recognized for its ability to inhibit microbial activity at 10 mg/L through interference with membrane function (Chidthaisong and Conrad 2000). Recently, members of the *Dehalobacter* genus have been isolated that can reduce chloroform to dichloromethane thereby reducing the solvent stress imposed (Grostern et al. 2010; Lee et al. 2012). These *Dehalobacter* strains show tolerance to chloroform above 100 mg/L. Whilst degradation is considered an important defense mechanism, the role of efflux pumps and membrane adaptation are yet to be determined for strictly anaerobic bacteria.

Indeed, the solvent tolerance of strictly anaerobic bacteria or bacteria grown under anaerobic conditions generally is an area of neglect in this research field. In a recent study, the effects of perchloroethene, carbon tetrachloride, chloroform and 1,2-dichloroethane on the growth of four fermentative, one nitrate-reducing, one

iron-reducing and one sulphate-reducing species was examined (Koenig and Groissmeier et al. 2014). The octanol-water partition coefficient or log $P_{o/w}$ of the solvents proved to be a generally satisfactory measure of their toxicity. Interestingly, toxicity also correlated well with growth rates observed in solvent-free cultures, with fast-growing organisms displaying higher tolerance. This suggests the ability to harvest energy from the environment and short doubling times underlies the ability to tolerate solvent stress in anaerobic bacteria. It stands to reason that the more energy and resources an organism has at its disposal the greater response it can mount via all known tolerance mechanisms.

Yung et al. (2016) recently conducted an interesting study on the transcriptional response (RNA sequencing) of *E. coli* to sub-growth-inhibitory concentrations of eight volatile organic compounds (n-butanol, N-cyclohexyl-pyrrolidone, cyclopentanone, N,N-dimethylacetamide, dimethyl sulphide, 1-methyl-2-pyrrolidone, N-methyl succinimide and toluene). Whilst each compound generated unique transcriptional responses in *E. coli* several general responses emerged.

Firstly, transcription of genes involved in Fe/S cluster biogenesis were upregulated in response to many of the solvents. The iron-sulfur cluster (ISC) and sulfur mobilization (SUF) systems which carry out biogenesis and maturation of all Fe/S clusters in prokaryotes, were variously upregulated. IscR, responsible for regulating Fe/S homeostasis and expression of a number of Fe/S proteins was upregulated in response to all compounds tested. Transcription of iscR itself is upregulated by diminishing Fe/S cluster availability but also by oxidative stress.

Secondly, transcription of genes involved in oxidative stress responses were upregulated suggesting the solvents tested at sub-growth-inhibitory concentrations impose stress on *E. coli* equivalent to oxidizing agents such as paraquat and other superoxide generators. Transcription of the SoxRS controlled genes encoding the PqiAB enzyme and genes encoding the YhcN and MntS enzymes associated with the presence of or resistance to hydrogen peroxide were upregulated.

Thirdly, transcription of genes encoding transport proteins were significantly upregulated. Whilst predictably this included multidrug efflux pumps encoded by *mdtI*, *mdtJ* and *emrB,* a swathe of transporters for importing amino acids (*oppABCDF*), inorganic ions (*feoA*, *feoB* and *efeO*) and siderophores (*exbBD*, *yncD* and *fhuF*) were upregulated at the transcriptional level. This interesting response appears to suggest that E. coli not only responds to solvents by exporting from the cytoplasm but by importing elements and compounds apparently essential to counter the solvent stress.

9.5 Conclusions

Solvent exposed environments are generally considered to be extreme. Solvents encompass a broad range of chemical compounds with relatively low polarity. These environments include natural oil or organohalide contaminated environments and industrial settings in which microbes are used to produce solvents or other

compounds in dual phase reactor systems where organic solvents are used to partition products out of the aqueous phase. Stress is exerted by interference with membrane function, the integrity of which is crucial for cell integrity, reproduction and activity. The ability of solvents to interfere with protein structure is perhaps an underestimated target for solvent stress, as implied by the central role of efflux pumps in solvent tolerance. It is a significant concern that selection for efflux pumps through exposure to solvents is likely to select for resistance to antimicrobials. Other solvent tolerance mechanisms include membrane adaptation and solvent biodegradation along with more generic strategies such as biofilm formation, motility and endospore formation. Whilst mechanisms of tolerance in aerobic bacteria have been extensively studied, less work has been done on anaerobic bacteria and archaea.

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Chapter 10
Clouds: A Transient and Stressing Habitat for Microorganisms

Anne Marie Delort, Mickael Vaïtilingom, Muriel Joly, Pierre Amato, Nolwenn Wirgot, Audrey Lallement, Martine Sancelme, Maria Matulova and Laurent Deguillaume

Abstract In this chapter, we synthesized the current knowledge about clouds as ecosystems which have been discovered very recently. First, we briefly described the cloud habitat. Cloud physics chemistry and microphysics are described, showing that this environment is extreme. Microorganisms are exposed to a dynamic medium changing extremely rapidly (evaporation/condensation of the cloud droplets, quick temperature and pressure changes, freeze/thaw cycle) and also to chemical stresses (strong oxidants, acidic pHs and toxics). Then the life cycle of microorganisms in the atmosphere is detailed showing that cloud is a transient habitat: microorganisms are aerosolized, transported in the air, integrated in cloud droplets and deposited back to the ground with precipitation. Finally the cloud microbiome is described; it appears that it remains largely unknown and based mainly on culture techniques. In the second part of the chapter, the abilities of these microorganisms to survive in this stressing environment are described in details. Microbes can adapt their metabolism as it was shown that the majority of the community is metabolically active and that they metabolize organic compounds in cloud water. They have also developed general strategies that help resisting to atmospheric constraints, such as the production of extracellular polymeric substances and pigments, or the formation of spores. Finally they can respond to specific stresses such as oxidative, osmotic and temperature

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stresses thanks to protecting metabolites such as osmo- and thermo-protectants, anti-oxidants or by using specific enzymes.

10.1 Introduction

Unlike most environments such as soil, freshwaters, and oceans, microbiology of the atmosphere has only recently been studied. Indeed, the presence of living cells in clouds opens new routes in understanding the biogeochemistry of this medium. This lack of knowledge is certainly related to the fact that this environment is not easy to study: clouds are difficult to sample, specific sites are needed to get in parallel all the meteorological and physico-chemicals parameters, the number of microbial cells is low making investigations particularly difficult. As cloud environment is very difficult to reproduce in lab conditions, microcosm experiments are not easy to design. Finally working on cloud microorganisms requires knowledge in atmospheric sciences and strong interactions with physicists in this field. However, for a long time, a gap existed between atmospheric sciences and biology, where physicists considered microorganisms as simple particles and studied them only for their microphysical properties. The fact that they can be alive and can actively impact the atmospheric processes was not yet considered.

The question remains to know whether the cloud microbiome is a real "ecosystem". Indeed its major specificity is the fact that it is transient and highly diluted. This means that microorganisms are dispersed in a few droplets for a rather short time, and are thus not very likely to communicate. However they can exchange metabolites thanks to evaporation-condensation mechanisms leading to concentration modifications and exchanges between droplets.

Cloud environment can be considered as "extreme" mainly because it is an unstable medium, where physico-chemical conditions changes are large and fast. In addition this medium is highly oxidative due to the presence of strong oxidants and UV light. To face these stresses, some microorganisms have developed specific features allowing them to survive during their journey in the atmosphere.

10.2 Clouds as a Transient and Stressing Habitat for Microorganisms

10.2.1 The Cloud Habitat

10.2.1.1 The Physics of Cloud

Cloud lifetime is controlled by the dynamics of the atmosphere at the synoptic scale and, in close interaction, by microphysical processes (i.e. nucleation of cloud

droplets, condensation and evaporation, collision/coalescence processes, sedimentation of hydrometeor etc.) at small scale.

Clouds are made of microscopic droplets of liquid water ("warm clouds"), crystals of ice ("cold clouds"), or both ("mixed phase clouds"). Cloud droplets are formed by the condensation of water vapor onto cloud condensation nuclei (CCN) when the supersaturation of air exceeds a critical value which is described by the "Köhler theory" (Köhler 1936). Cloud condensation nuclei are needed during the formation of cloud droplets because of the Kelvin effect, which describes the variation in saturation vapor pressure due to a curved surface. When the cloud droplet radius is small, the amount of supersaturation needed for condensation to occur is so important, that it is impossible to occur naturally in the atmosphere. The second important factor is the concentration of solute that is described by the Raoult's Law: at high solute concentrations, when the droplets are small, the supersaturation needed is smaller than without the presence of a nucleus. The cloud lifetime depends on the way the cloud is evolving in the atmosphere. A cloud droplet can evaporate or fall to the Earth as precipitation. In warm clouds, larger cloud droplets fall at a higher terminal velocity than the smaller ones; the large droplets then can collide with small ones and combine to form larger drops. When drops become large enough so that the acceleration due to gravity is much larger than the acceleration due to drag, the drops can fall down as precipitation. These processes are called "collision/coalescence". In mixed phase cloud, this effect is not as important because other complex mixed phase processes occurs and lead to precipitation formation. Mixed-phase clouds and cold clouds are composed of various iced hydrometeors (pristine, snow, graupel) with a large size range and with various complex shapes. Important processes that form precipitation in mixed-phase clouds are riming, when a supercooled liquid drop collides with a solid snowflake, and also aggregation, when two solid snowflakes collide and combine together.

At synoptic scale, the formation of clouds strongly depends on updrafts. Water droplets that group together are quickly pulled down to the ground by gravity, so that they would quickly dissipate and the cloud never forms. An updraft can form if warm air interacts with cold air which can be cause by topography. As the warm air rises in the atmosphere, the moisture in the updraft will condense into liquid form and add to the amount of water available for precipitation. Violent updrafts can reach speed up to 290 km/h. The droplets can also freeze during through one of these updrafts and can cycle through several updrafts before finally becoming so heavy that it falls to the ground.

In this context, clouds are harsh environments for microorganisms because they have to deal with rapid changes in osmotic pressure when the liquid water of the cloud varies and/or when evaporation of cloud droplets occurs. Pruppacher and Jaenicke (1995) estimated that atmospheric water condensates and evaporates in average 10 times before being removed by precipitation (Pruppacher and Jaenicke 1995). The solute concentration can vary by several orders of magnitude: assuming neither precipitation nor gasification of the solutes, a 20 μm cloud droplet that evaporates to a diameter of 2 μm would concentrate chemical species by a factor of 10^3. For instance, ion species measured at the puy de Dôme station, the observed

concentrations of total ion content ranged from 1 to 1.9 mM (Deguillaume et al. 2014). Evaporation of cloud droplets would thus increase ion concentration to up to 1 M and create high osmotic shocks for the microorganisms.

Clouds also have to deal with strong pressure and temperature variation as well as supporting several freeze-thaw cycles, especially when strong updrafts are present. For example, in the troposphere, temperature generally decreases with increasing altitude, by 0.6 to 1 °C every 100 m. This, associated with vertical winds of 50 km h^{-1} (14 m s^{-1}), potentially exposes airborne cells to thermal variations of up to approximately 1 °C every 7 s, and eventually also to freeze-thaw cycles.

10.2.1.2 The Chemistry of Cloud

The cloud aqueous phase is a very complex mixture of inorganic and organic chemical compounds. These chemical compounds found in cloud droplets originate from various sources: from the soluble fraction of the aerosol particles which can also act as cloud condensation nuclei (CCN), from the dissolution of soluble trace gases as well as from scavenging processes. Cloud reactivity also forms new chemical compounds. Since cloud water contains strong acids, potentially toxic molecules (formaldehyde for example), and strong oxidants like hydrogen peroxide or radicals, it can represent a stressful medium for microorganisms.

The major inorganic ions found in cloud water are the sulfate (SO_4^{2-}), chloride (Cl^-) and nitrate (NO_3^-) anions and the alkali and alkaline Earth metals cations (Na^+, K^+, Mg^{2+}, Ca^{2+}), in addition to ammonium (NH_4^+). These chemical compounds results from various sources. For example, the major fraction of the potassium, magnesium and calcium ions come from the mineral part of aerosol particles originating from soil; nitrate and ammonium can enter into cloud water as constituents of condensation nuclei as well as by gas to liquid scavenging of gaseous HNO_3 and NH_3; the sulfate arises from the oxidation of gaseous precursors that are dissolved into cloud droplets such as SO_2. In polluted region, the oxidation of SO_2 and NO_2 is a major source of strong acids H_2SO_4 and HNO_3 that control cloud water acidity i.e. the pH. In coastal regions and over the ocean, sodium chloride constitutes the largest part of all ions. The acidity of the cloud water strongly depends on the air mass origin. The pH can vary between 2.2 and 7 (Aleksic et al. 2009; Collett Jr et al. 2002; Deguillaume et al. 2014; Hill et al. 2007). For example, for polluted clouds, the pH at the puy de Dôme station (France) is the most acidic due to the higher amount of nitrate and sulfate (pH around 4). pH values encountered in clouds are far from the known limits of growth of microorganisms that can develop at pH close to 0 for certain species (Schleper et al. 1995). However, beyond these limits, their survival strongly depends of their capacity to maintain their pH cytoplasmic at values compatible to their metabolism. Most of the microorganisms have optimal pH for their growth between 5 and 9 (Padan et al. 2005). During the cloud lifetime, the microorganisms have to deal with strong variations of the pH of the cloud droplets due to the condensation/evaporation processes.

The organic matter also represents a major fraction of the soluble matter in cloud droplets (Herckes et al. 2013). The total Dissolved Organic Carbon (DOC) is highly variable depending on the history of the air mass. For highly polluted clouds, DOC values can reach 200 mgC L^{-1} as reported by Wang et al. (2011) at Mount Tai in China (Wang et al. 2011). Currently, DOC values are between 5 and 10 mgC L^{-1} in average for continental clouds (Anastasio et al. 1994; Löflund et al. 2002; Ervens et al. 2013; Hutchings et al. 2009) and below 5 mgC L^{-1} for clouds from marine origin (Marinoni et al. 2004; Deguillaume et al. 2014). Carboxylic acids represent around 10% of the dissolved organic carbon in the cloud droplets (Table 10.1). The carboxylic acids can be produced in the gaseous phase and dissolved in the aqueous phase (main source of acetic and formic acid); they can also result from the dissolution of soluble particles (main source of oxalic, succinic, malonic, and maleic acids); or produced via the aqueous phase reactivity (Herrmann et al. 2015). Due to the presence of free radicals such as the hydroxyl radical $^{\bullet}OH$ in the aqueous phase, the oxidation of organic matter is considered as an important source of carboxylic acids (Tilgner and Herrmann 2010); they also represent one of their main sinks. Carbonyl compounds are also present in cloud water and they essentially result from their dissolution from the gas phase into the aqueous phase depending on their Henry's law constants (Ervens et al. 2003). In the aqueous phase, the oxidation of aldehydes produces carboxylic acids but also lead potentially to the formation of oligomers (Ervens et al. 2003, 2015). Concentration levels of carbonyl compounds such as formaldehyde, acetaldehyde, glyoxal and methylglyoxal have been measured in cloud water (Houdier et al. 2011; van Pinxteren et al. 2005; Matsumoto et al. 2005). Formaldehyde in the gas phase is produced by biomass burning and fossil fuel combustion, and also by photochemical oxidation of methane and non-methane hydrocarbons. This compound is efficiently transferred into the aqueous phase due to its efficient Henry's law constant, explaining its higher concentration in cloud water (Table 10.1). In the case of foggy event occurring in polluted area, the aqueous formaldehyde concentration can reach up to 710 μM (Jacob et al. 1984). This kind of high concentration of formaldehyde can be partially toxic for the biological content of the cloud/fog droplets.

The proportion of undetermined organic matter in cloud water is still high and represents more than 90% of the total dissolved organic matter. Among this complex organic matter in cloud water, HUmic LIke Substances (HULIS) have been identified and correspond to large multifunctional compounds such as proteins, cellulose, dicarboxylic acids, polyols, amino acids, fatty acids, sugars, polysaccharides or aliphatic and aromatic hydrocarbons (LeClair et al. 2012; Ekström et al. 2010).

This description of cloud carbon content shows that this medium is rather poor, with low DOC concentrations (Table 10.1). In addition, methanol and formaldehyde (C1 compounds) could be toxic for many microbial species.

Cloud water is an oxidizing environment with a redox potential of up to more than 200 mV (Deguillaume et al. 2014), notably due to the presence of radicals ($^{\bullet}OH$ and $HO_2^{\bullet}/O_2^{\bullet-}$) and their precursors ($H_2O_2$, metals) (Table 10.1). H_2O_2 concentrations are ranging from 0 to 3.2 μM at Kleiner Feldberg [Germany; Sauer et al. (1996)], from 0.1 to 57.7 μM at the puy de Dôme, with an average of 7.8 μM

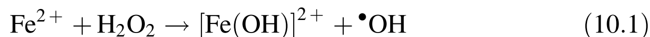
Table 10.1 Observed concentration of carboxylic acids, formaldehyde, hydrogen peroxide, iron and dissolved organic carbon in cloud samples

Chemical compounds	Concentrations (μM)	References
Acetic acid	30.0–84.0	Watanabe et al. (2001)
$CH_3CO(OH)$	4.0–37.8	Löflund et al. (2002)
	1.6–41.4	van Pinxteren et al. (2005)
	0.3–57.6	Deguillaume et al. (2014)
Formic acid	1.3–34.3	Löflund et al. (2002)
CHO(OH)	36.0–51.2	Decesari et al. (2001)
	4.9–39.1	van Pinxteren et al. (2005)
	0.2–52.8	Deguillaume et al. (2014)
Succinic acid	0.8–2.6	Löflund et al. (2002)
$CO(OH)CH_2CH_2CO(OH)$	0.1–4.1	Deguillaume et al. (2014)
Malonic acid	0.7–2.9	Löflund et al. (2002)
$CO(OH)CH_2CO(OH)$	0.4–1.8	van Pinxteren et al. (2005)
	0.3–7.0	Deguillaume et al. (2014)
Oxalic acid	0.7–12.6	Löflund et al. (2002)
CO(OH)CO(OH)	0.1–15.2	Decesari et al. (2001)
	2.4–11.6	van Pinxteren et al. (2005)
	0.2–19.4	Deguillaume et al. (2014)
Formaldehyde	8.0–14.0	Collett et al. (1990)
CH_2O	13.6–61.5	Igawa et al. (1989)
	0.1–4.8	van Pinxteren et al. (2005)
	0.1–14.2	Deguillaume et al. (2014)
Hydrogen peroxide	0–247	Olszyna et al. (1988)
H_2O_2	1–167	Richards (1995)
	0–14	Valverde-Canossa et al. (2005)
	0–19	Marinoni et al. (2011)
Iron	0.1–1.6	Deutsch et al. (2001)
Fe^{2+} and Fe^{3+}	0.6–6.3	Pehkonen et al. (1992)
	0.3–22.6	Erel et al. (1993)
	0.1–11.9	Parazols et al. (2007)
Disolved **O**rganic **C**arbon (**DOC**)	**Concentration** (**mgC L^{-1}**)	
	3.0–18.0	Anastasio et al. (1994)
	1.8–8.1	Ervens et al. (2013)
	2.0–35.0	Wang et al. (2013)
	0.3–25.0	Deguillaume et al. (2014)

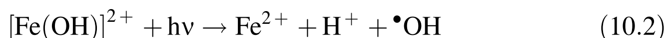
[France; Deguillaume et al. (2014)], and extremely high concentrations (up to 247 μM) were reported from Whitetop Mountain [U.S.A.; Olszyna et al. (1988)]. Their aqueous concentrations result from both various chemical interactions

(photolysis processes and chemical reactions) and from the phase transfer exchange between the gas and aqueous phase. $^{\bullet}OH$ radicals, can be either taken up from the gas phase or in situ produced in the aqueous phase.

One of the most relevant in situ sources of $^{\bullet}OH$ in the aqueous phase is the so-called "Fenton" reaction between H_2O_2 and iron(II) (Eq. 10.1). The produced iron(III)-hydroxy complexes are also photolyzed (mostly the $[Fe(OH)]^{2+}$ aqua-complexe that is dominant for atmospheric pH between 3 and 5) accelerating the formation of $^{\cdot}OH$ in the aqueous phase (Eq. 10.2).



$$Fe^{2+} + H_2O_2 \rightarrow [Fe(OH)]^{2+} + {}^{\bullet}OH \quad (10.1)$$



$$[Fe(OH)]^{2+} + h\nu \rightarrow Fe^{2+} + H^{+} + {}^{\bullet}OH \quad (10.2)$$

The photolysis of H_2O_2, NO_3^{-} and iron(III)-hydroxy complexes are also effective sources of $^{\bullet}OH$ in the aqueous phase (Bianco et al. 2015) together with the phase transfer from the gas phase. Additionally, the reactions of oxidized TMIs (Transition Ion Metals) with H_2O_2 and the photolysis of metal-organic acid complexes such as iron(III)-oxalate complexes can act as source for $HO_2^{\bullet}/O_2^{\bullet -}$ in the aqueous phase (Weller et al. 2014). The presence of such an oxidizing environment represents a strong stress that microorganisms have to cope with.

10.2.2 The Cycle of Microorganisms via the Cloud Habitat

The concept of "cloud microbiome" which was recently suggested is unique mainly due to the specific status of cloud microorganisms compared to the other stable environmental ecosystems (waters, soil, plants, etc.). Cloud is a transient habitat lasting from a few hours to a few days as it is part of the life cycle of the microorganisms in the atmosphere. Microorganisms are aerosolized, transported in the air and deposited further or integrated in clouds by nucleation and scavenging processes and can be back to the earth by wet deposition using precipitation as shuttles (Fig. 10.1). During their travel they are exposed to very strong stresses, especially in cloud itself, which likely alter viability (see Sect. 10.1).

The sources of microorganisms are very wide including water, soil and vegetation; Burrows et al. (2009a) have evaluated that, globally, $\sim 10^{24}$ bacterial cells are aerosolized from surface environments each year. Large spatial and temporal heterogeneities exist in the distribution of microorganisms in the air, notably in relation with the type of surface cover (rural, urban, forest, ocean, etc.) and it varies temporally with seasonal and daily periodicities (Burrows et al. 2009a; Lighthart 1997). The mechanisms of aerosolization are still not completely understood. In the case of oceans, microorganisms are emitted by bubble bursting. Bubbles are produced at the surface of the sea by whitecaps and breaking waves and rainfalls; since microorganisms are concentrated at the water-air interface, their concentration within jet drops can be increased by several orders of magnitude compared with

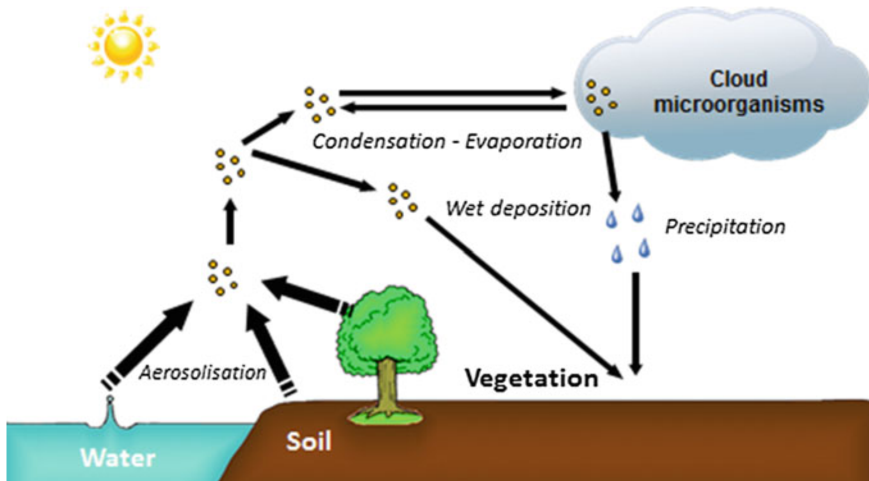


Fig. 10.1 Clouds acts as a transient habitat during the life cycle of microorganisms. Microorganisms are aerosolized, transported *vertically* and *horizontally*, integrated in clouds by condensing water and shuttled down to the ground mainly by precipitation

their concentration in the bulk liquid (Blanchard 1989; Marks et al. 2001; Aller et al. 2005; Mayol et al. 2014). Microorganisms can be also lifted up in the air by the wind as part of solid aerosols, particularly on dust particles (Griffin 2007). Fungi can emit spores specially designed to be aerosolized by the wind depending on meteorological factors including wind speed, temperature, humidity, (Jones and Harrison 2004). Finally vegetation is a major source of bioaerosols; the microbial density on plant leaves is $\sim 10^3$–10^8 bacteria g^{-1} (Lindow et al. 1978; Lindemann et al. 1982; Morris et al. 2008). In that case different mechanisms can be involved in aerosolization: microorganisms can be ejected by direct impact of rain drops on leaves or can be transported upward by turbulent flows (sensible heat flux); wind can lift dry leaves particles or dry biofilm fragments where bacteria are imbedded (Hirano and Upper 2000; Morris et al. 2004).

Measurement of emission fluxes is difficult and few data are available. It is supposed to vary largely with the type of sources: while Lindemann et al. (1982) have measured net upward fluxes in the range of 100–1000 CFU m^{-2} s^{-1} over agricultural areas, Burrows et al. (2009a, b) has evaluated emission rates as low as ~ 1 CFU m^{-2} s^{-1} from seas and glaciated ecosystems based on near-surface concentration measurements, however it should be noted that this evaluation is very rough and still questionable.

Once aloft, micron-sized particles like microorganisms are dispersed vertically and horizontally. They can go up in the atmosphere along the different layers (troposphere 0–12 km, stratosphere 12–50 km, and mesosphere 50–85 km). Although most studies have been performed in the troposphere, which is the major place where clouds form and exist; viable microorganisms have been collected up

to 77 km of altitude (Imshenetsky et al. 1978). Concerning horizontal dispersion microorganisms can be transported thousands of kilometers away from their emission source (Prospero et al. 2005; Kellogg and Griffin 2006; Smith et al. 2013), and eventually reach the most remote regions of the planet. For instance, Asian dust storms can take 7–9 days to cross the Pacific Ocean, while African dusts can reach the Caribbean and America within 3–5 days (Griffin 2007). The residence time of particles in the atmosphere depends on their size, large particles (>10 µm in diameter) can settle by dry deposition. For particles in the range of 0.1–10 µm the prominent process of deposition is wet: it requires the presence of condensed water. Finally small particles (from 10^{-4} to 10^{-1} µm) must aggregate within each other and reach a size large enough to be deposited (Renoux and Boulaud 1998). This means that the prominent manner to descend back to the surface for microorganisms, which are in the size range of 1 µm is wet deposition. The residence time of microorganisms in the atmosphere is thus largely dependent on meteorological conditions (dry vs. humid) and was modelled to be between 10 and 2 days, respectively (Burrows et al. 2009b). Very recently Amato et al. (2015) have measured the residence time of selected bacterial strains in a cloud simulated chamber (AIDA, Karlsruhe, Germany) which might improve the numerical models of bacterial dissemination in the future. The cultivability of airborne bacterial cells over time showed an exponentially decreasing function with a half-life time of about 3.5–4.5 h. In other words, considering the average residence time of 3.4 days of bacteria in the atmosphere estimated by models (Burrows et al. 2009b), our results indicate that the proportion of cells surviving aerial transport is only 1 cell out of 10^6 cells aerosolized from the surface. The distance of transportation of bioaerosols depends on the wind speed and can be simulated to be between several hundreds to thousand kilometers.

As explained above, microorganisms can be deposited to the ground using precipitation as shuttles. However falling rain drops are largely inefficient in scavenging particles of this size, so microorganisms have to be first integrated in cloud droplets by nucleation or scavenging processes into clouds. First microorganisms themselves can act as CCN (Cloud Condensation Nuclei) offering a surface to condensation of water vapour; it is as a particular case of aerosol particles presenting some specific physico-chemical properties due to their biological nature (Sun and Ariya 2006). Furthermore, some specific microorganisms, notably some bacteria belonging to the genus *Pseudomonas*, can initiate the formation of ice at relatively warm temperatures (−2 to −12 °C) thanks to a surface protein (Möhler et al. 2007; Ariya et al. 2009; Hoose and Möhler 2012). Such biological Ice Nuclei Active (INA) bioaerosols are present in precipitation (Christner et al. 2008a, b; Stephanie and Waturangi 2011). More recently they have also been described in cloud waters (Joly et al. 2013, 2014). Although the role of microorganisms as CCN is general and admitted, simply due to their particle size, the quantitative implication of INA bacteria in forming clouds and precipitations is still controversial (DeMott and Prenni 2010; Hoose et al. 2010).

10.2.3 The Cloud Microbiome

Before being precipitated, cloud is thus a transient habitat where microorganisms can live and survive for a few hours to a few days. However, it remains a rather unexplored extreme environment.

First descriptions of microorganisms in cloud droplets refers to the works of Sattler et al. (2001) and Bauer et al. (2002), it was then largely completed by recent studies (Amato et al. 2005, 2007d; Vaïtilingom et al. 2012). The concentration of microbial cells in warm clouds in the free troposphere was evaluated by microscopy or flow cytometry, it is in the range of 10^2–10^5 cells mL^{-1} depending on the sampling site, see Table 10.2.

The biodiversity of the cloud microbiome is still rather unknown. This is because only a few studies have been made due to the difficulties in sampling clouds in sterile conditions suitable for microbial study. In addition, most of the studies were performed using culture methods which is known to cover between <1% (of the bacteria) to ~10% (of the fungi) of the total communities. Fuzzi et al. (1997) first reported the presence of cultivable strains of *Pseudomonas*, *Bacillus and Acinetobacter* in fog water sampled in the Pô valley; Ahern et al. (2007) isolated a number of *Pseudomonas* strains in Hebridean clouds. The largest description of cloud isolates has been done from cloud waters sampled at the puy de Dôme station (1465 m a.s.l.) (Vaïtilingom et al. 2012) a site internationally recognized for atmospheric research (Global Atmospheric Watch, GAW labelled). 185 heterotrophic bacteria and 150 yeasts were isolated from 32 cloud events and identified, including -Alpha, -Beta and Gamma-Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria, and Basidiomycetous and Ascomycetous yeasts. The results show that major genera dominate among cultivable cells (Fig. 10.2). Concerning bacteria, the most frequently encountered genera are *Pseudomonas* (γ-Proteobacteria), *Sphingomonas*

Table 10.2 Microbial concentration in cloud waters

Sites	Altitude (m.a.s.l.)	Number of sampled clouds	Bacterial concentration (cells mL^{-1})	Fungal concentration (cells mL^{-1})	This study
Puy de Dôme, France	1465	34	3.3×10^3–2.5×10^5	8.9×10^2–3.2×10^4	Amato et al. (2007c), Vaïtilingom et al. (2012)
Mont Rax, Austria	1644	3	4.9×10^4–8.1×10^4	5.9×10^3	Bauer et al. (2002)
Mont Sonnblick, Austria	3106	12	7.9×10^2–2.5×10^3	–	Sattler et al. (2001)
Aircraft sampling, Michigan, U. S.A	2240–3320	5	9.2×10^4–4.3×10^5	–	Kourtev et al. (2011)

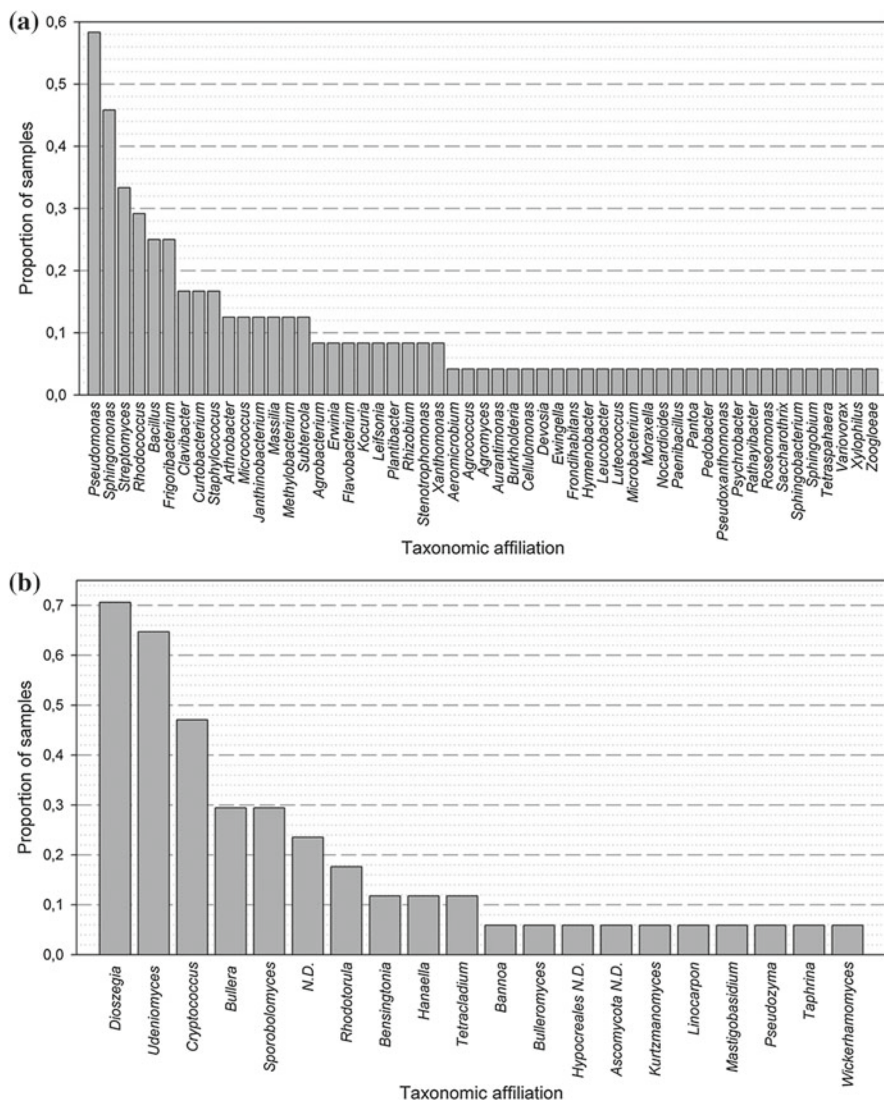


Fig. 10.2 The frequency of cloud events collected at the puy de Dôme submit in which the different genera of bacteria (**a**) or yeasts (**b**) were detected viable by culture over the period 2003–2010 (adapted from Vaïtilingom et al. 2012)

(α-Proteobacteria), *Streptomyces* (Actinobacteria), *Rhodococcus* (Actinobacteria), and *Bacillus* (Firmicutes), while for yeasts *Dioszegia* (Basidimycota), *Udeniomyces* (Basidimycota) and *Crytococcus* (Basidimycota) are the most frequent ones.

Although DNA-based analyses of air samples have been recently reported (Bowers et al. 2009; Brodie et al. 2007; Fierer et al. 2008; Gandolfi et al. 2013;

Garcia et al. 2012; Smith et al. 2013; Zweifel et al. 2012; Maki et al. 2010, 2013; Fröhlich-Nowoisky et al. 2009; Bottos et al. 2014; Fahlgren et al. 2010; Jeon et al. 2011; Maron et al. 2005; Després et al. 2007), very few studies have focused on cloud communities. Up to very recently it was very difficult to apply DNA-based techniques to assess this biodiversity due to sample volume limitations and a very low microbial density; new molecular tools which are very sensitive should provide more information. Kourtev et al. (2011) showed a high genetic diversity in Lower peninsula of Michigan (USA) by Denaturing Gradient Gel Electrophoresis (DGGE); they found the same major bacterial phyla (Proteobacteria > Actinobacteria > Firmicutes) than in clouds collected at the puy de Dôme (Vaïtilingom et al. 2012), but the major difference was the prevalence of Cyanobacteria (around 50% of the sequences). Actually cyanobacteria were not identified using culture-based methods as there were not looked for. More recently Šantl-Temkiv et al. (2013a, b) described the microbial population in a storm cloud (hailstones); the major bacterial groups belonged to Actinobacteria, Bacteroidetes, gamma and alpha Proteobacteria phyla as described in warm clouds (Vaïtilingom et al. 2012), however the major genus of alpha Proteobacteria in hailstones was *Mycobacterium* instead of *Sphingomonas.* Using SSU rRNA gene sequence analysis of microorganisms living in cloud-free, cloudy and tropical storm environments of the upper troposphere (8–15 km altitude) also revealed the prevalence of Proteobacteria (alpha and beta Proteobacteria) (DeLeon-Rodriguez et al. 2013). Interestingly two of the core families of these different samples were the *Methylobacteriacae* and *Oxalobacteraceae*. The difficulty to compare the biodiversity described in all these studies lays in the fact that of course the techniques used are different (culture *versus* uncultured-based approaches) but also because the samplings were performed in different geographical sites or altitudes. The heterogeneity at low altitude can result directly from the sources influences as described by Burrows et al. (2009b). However when the whole atmosphere is considered, one could think microorganisms circulate freely around the earth, opposite to microorganisms present in other closed environment compartments such as soil, lakes and rivers. The question of the biogeography of the atmosphere is still a non-resolved question (Womack et al. 2010).

These studies show that although the cloud microbiome is diverse, some major groups are dominating; this could result of a selection of the type of microorganisms occurring during the aerosolization process or thanks to their survival abilities in clouds.

10.3 Microorganisms Are Surviving in Clouds

10.3.1 Microorganisms Are Metabolically Active in Clouds

Physicists of the atmosphere had considered for a long time that microorganisms were just inert particles, however it has been discovered recently that at least a

fraction of the microorganisms are actually alive in clouds. Although a small fraction of cloud microorganisms can be cultivated, most of them were shown to be still viable. Sattler et al. (2001) measured the incorporation of [methyl-^{3}H]thymidine and [^{14}C]leucine demonstrating for the first time that bacteria could be maintained and grow in super-cooled cloud droplets. Amato et al. (2007a, b) showed the ability of microorganisms isolated from cloud to grow in cloud water incubated in laboratory. Looking at individual strains, their doubling times at 17 and 5 °C were in the range of 5–20 h depending on the strains. It is rather long compared to cloud life time, meaning that the multiplication of cells only happens during long cloud events, and probably not more than once in the cloud's lifetime. Cloud medium thus allows microorganisms to maintain a metabolic activity and develop by sustaining organic substrates to the cells, as suggested by Fuzzi et al. (1997). This metabolic activity was largely confirmed by assaying the ATP (Adenosine triphosphate) content directly in cloud water samples. The theoretical concentration of ATP in bacteria and fungi while taking into account their total number, was compared to those measured in clouds. The similar results suggest that most of the microorganisms were active (Amato et al. 2005). A long term survey of these in situ measurements at the puy de Dôme station showed that the ATP content is rather stable, independently from the season or the geographical origin of the air mass (Vaïtilingom et al. 2012). Hill et al. (2007) also demonstrated that 76% of the bacteria were alive in cloud waters as they were able to uptake the dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride). The existence of metabolic activity in clouds implies the uptake of molecular compounds by cells as nutrients, and so of their contribution to cloud chemical reactivity.

A few studied have focused on the biodegradation of some of the cloud carbon sources by cloud microorganisms. The first type of compounds investigated were carboxylic acids which constitute one of the major classes of organic compounds in cloud waters (Deguillaume et al. 2014). The group of Ariya studied the degradation of series of di-carboxylic acids (malonic, oxalic, succinic, glutaric, adipic, pimelic and pinic) by airborne fungi (Ariya et al. 2002; Côté et al. 2008). They showed that these were all good substrates except oxalate. Other studies confirmed that formic, acetate, succinate and malonic were degraded by cloud bacteria but not oxalate (Amato et al. 2007a; Vaïtilingom 2011; Vaïtilingom et al. 2013). Experimental data concerning the non-degradability of oxalate are not consistent with the description of the oxalate-degrading bacteria (*Oxalobacteraceae*) described by DNA-based methods (DeLeon-Rodriguez et al. 2013). This could result from the fact that this genus is not active in real atmospheric conditions or that it is not present in cloud samples collected at the puy de Dôme contrarily to sample collected at high altitude over the Atlantic Ocean. Mono-carboxylic acids such as formate, acetate and lactate are also easily degraded by cloud microorganisms (Amato et al. 2005, 2007a; Vaïtilingom et al. 2010, 2011, 2013). Herlily et al. (1987) also showed some years ago that acetate and formate were biodegraded in rainwater. Between 2 and 9 carboxylic acids (lactate, acetate, glycolate, propionate, formate, glyoxylate, α-keto-glutarate, succinate, tartrate) could be also used by seven strains of

Methylobacterium and one strain of *Bradyrhizobium* isolated from hailstones (Šantl-Temkiv et al. 2013a).

C1 compounds such as methanol and formaldehyde also represent valuable carbon sources for cloud microorganisms; the metabolic routes can be variable according to the strains. Among the theoretical potential pathways the transformations of formaldehyde into methanol (reduction) and/or into formate and CO_2 have been identified in 60 cloud strains using ^{1}H-NMR (Amato et al. 2007b). A more detailed study on a *Bacillus* strain by ^{13}C NMR showed that ^{13}C-formaldehyde was both reduced to methanol, oxidized to formate and CO_2 and also integrated in the serine metabolism leading to the production of glycerol, 1,2- and 1,3-propanediol (Husarova et al. 2011). The presence of facultative methyloptroph strains, belonging to the genus *Methylobacterium*, have been described as important in hailstones (Šantl-Temkiv et al. 2012). These strains were actively using methane under simulated cloud conditions, as demonstrated by enrichment techniques, and could thus represent a sink for atmospheric methane.

Finally, a recent paper reports the efficient degradation of sugars present in aerosols by a *Bacillus* strain isolated in cloud waters, as a model of cloud microorganisms (Matulová et al. 2014). These sugars have a biogenic origin; they include alditols (manitol, glucitol, arabitol), monosaccharides (arbinose, xylose, ribose, fructose, glucose, galactose, mannose, rhamnose), disaccharides (lactose, sucrose, maltose, tetrahose, cellobiose), oligosaccharides and polysaccharides (cellotetraose, cellulose, arabinogalactan, glucuronoxylan, inulin, starch). The degradation rates of theses sugars measured by ^{1}H-NMR was in the same range of order than those measured for C1 compounds by the same strain.

Most of these experiments have been performed on simplified microcosms, starting from a single strain incubated with a single substrate (Ariya et al. 2002; Côté et al. 2008; Husarova et al. 2011; Amato et al. 2007a; Vaïtilingom et al. 2010; Matulová et al. 2014; Šantl-Temkiv et al. 2012), but more complex systems closer to cloud environment have been used including artificial cloud water tested with 17 representative strains incubated at temperatures relevant for clouds (5° and 17 °C) (Vaïtilingom et al. 2011) or real cloud waters containing the whole endogenous microflora (with or without solar light) (Vaïtilingom et al. 2013). To conclude cloud waters provide numbers of carbon sources to maintain a high microbial metabolic activity.

10.3.2 Microorganisms Can Resist to Atmospheric Stresses

Cloud medium is clearly a harsh environment as fully explained in Sect. 1 both because it is constantly in evolution and thus under different micro-physical status and because cloud is a very active chemical reactor. However the fact that metabolically active microorganisms are present in clouds suggests that they have elaborated strategies to survive in clouds and resist to these stressing conditions.

10.4 Anti-stress General Features: Pigments, Spores, ExoPolymeric Substances (EPS)

First some general features characterize these microorganisms; they are related to stress protection. For instance about 50% of the cloud isolates are pigmented (yellow, red, orange) (Amato et al. 2005, 2007d; Vaïtilingom et al. 2012), as those recovered from permanently cold regions (Fong et al. 2001; Mueller et al. 2005; Dieser et al. 2010). These pigments are probably carotenoïds that could be incorporated in the microbial membrane and might have a double role: (i) they help maintaining membrane fluidity in the cold; (ii) they could also act as free radical scavengers (Gourmelon et al. 1994). Interestingly, these pigments are produced by bacteria exposed to UV light on plant leave surfaces, so these are "primed" for cloud conditions before being aerosolized. Some microorganisms resist against stresses being in the form of spores, it can be the case of fungal spores but also of some specific bacteria such as *Bacillus* species. Spore forming microorganisms have been found in large number in the atmosphere. *Bacillus* are widely present in the air (Maki et al. 2013; Šantl-Temkiv et al. 2012). Previous studies (Elbert et al. 2007; Heald and Spracklen 2009) estimated the fungal spore concentrations from 10^4 to 10^6 m^{-3} of air with a huge spatial and temporal variability. Elbert et al. (2007) report an average of 35% of the total aerosol mass to be fungal spores in the Amazon region. Fungal spores contribute to 0.9% of the total OC (Organic Carbon) mass in the Austrian Alps and could reach up to 14% of the OC mass concentration in summer at a suburban site (Bauer et al. 2002, 2008). More recently, different groups report on-line detection of bio-aerosols and particularly of fungal spores. This detection is based on the analysis of the fluorescent properties of biological compounds (including NADH and tryptophan) [see Pan (2015) for review]. Two main types of instruments are used: Ultraviolet Aerosol Particle Sizer (UV-APS) and Waveband Integrated Bioaerosol Sensor (WIBS). Although the global trends measured by these techniques are in accordance with the already published values of bioaerosol concentrations, these approaches are still under development to be able to discriminate between fungal spores, bacteria and yeasts. Interesting papers have precisely compared these real-time fluorescent techniques with classical microscopy: Gabey et al. (2013) described some disparity between bacteria numbers and fluorescent particles concentration, while Healy et al. (2014) reported a good correlation between spore numbers and their fluorescent measurements; however they pointed the difficulty to detect *Cladosporium* spores because of their dark, highly absorptive cell-wall. Unfortunately these on-line techniques cannot be applied to analyse atmospheric water contents. Microscopy techniques have shown that yeast, fungi as well as spore forming bacteria such as *Bacillus* strains are present in cloud and fog waters (Fuzzi et al. 1997; Amato et al. 2007d; Vaïtilingom et al. 2012). Their concentration was higher when the water pHs were lower suggesting an adaption to this polluted media (Fuzzi et al. 1997; Amato et al. 2007d).

Finally another global protective strategy against numerous stresses relies on the formation of a biofilm or aggregates using the synthesis of ExoPolymeric Substances (EPSs). Actually the formation of biofilms seems to be the typical way for bacterial cells to grow in nature as it confers many ecological advantages (Davey and O'toole 2000; Flemming and Wingender 2010). This highly hydrated layer surrounding the cells can protect them against desiccation and UV exposure (Davey and O'toole 2000) or protect them in extreme marine habitats (Poli et al. 2010). Monier and Lindow (2003) showed a differential survival of solitary and aggregated bacterial cells on leaf surfaces, aggregates were increasing drastically bacterial survival when exposed to desiccation. This study was performed using *Pseudomonas syringae* as a model strain. It is worth noting that this bacterial species is one of the most abundant cultivable species found in clouds, these bacteria might be aerosolized in the atmosphere under aggregated forms and therefore particularly well adapted to survive to atmospheric stresses (Vaïtilingom et al. 2012). It is also likely that microorganisms which are transported on dust storms are under the form of biofilms. Tong and Lighthart (1998) reported that cell aggregation or association with particles increased survival during the day due to a shielding effect. Although bacterial EPS synthesis has been studied in many environments, only one paper is related to the atmospheric environment. Matulova et al. (2014) showed that a cloud bacterium (*Bacillus* sp. 3B6) could produce two types of EPSs from various saccharides (L-arabitol, D-fructose, sucrose, D-glucose, cellotetraose, cellulose, starch) present in the atmosphere. Their structures were identified as 1,6-α-galactan and partially acetylated polyethylene glycol (AcPEG) (Matulová et al. 2014).

10.5 Facing Specific Stresses: Oxidative, Osmotic and Temperature Stresses

Apart from these general strategies, microorganisms can modify specifically part of their metabolism to face specific stresses encountered in clouds.

One major problem for microorganisms is the presence of oxidants or their sources, including $^{\bullet}OH$ radicals, H_2O_2, iron, and solar light. Joly et al. (2015) have investigated the survival of 5 strains isolated from cloud waters towards 100 μM H_2O_2 in artificial cloud water; this concentration is realistic for H_2O_2 in cloud waters as it was previously detected at concentrations ranging from 0 to 247 μM (Table 10.1). The strains were chosen as models of strains collected at the puy de Dôme station representative of the cloud cultivable microbiome: 2 *Pseudomonas*, 1 *Sphingomonas* and 1 *Arthrobacter* among bacteria and 1 Basidiomycota yeast related to the genus *Dioszegia* (Vaïtilingom et al. 2012). The results showed that under these conditions close (or slightly overestimating) to what is found in clouds, i.e. 100 μM H_2O_2, the five strains were not affected (Table 10.3). Joly et al. (2015)

Table 10.3 Survival rates observed for the experimental conditions the most relevant for clouds and proportion of cloud samples from which representatives of the corresponding genus were detected by culture

	Survival rates to stresses				Occurrence in clouds[a] (%)
Taxonomic affiliation and type of microorganism	H_2O_2 (90 min, 100 µM) (%)	Solar light (10 h) (%)	Osmotic shock (1 M NaCl) (%)	Freeze-thaw (10^6 cells mL^{-1}, per cycle) (%)	
Dioszegia hungarica Yeast	84	37	56	80	71
Sphingomonas sp. Gram negative bacterium	70	101	15	71	46
Pseudomonas syringae 32b-74 Gram negative bacterium	77	104	78	51	58
Pseudomonas syringae 13b-2 Gram negative bacterium	94	104	63	45	
Arthrobacter sp. Gram positive bacterium	129	96	101	16	13

Adapted from Joly et al. (2015)

[a]Proportion of clouds samples from which representatives of the corresponding genus were detected by culture (data from Vaïtilingom et al. 2012)

also exposed the same strains to artificial solar light mimicking the natural light inside a cloud at the top of the puy de Dôme station (Joly et al. 2015) and demonstrated that the light available inside a cloud does not significantly impact bacterial viability (Table 10.3).

These previous experiments were performed under simplified conditions and using model strains. The results were confirmed using real cloud water samples incubated in a photo-bioreactor, specifically designed to reproduce cloud environment (Vaïtilingom et al. 2013). 3 cloud samples from independent cloud events were collected at the puy de Dôme station where they contained a very complex mixture of organic compounds and a whole biodiversity of the cloud microbiome. Each sample was divided into four parts: two parts were filtered through 0.22 µm porosity and while the two other remained intact, generating sterile or non-sterile samples, respectively. A sterile and a non-sterile subsamples were exposed to artificial solar light. At the same time, the other sterile and non-sterile subsamples were incubated in darkness. This experimental design allowed us to demonstrate:

– *abiotic reactions*: typically Fenton reactions (iron + H_2O_2) and photochemical reactions (iron + H_2O_2 + light); these reactions generate hydroxyl radicals from H_2O_2 source.
– *biotic* + *abiotic reactions:* iron + H_2O_2 + microorganisms with or without light. These conditions allow studying the interactions between these oxidants (including radicals) and cloud microorganisms. Biotic reactions could be inferred from the difference between the reactions observed in the 2 conditions.

ADP/ATP ratio which reflects the metabolic status of cloud microorganisms was monitored over time; no change was measured in the presence or the absence of light. This clearly means that microorganisms were not impacted by the generation of radicals and by solar light. In parallel it was shown that the endogenous cloud microflora was able to degrade H_2O_2 present in the cloud samples, suggesting the presence of very active catalases. In addition the degradation rates of H_2O_2 were not decreased in the presence of light, showing no inhibition of the biological process by light and radicals.

Solar light is actually composed of two types of highly energetic wavelengths (UV-C, ~190–290 nm, and UV-B, ~290–320 nm) that can potentially affect microorganisms mainly by causing DNA damage (λ = 260 nm; see the review by Witkin (1976)). However UV-C radiations are mostly absorbed by stratospheric ozone, i.e. above the highest altitudes and thus have no effect on tropospheric clouds where microorganisms live. If microorganisms present in the air reach this stratospheric zone, they can be indeed damaged. This was shown by Smith et al. (2011) who exposed spores of *Bacillus subtilis* to a series of stratospheric simulations combining temperature, desiccation, pressure and UV light. UV light was the only stress to have an impact on *Bacillus* survival. For UV-B radiations, the deleterious effects are limited and can be balanced by repair mechanisms. Solar light is also composed of longer UV wavelengths (UV-A, ~320–400 nm) and visible light (~400–800 nm) which can indirectly alter cell viability by producing Reactive Oxygen Species (ROS) that include $^{\bullet}OH$ and $O_2^{\bullet -}$ radicals (Fig. 10.3). These extracellular radicals are produced mainly by direct photolysis of H_2O_2 or by photo-Fenton reactions (Fe + H_2O_2) and can diffuse into the cell across the cytoplasmic membrane. The same type of radicals can also be produced intracellularly when O_2 diffuses inside the cell during the respiration process of aerobic microorganisms. These radicals are extremely deleterious for cells as they can damage the major cellular components (Proteins, DNA, lipids, etc.) and induce cell death. Hopefully microorganisms are protected from these ROS with the assistance of various mechanisms involved in the oxidative stress metabolism [see Davey and O'toole (2000) for review]. First radicals can be scavenged by antioxidant molecules such as vitamins (ascorbic acid, α-tocopherol.), gluthatione, and pigments (carotenoïds) (Tong and Lighthart 1998; Dieser et al. 2010). As already stated the majority of atmospheric microorganisms is pigmented: 50% in clouds and 80% in the air (Amato et al. 2005; Fahlgren et al. 2010; Vaïtilingom et al. 2012; Zweifel et al. 2012). Another protection mechanism to fight the presence of ROS is based on the activity of specific enzymes of the oxidative metabolism (Fig. 10.3): Super

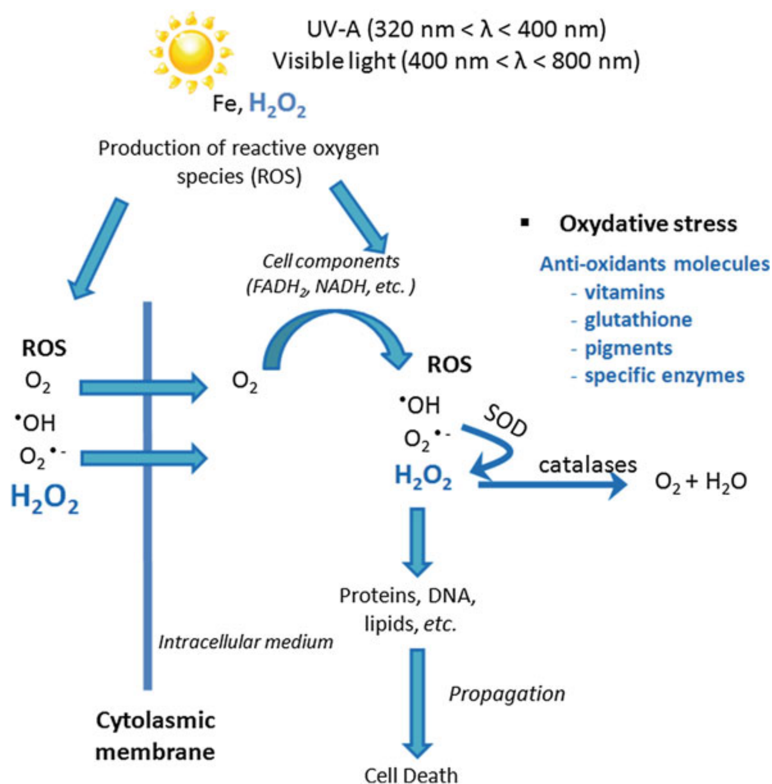


Fig. 10.3 In clouds, microbial cells are exposed to reactive oxygen species (ROS) produced by the uptake of O_2 during respiration or by chemical reactions (photolysis of H_2O_2, Fenton or photo-Fenton reactions) occurring in cloud water. Cells react to this oxidative stress thanks to the production of vitamins, glutathione, pigments or specific enzymes

oxide dismutase (SOD) is able to transform $O_2\bullet^-$ into H_2O_2 which again can be transformed in O_2 and H_2O as non-toxic compounds by catalases (Vorob'eva 2004; Davies 2000). Catalases can also act directly on H_2O_2 which has been transferred in the cell from the extracellular medium (cloud water).

A second important stress is osmotic stress. Once in clouds, microorganisms are protected against desiccation by the presence of condensed water; however they are then exposed to rapid variations of osmolarity due to repeated condensation-evaporation cycles. In clouds, microorganisms are subject to osmotic variations when water condensates or evaporates (Fig. 10.4). Evaporation will induce an increase of the extracellular concentration and thus a hyper-osmotic shock, while the reverse happens during the condensation phase and creates a hyper-osmotic shock. To mimic this evaporation-condensation phenomenon, Joly et al. (2015) have exposed the 5 cloud microbial strains to a hyper-osmotic shock (1 M NaCl, realistic concentration for cloud conditions), followed by a hypo-osmotic shock, and they measured viability by culture (Table 10.3). The survival rates for the 2 *Pseudomonas*

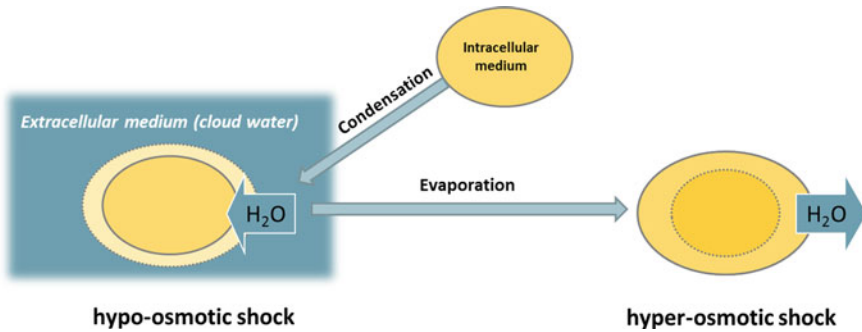


Fig. 10.4 During the evaporation-condensation cycles of cloud droplets, microbial cells experience hyper-osmotic and hypo-osmotic shocks due to the change of solute concentrations surrounding the cells. Cells answer by water efflux or influx modifying the cellular volume

and the *Arthrobacte*r strains was quite high, that of the yeast *Dioszegia* was also important, showing that these microorganisms can resist to osmotic stress. However, the *Sphingomonas* strain was very impacted by such osmotic shock. Although these model strains might give a limited view of the whole cloud microbiome, this work suggests that the adaptation to osmotic stress is not a common feature to microbial cells living in clouds, and it is more likely strain dependent.

Microorganisms have developed different processes to fight against osmotic shocks (see *Csonka*, 1989 for review). When the extracellular concentration is increased (hyperosmotic shock), microorganisms have to equilibrate internal and external osmolarities by immediate efflux of water molecules (Fig. 10.4). This response leads to increased intracellular concentration of metabolites, decreased metabolic activity, and shrinkage of the cell. To reestablish influx of water, cells increase cytoplasm osmolarity by synthesizing or up-taking compounds known as "compatible solutes" or "osmoprotectants": potassium ions, amino acids (proline, glutamate…), sugars (trehalose, sucrose…) or peptides. A recent publication demonstrated the production of compatible solutes including betaine, ectoine, *N*-acetylglutaminylglutamine amide (NAGGN), and trehalose by a strain of *Pseudomopnas syringae* under osmotic stress conditions (Kurz et al. 2010). This is of interest as this species is one of the major ones encountered in clouds.

Further stress to be encountered by cloud microorganisms is temperature; this includes living in the cold, and enduring cold shocks as well as freeze-thaw cycles. Cloud temperature varies with the altitude, the latitude and the season. Although some microorganisms have been collected at 77 km of altitude where the temperature is extremely low, this review focuses on microorganisms in low altitude clouds called "warm clouds", which have been examined in more detail than the ones in high atmosphere. In such clouds collected at the puy de Dôme station in France at 1465 m a.s.l., the annual average temperature is around 5 °C but can reach 17 °C or more during the summer. It has been shown that a great part of the cloud microbial isolates are psychrophile or psychrotolerant and are clearly adapted for growth at the temperatures existing in warm clouds (Amato et al. 2007d; Vaïtilingom et al. 2012).

In addition, as explained earlier, more than 50% of these isolates are pigmented, they can also be embedded in biofilms or aggregates, all these factors protect them against the cold. This ability to grow and survive at low temperatures is actually not surprising as microorganisms have been found in many cold environments including snow and ice, particularly in polar environments (Carpenter et al. 2000; Foght et al. 2004; Dieser et al. 2010; Amato et al. 2007b; Junge et al. 2006; Groudieva et al. 2004; Christner et al. 2001, 2003; Price 2000). The specificity of the atmospheric medium is that, because it is a transient habitat, microorganisms have to adapt from a terrestrial environment to an atmospheric one, they have to cope with cold shocks. In our group, we have studied this cold shock impact on a model strain, *Pseudomonas syringae* 13b2 using metabolomics (unpublished data). Incubations of the strain at 17 and 5 °C were compared; key biomarkers were identified by Nuclear Magnetic Resonance and Mass Spectrometry. These markers indicate that at 5 °C, many metabolic changes occur to compensate the effects of low temperature. The first important feature is to re-establish membrane fluidity by changing membrane composition which allows the membrane functionality (Shivaji and Prakash 2010). Also we observed that cryo-protectants, in particular trehalose, carnitine, glutamate, glycine, and glycerol were produced, and the energetic state was boosted (increase of ATP) as well as the sulfur metabolism (including glutathione over production). This global answer of the strain shows that a single stress such as a cold shock can also induce responses common with other stresses like osmotic shocks (osmo-protectants are often similar to cryo-protectants) and oxidative stress (gluthatione). These crossed answers have been often reported in the literature (Mikami et al. 2002; Tanghe et al. 2003). The regulation systems to cold and osmotic stresses present many similarities (Suzuki et al. 2001; Mikami et al. 2002), and osmoprotectant compatible solutes such as trehalose, glycerol and saccharose also serve as cryoprotectants (De Antoni et al. 1989; Panoff et al. 2000). Tanghe et al. (2006) highlighted the importance of aquaporins, channel proteins devoted to the transport of water through the cell membrane, in the resistance to freezing. Many studies have reported a close relationship between cold stress and oxidative stress in bacteria. Indeed it was shown that the production of free radicals ($O_2^{\circ -}$ and °OH) and H_2O_2 is increased when the temperature is decreased (Zhang et al. 2012).

In addition to cold shocks, microorganisms can experience repeated freeze-thaw cycles due to the temperature gradient existing in the troposphere associated with rapid vertical winds. To test the resistance of cloud microorganisms to such a stress, Joly et al. (2015) have subjected the 5 model strains mentioned above to 6 consecutive freeze-thaw cycles (−40 °C/+5 °C) and measured their survival (Table 10.3). The resistance was highly strain dependent, ranging from high to very low survival rates (1–80% per freeze-thaw cycle). Interestingly when cell concentration was increased the survival rate was improved, this is consistent with the observed improved resistance of aggregated cells (Monier and Lindow 2003). Also the ice nucleation activity of the *Pseudomonas* strains did not seem to be a protection.

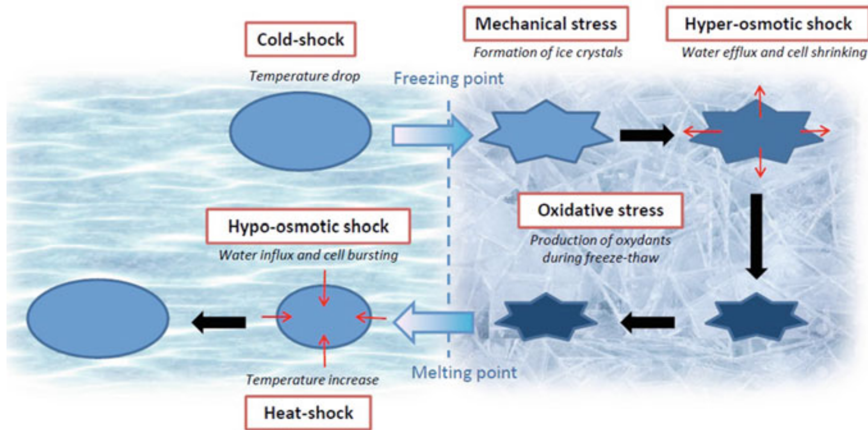


Fig. 10.5 Freeze-thaw cycles: a combination of multiple stresses

Freeze-thaw cycle is actually a complex mechanism involving a combination of different stresses (Fig. 10.5). The first step is directly related to a cold shock, as the temperature decreases very quickly, leading to the formation of ice. The formation of ice crystals represents then a mechanical stress for the cells which is combined with an osmotic stress as the osmolites are concentrated in the extracellular medium (hyper-osmotic shock). Indeed the formation of ice results in a supersaline liquid network of microveins where microbial cells are trapped (Price 2000; Mader et al. 2006; Amato et al. 2009). It is also combined with a change of the cell shape and possible mechanical damages. When ice is thawing, again a temperature shock (heat shock) is experienced by the cells in addition to another osmotic stress as the solutes are diluted in the extracellular medium (hypo-osmotic shock). During freezing-thawing, ROS are produced, inducing thus an additional oxidative stress in cells (Stead and Park 2000). We can thus hypothesize that the cells will adapt by modifying their metabolism to face these different stresses. For counteracting the mechanical damages caused by freezing, microorganisms can produce antifreeze proteins (Duman and Olsen 1993). For the other stresses (temperature, osmotic, and oxidative), as shown above, common response pathways can be induced (Mikami et al. 2002; Tanghe et al. 2003). In some cases it has been shown that pre-exposition to one stress can eventually help the cell to resist to other stresses; for instance oxidative stress resistance was shown to be improved by pre-exposition to low doses of oxidants as the result of the induction of oxidative (Storz et al. 1990) and general stress response (Tanghe et al. 2003).

Because the mechanisms of stress resistance are so complex, it is no possible to just sum the impacts of the different individual stresses for estimating the chances of survival in clouds. However it can be noted that the survival rate of the tested microorganisms facing freeze-thaw cycles roughly matches, perhaps coincidentally, the frequency of cloud events collected at the puy de Dôme station in which the different genera of bacteria or yeasts were detected viable by culture (Table 10.3).

For instance the yeast *Dioszegia hungarica* which was almost insensitive to freeze-thaw cycles (survival rate of 80%) belongs to the yeast genus the most represented in cloud water samples (71%). The Actinobacterium *Arthrobacter* which highly suffered from repeated freeze-thaw (survival rate of 16%) was detected as alive much less frequently (13% of the clouds). The Proteobacteria *Pseudomonas syringae* and *Sphingomonas* sp. survived freeze-thaw cycles with a rate of 45–71% were the most represented bacterial genera, present in about 40–60% of the samples.

10.6 Conclusions and Perspectives

In conclusion we know now that metabolically active microorganisms are present in clouds which represent a transient habitat. They can maintain viability to different major stresses present in this harsh environment thanks to various general or specific strategies. The recurring presence of some groups of microorganisms in clouds can possibly result from two mechanisms: (i) these microorganisms are aerosolized from major specific sources on Earth's surface (ii) these microorganisms have elaborated strategies to improve their survival in clouds. Previous studies suggest that stress response is very often strain dependent; therefore it is difficult to make a general assessment correlating the occurrence of specific microorganisms in clouds with their stress resistance. However our work, although it is restricted to a limited number of model strains (Table 10.3) and thus is still speculative, tends to give some tendencies: the resistance to freeze-thaw cycles seems to be a major factor driving the final frequency of microbial strains in clouds.

Future work is needed to better describe and understand this unexplored world which is the cloud microbiome. Thanks to the development of new technologies such as devices allowing high sampling volumes and sensitive molecular tools, it is hoped that "omics" studies will be available soon. Metagenomics and metatranscriptomics could give new insights into the structure and function of cloud communities. metabolomics could also bring valuable information about the response and adaptation of cloud microorganisms to atmospheric stresses. Finally, more sampling site should be considered as it is to date mainly restricted to a few sites over the world. For that new collaborations and interdisciplinary teams between biologists and physicists of the atmosphere should be established.

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