Rosa Margesin
Franz Schinner
Jean-Claude Marx
Charles Gerday
Editors

Psychrophiles

From Biodiversity to Biotechnology

Psychrophiles: from Biodiversity to Biotechnology

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Psychrophiles: from Biodiversity to Biotechnology

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Cover illustration: Ice floe (upside down, about 80 cm thick) with dense populations of pennate diatoms at the sea-ice water interface, indicated by brown color that is caused by their main light harvesting pigment fucoxanthin (photo by David N. Thomas, see Chap. 17)

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Preface

Most scientists in the middle of the twentieth century would probably not have believed that life was possible at extreme values of environmental factors, such as pH values close to 0 (e.g. sulfurous environments) or to 14 (e.g. soda lakes), salinities of 6 M NaCl (e.g. Dead Sea), hydrostatic pressures approaching 0.1 MPa (deep sea) and temperatures exceeding 100°C (thermal vents or hot springs) or as low as –20°C (e.g. polar regions). Of the current studies on extremophiles, approximately 30,000 articles by the year 2007, almost two-thirds have been performed on organisms adapted to outstanding temperatures, but much more attention has been paid to thermophiles than to psychrophiles. However, over the past 10 years, scientific publications on cold-adapted microorganisms have increased by a factor of ten.

If one considers the extent of cold habitats, psychrophiles, i.e. cold-loving organisms, should largely lead in this comparison with thermophiles because a great proportion of the Earth's biosphere never reaches temperatures above 5°C. Nearly three-quarters of the Earth is covered by oceans whose deep water masses, irrespective of latitude, are constantly between 2 and 4°C. The large continent of Antarctica also provides a permanently cold terrestrial environment as well as an aquatic niche in the surrounding ice that melts during the summer. Other examples of cold habitats are permafrost soils, high alpine soils, cold deserts, cold caves, marine sediments, snow, glacier and sea ice. Cold ecosystems host a wide diversity of psychrophiles, including bacteria, archaea, yeasts, filamentous fungi, and algae. These microorganisms have evolved a number of strategies to thrive successfully in cold habitats where they play key roles in nutrient cycling, such as nitrogen fixation, nitrification and denitrification, photosynthesis, sulfur oxidation and reduction, methanogenesis, and transformation of organic compounds.

This book is focused on psychrophiles and describes, at the edge of knowledge, representative groups of cold-adapted microorganisms as well as the habitats in which they live and their strategies to cope with the cold. It is subdivided into four main sections:

(i) boundary conditions for microbial life at low temperatures
(ii) biodiversity

(iii) molecular adaptations
(iv) biotechnological aspects

thus covering almost all the fields of knowledge in “cold” microbiological research.

It is certainly not by chance that this book is published during the International Polar Year 2007-2008, which is the fourth polar year following those in 1882-1883, 1932-1933 and 1957-1958 and involving over 200 projects, with thousands of scientists from over 60 nations examining a wide range of physical, biological and social research topics. Therefore, this book perfectly matches the current demands and trends and provides an additional source of information to all those scientists who are interested in “cold” microbiology.

Last but certainly not least, the editors of this book want to thank all the authors, who are the leading scientists in the respective field, for having accepted to write a chapter of this book, even though all these persons are also very busy and highly solicited scientists. We also thank Springer - Life Sciences, especially Dr. Dieter Czeschlik and Dr. Jutta Lindenborn, for their continuous support and trust in our capacity to successfully achieve the editing of this book.

Innsbruck, Austria — Rosa Margesin, Franz Schinner
Liège, Belgium — Jean-Claude Marx, Charles Gerday
July 2007

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Part I
Boundary Conditions for Microbial Life at Low Temperatures

Chapter 1
The Climate of Snow and Ice as Boundary Condition for Microbial Life

Michael Kuhn

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1.1 The source of energy: solar radiation

The Earth's surface is the place where the biosphere, the atmosphere, the hydrosphere and the lithosphere interact most extensively. In mountains and polar regions, the cryosphere adds more facets to this multiple interaction. The biogeochemical cycles at the Earth's surface are driven by the vertical exchange of energy and water locally, and by the horizontal motion of air and water in the global circulation. The energy absorbed from the incident solar radiation is used to heat the ground, snow, or water, which in turn heat the overlying air by turbulent convection; to evaporate water, melt or sublimate ice; and in part is re-emitted as infrared radiation.

Solar radiation, the prime energy source of all climatic and biotic processes, has a strong daily and seasonal variation in mid and high latitudes. This is best illustrated by its reference value, the extraterrestrial irradiance, the amount of energy that would be received if there were no atmospheric extinction. Daily sums of extraterrestrial irradiance are displayed in Fig. 1.1 in response to geographical latitude and time of the year. While the tropics have the highest annual sums, the two polar regions reach the highest daily totals in their respective summers, with Antarctica receiving more than the Arctic since the Earth is closest to the Sun in the Austral summer.

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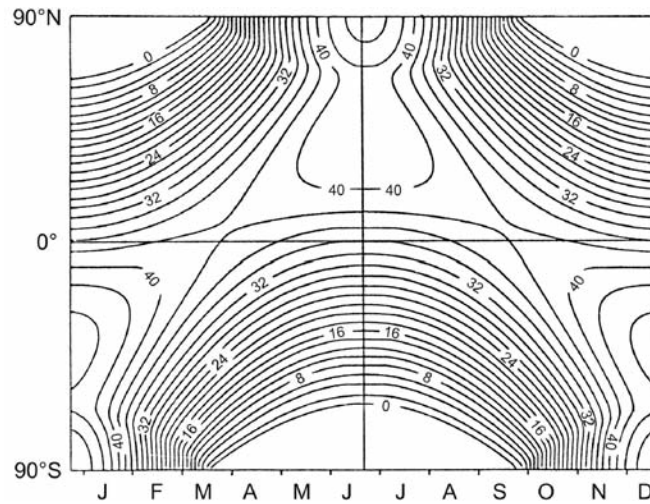

Fig. 1.1 Daily sums of extraterrestrial solar irradiance, the reference amount of energy that would be received without atmospheric extinction. Values are given in MJ m^{-2} day^{-1} computed for a solar constant of 1,368 W m^{-2}

Extinction by the air and its trace gases and by clouds and aerosols gives it a change with altitude as well. Global irradiance, the sum of direct and diffuse solar irradiance, was compiled from records at Austrian stations in Fig. 1.2, as a function of cloudiness and altitude. There is an increase of global irradiance of the order of 1% per 100 m altitude at mean cloudiness, and a decrease by 50% when comparing cloudless and cloud covered sky at an altitude of 3,000 m.

The maximum daily average of 400 W m^{-2} in Fig. 1.2 is associated with an instantaneous maximum of ca. 1,000 W m^{-2} at noon. 400 W m^{-2} is identical to the maximum mean daily irradiance reached in the Dry Valleys of Antarctica, close to sea level, although the solar geometry at that high latitude is very different from that of the Alps. This daily average amounts to 83% of the extraterrestrial irradiance in alpine conditions, a fraction that is nearly identical to the 85% found in the central Antarctic at the time of summer solstice. It is obvious from Figs. 1.1 and 1.2 that this fraction decreases at lower solar elevations.

A large part of this incident solar radiation is reflected back to the atmosphere. The broad band albedo of dry alpine or polar snow exceeds 80%, reaching 90% in the visible and UV parts of the spectrum and dropping to less than 20% in the near infrared; in the thermal infrared, snow is essentially a black body with an emissivity close to 0.98.

At low angles of solar elevation, as typical for polar regions, forward scattering in the snow increases albedo to values >90%. The albedo of snow and ice decreases with increasing grain size and increasing liquid water content so that clean alpine snow that survives into summer displays albedo values between 60 and 70%. The presence of dust or other admixtures reduces the albedo further.

The albedo of ice depends largely on the presence of cracks and air bubbles: typical clean ice of alpine glaciers would reflect about 40%, dust and dirt covered ice may reflect

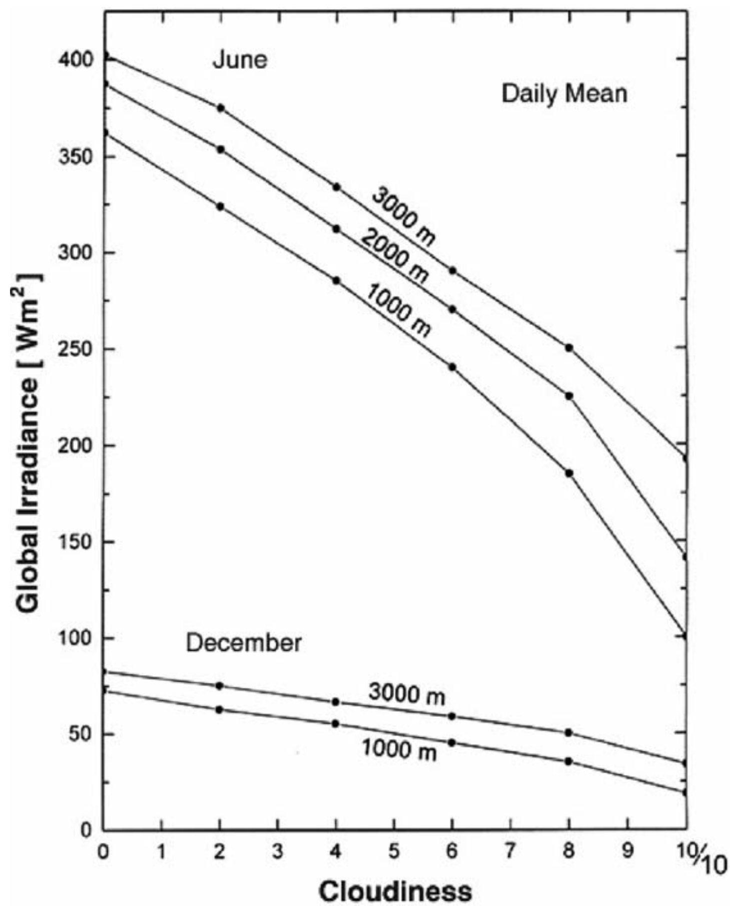

Fig. 1.2 Daily averages of global irradiance at eastern alpine stations, according to altitude and cloudiness, based on data by Dirmhirn (1964)

as little as the surrounding rocks, i.e. 15–20%. The so-called blue ice of Antarctica has emerged to the surface after having been subjected to very high pressure in the deepest part of its trajectory: 1,100 m of ice exert a hydrostatic pressure of 100 bars (100 atmospheres), a pressure under which air bubbles become dissolved in the crystal lattice of the ice. This bubble free, blue ice has the darkest appearance of any naturally occurring ice.

1.2 Distribution of energy: the energy balance of snow and ice

Solar radiation is the prime source of energy for planet Earth. It supplies a global, annual average of 240 W m^{-2}. Geothermal heat supplied by the hot interior of the Earth and by radioactive decay amounts to only 60 mW m^{-2}, negligible compared

to solar radiation, but of vital importance at the base of ice sheets. When discussing the distribution and balance of energy fluxes, we clearly need to distinguish the atmosphere, the surface of snow and ice, its interior and its base.

The principle of the energy balance is most clearly and generally demonstrated for the case of the snow surface, the cryosphere/atmosphere interface. Here, solar (or shortwave) radiation $S\downarrow$ is supplemented by atmospheric infrared (longwave) radiation $L\downarrow$ emitted by greenhouse gases, clouds and aerosols, while part is reflected $S\uparrow$, or emitted from the surface $L\uparrow$. The net value of the four fluxes is called the radiation balance. The amount delivered, or lost by the surface, is distributed in four ways:

(1) heat supply C to or from the snow by conduction, penetration of solar radiation, or convection in the pore space of the snow, which all may change the snow temperature;
(2) turbulent transfer of sensible heat H to or from the atmospheric boundary layer;
(3) turbulent transfer of latent heat of evaporation, sublimation or condensation LE;
(4) the latent heat of melting or refreezing LM.

All fluxes are defined positive if they deliver energy to the surface so that, at the surface, their total must be zero.

$$S\downarrow + S\uparrow + L\downarrow + L\uparrow + C + H + LE + LM = 0$$

These quantities are usually expressed as energy flux densities in W m^{-2}. As they depend on atmospheric variables that are not locally determined we first need information on the local climatic boundary conditions.

1.3 Air temperature: effects of altitude and latitude

The change of temperature and other environmental conditions with altitude in mid latitude mountains has often been compared to their change with latitude: a 1,000-m higher altitude in the Alps may roughly be equivalent to a 1,000-km move northward. In the case of temperature, however, the reasons for the decrease with altitude are basically different from those for the decrease with latitude. If a parcel of dry air is moved upward, it loses pressure, expands and thereby cools at a rate of 1° per 100 m altitude; in the case of moist air, condensation may reduce this figure to 0.6° per 100 m. In both cases, the cooling is the consequence of vertical motion. The decrease of temperature with increasing latitude, on the other hand, follows from the decreasing annual supply of solar radiation (Fig. 1.1).

From five pairs of mountain and valley stations in the Eastern Alps, situated respectively above 1,800 m and below 800 m, typical values of altitudinal temperature gradients are given in Fig. 1.3.

The low negative values that prevail in alpine winter are primarily due to temperature inversions above the valley stations. Highest negative values approaching saturated adiabatic conditions occur in spring with intense vertical mixing of the atmosphere. Values in Fig. 1.3 are valid for near surface air temperatures; they differ

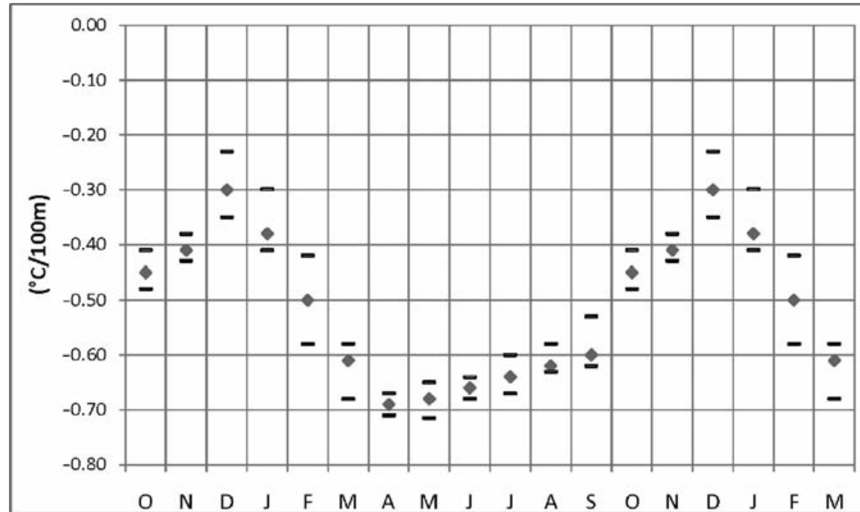

Fig. 1.3 Temperature gradients between five pairs of stations in the Eastern Alps; highest, mean, and lowest values in °C per 100 m

Table 1.1 Mean diurnal and annual temperature ranges at an alpine valley station (Gastein, 1,100 m above sea level) and a mountain station (Sonnblick, 3,200 m)

Location	Parameter	January	July	Annual range
Gastein	T_{min} (°C)	−7.4	12.5	19.9
	T_{max} (°C)	−1.5	21.1	22.6
	Daily range (°C)	5.9	8.6	
Sonnblick	T_{min} (°C)	−14.2	0.5	14.7
	T_{max} (°C)	−12.7	2.7	15.4
	Daily range (°C)	1.5	2.2	

from gradients in the free atmosphere. Although based on alpine records, they are representative for many mountain areas around the world.

Mountain stations generally have smaller diurnal and annual temperature ranges as illustrated by Table 1.1. This is a ventilation effect due to higher wind speeds at mountain peaks, i.e. due to relative topography rather than to absolute altitude.

The decrease of temperature with increasing latitude φ is very obvious in Fig. 1.4. The station Decepción is situated at sea level on an island at 63° S. It displays the low annual variation of temperature typical for oceanic situations. The extreme latitude of South Pole Station (2,800 m) does not give it extreme temperatures; it is exceeded by Vostok Station at 3,400 m. The annual range of temperatures increases with latitude and altitude due to both solar geometry and decreasing cloudiness at continental stations.

The daily range of temperature, on the other hand, is determined by the daily range of solar elevation which is $2(90 - \varphi)$, limited by a culmination at $90 - \varphi + \delta$ where δ is the solar declination. This means that there is no daily range of solar elevation and temperature at the poles.

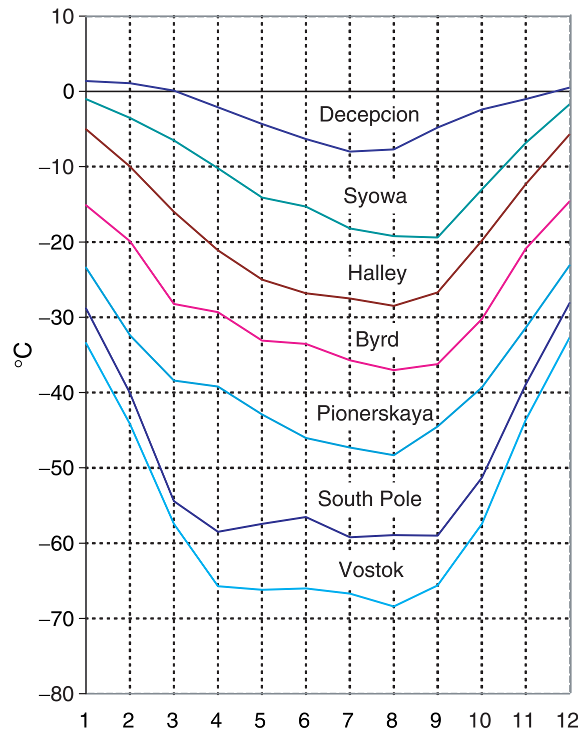

Fig. 1.4 Mean monthly temperature at antarctic stations, determined by a mix of latitude and altitude of the locations. Note the coreless winters at South Pole and Vostok. From data by Schwerdtfeger in Orvig (1970)

The change from polar day to polar night at latitudes beyond the polar circles (66.5 degrees) causes an asymmetry of solar forcing and an asymmetry in the annual march of temperature. Figure 1.4 shows how the annual march of temperature changes from a nearly sinusoidal T(t) at Decepción to a so-called coreless winter in which T reaches low values in April and then slowly decreases to an August minimum at the inland stations South Pole and Vostok.

1.4 Atmospheric humidity and precipitation

Atmospheric humidity is strongly controlled by air temperature, and to a lesser degree by the distance to the open sea. Table 1.2 gives figures relevant to humidity and phase transitions in the cryosphere.

Values of saturation vapour density (absolute humidity), and saturation pressure are given with respect to ice (i) and to supercooled water (w). Cloud droplets may stay liquid (supercooled) far below 0°C. Statistically, –15 to –20°C seems to be the modal temperature for the transitions from liquid water to ice in clouds, and super-

Table 1.2 The change with temperature T of density ρ, saturation vapour density ρ_v^*, saturation vapour pressure e*, specific heat c and latent heat L. Indices i stand for ice, a for air, w for liquid water, v for vapour, m for melting (data from Linke and Baur 1970 and Brutsaert 1982)

T	Density ρ (kg m^{-3})		Saturation vapour density ρ_v* (g m^{-3})		Saturation vapour pressure e* (hPa)		Specific heat c (J kg^{-1} K^{-1})				Latent heat L (MJ kg^{-1})	
(°C)	ρ_i	ρ_a	ρ_{vi}*	ρ_{vw}*	e_i*	e_w*	c_i	c_w	c_a	c_v	L_v	L_m
+10		1.24		9.39		12.27		4,192			2.477	
0	916.4	1.29	4.85	4.85	6.11	6.11	2,105	4,218	1,005	1,846	2.501	0.334
−10	917.4	1.34	2.14	2.36	2.60	2.86	2,030	4,271			2.525	0.312
−20	918.3	1.39	0.88	1.07	1.03	1.25	1,959	4,354			2.549	0.289
−30	921.0	1.45	0.39	0.45	0.38	0.51	1,884	4,520			2.574	0.264
−40	922.0	1.51	0.12	0.18	0.13	0.19	1,812	4,772			2.602	0.236

cooled droplets have been postulated for temperatures down to −38°C, a limit which is difficult to prove in real conditions.

It is useful to introduce the terms “temperate ice” which is at the melting point, and “cold ice” which is below freezing. The melting point is reduced below 0°C by ambient pressure, by chemical admixtures, and by the radius of curvature of snow grains in the sub-millimeter range.

From Table 1.1, it is obvious that saturations vapour pressure at 0°C is 47 times as large as that at −40°C which explains the decrease of atmospheric humidity and precipitation with latitude. While mean annual accumulation is between 1 and 2 m of water equivalent in the Alps, it is around 500 mm at the Antarctic coast and drops below 30 mm on the East Antarctic Plateau. A similar, but less impressive, decrease goes from subarctic mountains to the centre of the Arctic Ocean.

The change of precipitation in the Alps and other mountain ranges is controlled by altitude and may increase by a factor of three from the dry, screened interior to the wet, exposed margins at either side of the Alpine range. Screening effects are best developed in mid-latitude mountain ranges of N–S extent: Scandinavia, Pacific Coastal Ranges, Chilean Andes, Southern New Zealand.

1.5 The cryosphere: a matrix for life

The total extent, or an inventory of the cryosphere, is of little importance to microbiology; rather, it is the availability of solar radiation, liquid water and nutrients (Kuhn 2001; Psenner et al. 2003). These conditions in turn depend on altitude, latitude, and on the cryospheric stratum, as there are: seasonal and perennial snow; glaciers, ice caps and ice sheets; lake and river ice, sea ice; and permafrost and various kinds of ground ice. These four groups differ primarily in their structure and in their connection to other parts of the biosphere like water and soil, or in their response to

climatic changes: while the seasonal snow pack receives an atmospheric forcing first, ground ice or the base of ice sheets are last. The structure of the cryospheric strata determines the transport of energy, water and nutrients, where both energy and nutrient fluxes may be connected to liquid water.

Of the four groups, lake, sea and ground ice are frozen water (congelation ice), while snow and glacier ice is of atmospheric origin (meteoric ice). Airborne crystals of a large variety of shapes, that are systematically determined by temperature and supersaturation of atmospheric water vapour, precipitate to build the snow pack at the Earth's surface. The original, delicate snow crystals immediately start changing into rounded grains by what is called destructive metamorphism that transfers molecules from tips and convex sites on the crystal surface to the grooves and concave sites. The various stages of transformation are very well illustrated in the International Classification of Snow on the Ground (Colbeck et al. 1990). The granular shape makes for a denser packing of the snow, a density of about 300 kg m^{-3} being typical for old, dry alpine as well as polar snow.

Early winter snow packs experience heat flow from the ground beneath and heat loss to the atmosphere from the top layer, which may result in temperature gradients of several degrees per meter. Associated with the temperature gradient there is a strong gradient of vapour pressure in the pore space, which induces upward diffusion of water vapour and the build-up of facetted crystals of a size of several mm, the so-called constructive metamorphism.

In polar snow, in the absence of melting and with weak temperature gradients, the further growth of snow grains and the final, gradual conversion to ice proceeds slowly. Under alpine conditions with frequent melt-freeze cycles, the grains become repeatedly surrounded by liquid water films. In that stage of metamorphism, the melting point of small grains is lower than that of the larger ones which will attain a modal size of 1–2 mm. This is the uniform snow that skiers indulge in, calling it firn in their terminology (in glaciological terms, firn is the snow that has survived one hydrological year).

The snow matrix in the most general case consists of ice, liquid water, water vapour, air. It has peculiar properties, being permeable to both air and water flows at bulk snow densities of up to 830 kg m^{-3}, and permeable to short wave radiation at any density. Its pore space, that is the volume not filled by ice, may contain water or gases. Convection in the pore space may transport latent heat of evaporation/sublimation, and sensible heat. There is a net radiative transfer of long wave (infrared) radiation from one pore wall to the opposite wall in a general temperature gradient. There is, of course, molecular conduction of heat through the snow matrix.

In summary, there are fluxes of air and liquid water through the snow pack, both including soluble and insoluble impurities. Electromagnetic radiation penetrates the snow pack, directly in the short wave range and wall-to-wall in the infrared. The molecular conduction becomes increasingly important in the deeper layers with increasing bulk density. It is usually formulated as

$$C = \lambda \, dT/dz$$

where the depth z is positive into the ground and C is positive when directed towards the surface. The thermal conductivity λ and the thermal diffusivity K effectively

Table 1.3 Thermal conductivity λ and thermal diffusivity K of snow and ice at various bulk densities ρ

	Bulk density (ρ; kg m^{-3})				
	100	200	300	500	917
Thermal conductivity (λ; W m^{-1} K^{-1})	0.0003	0.12	0.27	0.74	2.47
Thermal diffusivity (K; 10^{-6} m^2 s^{-1})	0.0014	0.28	0.42	0.70	1.28

include all effects mentioned and thus strongly depend on density and associated permeability as shown in Table 1.3.

The extinction of solar radiation in snow and ice follows an exponential law according to

$$S{\downarrow}(z) = S{\downarrow}(0)\, e^{-\kappa z}$$

where the extinction coefficient κ for shallow layers of dry snow has typical values of about 10–20 m^{-1} for spectrally broad bands (Meirold-Mautner 2004), corresponding to a reduction of the radiation below 10 cm of snow to a fraction of 37–14% of the value incident at the surface. The value of κ strongly depends on wave length so that broad band extinction coefficients are not constant with depth. Extinction is least for the blue part of the spectrum, giving a blue shade to light that returns upward from deeper layers or crevasses. (Actually, it is the spectrum of the incident radiation, the spectral extinctions coefficient and the spectral sensitivity of the observer's eye that together give snow its colour.)

1.6 Liquid water in the cryosphere

As liquid water is an essential asset of the biosphere, it is of particular interest to investigate the conditions under which it may occur in an environment of 0°C or less.

In the atmosphere, there are the supercooled cloud droplets mentioned in Sect. 1.4 that remain liquid in the absence of a freezing nucleus. Once they touch an ice crystal or splinter, or any other efficient nucleating agent, they freeze spontaneously. At subfreezing temperatures, liquid water in contact with solid surfaces has little chance to remain liquid.

At the surface of an ice crystal there is a layer of several molecules thickness in which the ice molecules are much more mobile than in the deeper layers of the crystal lattice, the so-called quasi liquid layer. It is best developed at freezing temperature and solidifies around −5°C. It is vital for the reduction of total surface free energy in the destructive metamorphism, the rounding of snow grains, as it allows for a much higher rate of transfer of molecules from the convex to the concave sites on a crystal than would be possible by diffusion in the vapour phase.

Surface melting supplies most of the water found in the snow pack and on the glacier surface. At an atmospheric pressure of 1,013 hPa and with an energy supply of 334 kJ kg^{-1}, ice melts when it reaches 0°C, independent of ambient air temperature. Ice may in fact melt at air temperatures down to at least −5°C and may stay frozen at air temperatures of at least +5°C, depending on micrometeorological conditions (Kuhn 1987).

Proceeding downwards on a glacier that spans the thermal regime from cold at the top to temperate at the terminus, one first walks on the dry facies, where melting never occurs. This is followed by a zone where snow melts at the surface but melt water does not percolate through the entire annual layer, leaving a dry layer between layers of refrozen snow. Farther below, melt water percolates the entire annual layer and refreezes as superimposed ice on the impermeable surface of the ice body. This form of latent heat transport is the reason why most alpine glaciers below 3,400 m are temperate in spite of mean annual ambient air temperatures far below freezing.

Below that zone, melting and net ablation of glacier ice prevails. Melt water produced at the glacier surface penetrates the ice in cracks, crevasses and in the funnels of moulins and leaves it in a system of braided channels.

Surface melting may produce extended areas of water-soaked firn where algal growth is promoted and further enhances melting on account of its low albedo (Meirold-Mautner 2004). When the firn pore space is saturated with melt water, the surface layer may accelerate downwards as a slush flow or may stagnate and form supraglacial lakes. These lakes have a lower albedo than the surrounding ice, absorb solar radiation and heat from the surface and throughout. Thereby, they attain water temperatures above freezing which are associated with increasing density up to 4°C. Where in contact with the underlying ice, their water stays at 0°C, which induces thermal downward convection of warmer, denser surface water and the positive feed back of enhanced melting.

A variation of this thermodynamic situation is displayed in the cryoconite holes, a sample of which is shown in Fig. 1.5. The biological importance of these features was first described by Steinböck (1936) of my home University of Innsbruck and

Fig. 1.5 Cryoconite holes in the ablation zone of an alpine glacier. Note the absorbing sediments and the circular northern rim caused by the daily march of solar irradiance. The hole on the left has a length of about 20 cm

has recently received revived interest (Margesin et al. 2002). When insoluble organic and inorganic particles move in the water film on top of a melting glacier, they may happen to concentrate and build a dark sediment that absorbs solar radiation at a high rate, heats up and melts down into the ice. The circular shape of the cryoconite hole in Fig. 1.5 reflects the daily circle of solar radiation and its melting power. A radially small plaque of cryoconite or a small stone will melt vertically downward in the top centimeters of the ice; larger bodies like the one displayed in Fig. 1.5 will have an oblique melt channel of decimeter depth. Absorption of solar radiation that heats the bottom sediments up to a temperature of 4°C creates stable layering in the cryoconite hole and thus promotes downward melting.

Yet another thermodynamic situation is seen in the lakes of the Antarctic Dry Valleys which have a permanent ice cover of 3–6 m thickness (McKay et al. 1985). Here, water is kept liquid under an ice cover that separates it from air of mean annual temperature of −20°C. This is accomplished by a minor contribution of geothermal heat and a major energy gain from seasonal, or ephemeral, lateral influx of melt water from the ice free surroundings (Lewis et al. 1998), comparable in a particular way to the maintenance of temperate conditions in alpine glaciers versus an ambient temperature below freezing.

At the base of glaciers or ice sheets, the load of ice exerts an overburden pressure of 1 bar (10^5 Pa) per 11 m of ice thickness. The increased pressure in turn reduces the melting point by 0.76×10^{-6} per Pa so that the pressure melting point below 4,000 m of ice is −2.8°C. These are conditions prevailing at a number of subglacial lakes in Antarctica (Siegert et al. 2001). Regardless of the extremely low surface temperatures of Antarctic ice, melting at the base of the ice sheet is powered by the geothermal heat flux which has a global average of 60 mW m^{-2}, sufficient to melt 7 mm of ice per year, a value that applies to the base of shallow, temperate glaciers as well.

1.7 Hot spots in the ice

The differential absorption of solar radiation by dark rocks may create isolated spots of temperatures far above freezing. While cryoconite, sand or small stones absorb more solar radiation than ice or snow, heat up and melt down into the ice, large rocks absorb similar amounts of energy per unit surface area, but due to their thickness have smaller temperature gradients and less heat conduction downward. Their energy gain is thus used to raise their temperature and, under alpine summer conditions, this may result in rock surface temperatures in excess of +40°C. One rock of 2 m diameter, protruding 1 m above the surrounding snow surface at 3,000 m above sea level, was measured with an IR thermometer in early summer in the Austrian Alps. Its surface temperature rose to 42°C in the early afternoon on its southerly aspect, and to a similar temperature on its western side about 1 h later.

A profile of surface temperatures from the glacier tongue of Hintereisferner across the moraine was recorded in late May, with peak temperatures again exceeding

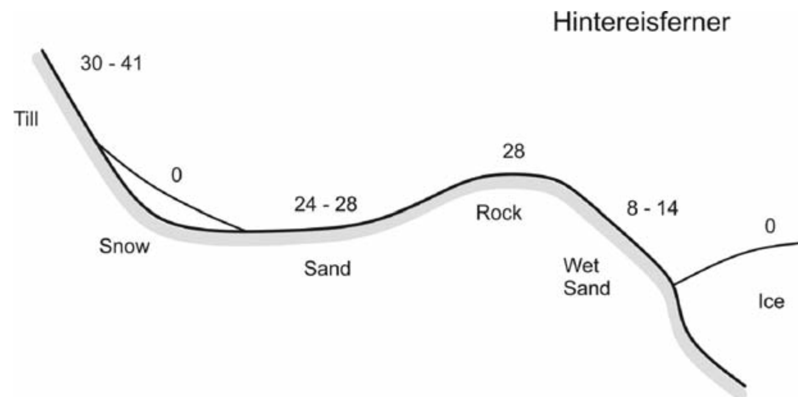

Fig. 1.6 Surface temperatures in the surrounding of the glacier tongue of Hintereisferner in the Austrian Alps at an altitude of 2,500 m, measured by ground based infrared thermometer at a solar elevation of 60°. The cross section is approximately 100 m long

40°C. The values given in Fig. 1.6 show that wet sand is colder than dry sand as is to be expected due to the loss of latent heat; rocks in the vicinity of the glacier are cooler than those farther up due to the cooling effect of the katabatic glacier wind.

1.8 Conclusions

Contrary to common expectations the cryosphere harbors abundant life. While from the side of the biosphere this is the success of adaptive strategies, the geosphere supports these by environmental conditions that supply light and energy, provide shelter in and under the snow and ice matrix, and enable the circulation of nutrients and liquid water.

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Chapter 2
Limits for Microbial Life at Subzero Temperatures

Corien Bakermans

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2.1 Introduction

The limitations of terrestrial life are not well-defined or understood and have primarily been advanced through exploration and discovery of organisms living in "extreme" environments where life was not thought possible. Identifying the limits of life is hampered by our inability to define the essential nature of life, rather than just describing its properties (organization, energy use, growth, adaptation, response to stimuli, reproduction). Similarly, until the twentieth century, we could not define the essential nature of water (a molecule composed of two atoms of hydrogen and one atom of oxygen joined by covalent bonds), only its properties (colorless, solvent, liquid at specific temperatures and pressures). Additionally, when examining the limits of life, defining what is "alive" becomes more difficult. The metabolism of life is often separated into three classes: growth, maintenance, and survival (Morita 1997). Clearly an organism that is metabolizing actively enough to reproduce itself exhibits *growth* and is considered living. But what about organisms that are actively metabolizing but not reproducing? In this case, energy is consumed for repair and *maintenance* of cellular structures to preserve the integrity of the organism, but cannot sustain reproduction. Maintenance may also allow adaptation and evolution to changing conditions over very long time spans (other definitions of maintenance do not generally include evolution). Finally, organisms routinely *survive* conditions at which they cannot actively metabolize by forming completely inactive dormant

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states (such as spores) or with very weak intermittent metabolism for the repair of accumulated damage.

This review is primarily interested in microorganisms that are actively metabolizing for growth or maintenance, not merely survival, at extreme low-temperature conditions. Microorganisms that actively live at subzero temperatures must evolve mechanisms to deal with the accompanying thermodynamic constraints. These constraints include the reduction of available liquid water accompanied by the formation of ice crystals, lower rates of catalysis and transport, decreased membrane fluidity, and stabilization of molecular structures (Cavicchioli et al. 2000). Most of these thermodynamic constraints apply to all microorganisms at the lower limits of their growth temperature ranges; while the most severe constraints affect those microorganisms (psychrophiles) that grow at the lowest temperatures. Only recently have microbiologists begun to realize the potential for bacteria to actively survive, and even reproduce, at low temperatures (Graumann and Marahiel 1996; Russell and Hamamoto 1998; Deming 2002; Bowman 2004). Recent exploration and investigations of low-temperature environments are redefining the known limits of microbial activity and are fueled by new techniques and capabilities. This chapter reviews the physical chemical effects of low temperatures and the activity of microorganisms at subzero temperatures.

2.2 Physical chemical effects of low temperatures

The effects of temperature derive from its essential nature: the energy of motion of atoms. Temperature is a measure of the average energy of motion (vibration, rotation, or translation) of the atoms or molecules of a substance. Hence, cool molecules move, vibrate, and rotate less than hot molecules. Thermal energy promotes phase changes from solid to liquid to gas, diffusion of molecules within the gas or liquid phase, and conformation changes of enzymes and their substrates allowing chemical transformations to occur. As temperature decreases the available thermal energy (enthalpy) of a system decreases. The effects of temperature on liquid water, reaction rates, and molecular stability are discussed in the following sections.

2.2.1 Liquid water

Liquid water is essential to life, yet water freezes at the relatively high temperature of 0°C. Without liquid water, there is no solvent system for enzymes, membranes, etc., to function in or for substrates to diffuse in. Ice crystals can also pierce cell membranes causing extensive damage. Liquid water can be maintained below 0°C by a variety of physical chemical processes, such as: freezing point depression, ordering effects, supercooling, and pressure (Table 2.1).

In environmental systems, freezing point depression is the dominant process for sustaining liquid water at subzero temperatures. Freezing point depression occurs

Table 2.1 Liquid water

T_m[a] (°C)	$T_{in\ situ}$[b] (°C)	System	Film or vein thickness (μm)	a_w[c]	Reference
0	-	H_2O	n/a[d]	1.0	-
-	−1.5	Permafrost	0.015	0.99	Rivkina et al. (2000)
−1.9	-	Seawater (3.5% salinity)	n/a	0.98	-
-	−3 to −5	Lake Vostok accretion ice	250–1000	0.92–0.96	Price (2000)
-	−5 to −15	Ancient glacial ice	6–12	0.85–0.95	Price (2000)
-	−10	Permafrost	0.005	0.9–0.99	Rivkina et al. (2000)
-	−15	Sea ice	1–5	~0.87	Bock and Eicken (2005)
-	−20	Soil	0.005	0.99	Jakosky et al. (2003)
−21	-	Saturated NaCl soln.	n/a	0.75	-
−22	-	H_2O under 207.5 MPa pressure	n/a	1.0	-
-	−28	Sea ice	<1	0.7–0.8	Bock and Eicken (2005)
−42	-	Supercooled H_2O	n/a	1.0	-
-	−52	Don Juan Pond, Antarctic; $CaCl_2$ and NaCl brine	n/a	0.45	Beaty et al. (2006)
-	−56	Glacial ice	1–2	<0.67	Price (2000)

[a]Melting and freezing point temperature; [b]environmental temperature; [c]water activity; most values are estimates based on the solute concentration required to achieve freezing point depression; [d]not applicable

through the addition of solutes and is proportional to molal concentration. Hence, seawater (3.5% salinity) freezes at −1.9°C, while a saturated (~30%) NaCl solution freezes at −21°C. As water freezes, pure water crystallizes first leaving a more concentrated solution that causes the freezing point to drop further. This will continue until the solution becomes saturated with solute, at which point further decreases in temperature result in crystallization of both solute and solvent. Freezing point depression allows for the presence of thin films of water in soils, sea ice, and glacial ice down to temperatures of −20, −26, and −56°C, respectively (Price 2000; Jakosky et al. 2003; Bock and Eicken 2005). Unless significant concentrations of solutes are present, the thin films present in soils may only correspond to water adsorbed to the surface of mineral grains and ice crystals.

Adsorbed water has a lower freezing point than bulk water due to ordering effects associated with being in close proximity to an ordered surface, and can extend as far as 1 μm from the surface (Drost-Hansen 2001). Layers of adsorbed water may be too thin to allow for the movement of bacteria. However, adsorbed water may allow the transport of substrates and wastes (Cary and Mayland 1972;

Ugolini and Anderson 1973). Ordering effects will also influence the freezing of intracellular water (Mindock et al. 2001). Because liquids require a seed crystal or nucleus to begin the freezing process, in the absence of nuclei water can be "supercooled" to temperatures far below 0°C. Microorganisms take advantage of this phenomenon by preventing nucleation within cells through the use of antifreeze proteins (Kawahara 2002; Scotter et al. 2006). Increased pressure also reduces the freezing point, but only slightly, and comes into play primarily beneath ice sheets and glaciers. Additionally, water need not enter a crystalline state at low temperatures but can form a glass through the process of vitrification which occurs through rapid cooling or the addition of substances, such as organic polymers, that prevent the formation of ice crystals.

Water can remain liquid at temperatures from 0 to −56°C through a variety of processes. However, whether or not this water is still biologically useful remains questionable, particularly when only very thin films of adsorbed water remain liquid. The availability of liquid water is often measured as the water activity ($a_w = rh/100$, where *rh*=relative humidity). Most low temperature environments have a_w well above the recently proposed minimum water activity capable of sustaining life, 0.62 (Beaty et al. 2006).

2.2.2 Reaction rates

Reaction rates are particularly hard hit by decreases in thermal energy since rates decrease exponentially with decreasing temperature as defined by the Arrhenius equation:

$$k = Ae^{\frac{-E}{k_B T}}$$

where *k* is the reaction rate, *A* is the pre-exponential term, *E* is the activation energy, k_B is Boltzmann's constant and *T* is the absolute temperature in Kelvin. Despite the exponential decrease in rates with falling temperatures, bacterial metabolism does not appear to be limited by low rates. Very low rates of metabolic activity appear sustainable for long periods of time (10^4 to 10^6 years) in various low-temperature ecosystems (Johnston and Vestal 1991; Sun and Friedmann 1999; Sowers 2001; Price and Sowers 2004; Tung et al. 2005, 2006). In addition, very low rates that support significant microbial communities have been reported in other ecosystems independent of low temperatures (D'Hondt et al. 2004; Lin et al. 2006). Because rates of reaction, diffusion, and chemical degradation decrease coordinately, bacteria appear to continue metabolizing at low temperatures, albeit slowly. Despite significant differences in rates between bacterial growth, maintenance, and survival (several orders of magnitude), metabolic activity capable of sustaining growth has been extrapolated down to −40°C based on *in situ* measurements (Price and Sowers 2004).

2.2.3 Molecular stability

The decrease in thermal energy of molecules at low temperatures also leads to increased stability and rigidity. For example, membrane lipids will become less fluid, while secondary structures of DNA and RNA will become less flexible. As temperature decreases, proteins will also become less flexible. However, below a threshold point, cold denaturation of proteins will occur (D'Amico et al. 2006). As a general mechanism, cold-adapted microorganisms increase the disorder within macromolecules to maintain fluidity or flexibility and hence function at low temperatures (Feller 2007).

At low temperatures, membrane lipids undergo a transition from liquid crystal to gel phase. As membranes stiffen, embedded proteins cannot function and transport nutrients effectively, causing starvation (Nedwell 1999; Pomeroy and Wiebe 2001). When 50–90% of membrane lipids are in the gel phase, bacteria will stop functioning (Jackson and Cronan 1978; Melchior 1982). Microorganisms can maintain fluidity of cell membranes at low temperatures by increasing unsaturated lipids, decreasing acyl chain length and branch-chained lipids, or altering polar head groups and by producing compatible solutes (Russell and Fukunaga 1990). For example, psychrophilic bacteria commonly increase the proportion of C18:1 and/or C16 fatty acids to maintain membrane fluidity at low temperatures (Russell and Fukunaga 1990; Russell 1997). These fatty acids have very low melting points: for example, oleic acid (C18:1ω9) melts at 14°C, while palmitoleic acid (C16:1ω7) and linoleic acid (C18:2ω9,12) melt at −0.1 and −5°C, respectively (Williams and Frausto da Silva 1996). Considering the low melting points of unsaturated fatty acids, the functional limits of membrane lipids do not appear to have been reached.

Low temperatures reduce the activity of proteins through decreased flexibility of protein structure. Thermal energy from the environment fuels the conformational motions of enzymes that are the basis for enzymatic function. Consider that, "If the environment is too cold, the enzyme may move so slowly that catalysis no longer occurs at a metabolically useful rate" (Fields 2001). Cold adaptation of enzymes is commonly achieved by reducing weak stabilizing interactions (ion pairs, hydrogen bonds, hydrophobic and intersubunit interactions), increasing solvent interactions with apolar or interior residues, reducing proline and arginine content, and/or clustering of glycine residues (Feller et al. 1996; Russell 2000). But have the low-temperature functional limits of proteins been reached? While *in vitro* enzyme activity has been reported at 190°K (Daniel et al. 1998, 2003), very few studies of the functional limits of cold-adapted proteins at low temperatures have been conducted. A mutation (N288V) of a psychrophilic α-amylase decreased the melting temperature but also decreased the activity to half that of the wild-type enzyme, and the protein was only marginally stable at low temperatures, suggesting that the low-temperature limits have almost been reached for this enzyme (D'Amico et al. 2001). In contrast, directed evolution of psychrophilic subtilisin S41 created a protein

that was more active at low temperatures and less thermolabile than wild-type, but only towards a specific substrate, suggesting that this protein may not have reached its functional low-temperature limit (Miyazaki et al. 2000). In addition, it could be argued that, because the specific activity of psychrophilic and mesophilic enzymes is not always the same at their respective optimal temperatures, adaptation to cold in psychrophiles is not complete (assuming that mesophilic enzymes are optimized). Currently, there is insufficient data to determine if further cold adaptation is incomplete or not possible.

Low temperatures stabilize secondary structures of nucleic acids which will inhibit the processes of transcription, translation, and DNA replication. Cold-adapted microorganisms alleviate stress on these processes with specialized helicases and chaperones (Jiang et al. 1997; Chamot and Owttrim 2000; Phadtare et al. 2002). To maintain conformational flexibility of functional RNA (transfer and ribosomal RNA) at low temperatures, cold-adapted microorganisms increase the dihydrouridine content of tRNA and decrease the GC content of 16S rRNA (Dalluge et al. 1997; Noon et al. 2003; Khachane et al. 2005). Whether or not microorganisms have already maximized their capacity to alleviate low-temperature stress on these processes remains to be determined.

While examining individual biomolecules is useful in defining functional low-temperature limits, the complex interactions that occur between biomolecules will certainly contribute to the functional low-temperature limits of cells. Cold-adapted microorganisms must coordinate the low-temperature functionality of many biomolecules and their interactions with each other and the environmental. Hence, by examining the capabilities of microorganisms inhabiting low-temperature environments on Earth, the functional low-temperature limits of cellular systems can be explored, while recognizing that the low-temperature capabilities that have evolved depend on the particular conditions of the environment and selection pressures experienced.

2.3 Activity of microorganisms at subzero temperatures

A variety of low-temperature environments on Earth such as sea ice, glaciers, permafrost, and snow have proven suitable for microbial habitation. Exploration of these environments and the microorganisms that inhabit them is rapidly providing evidence for microbial activity at subzero temperatures (Table 2.2). While this review focuses on microorganisms due to their ability to reproduce at low temperatures, some macroscopic eukaryotes can also actively metabolize at subzero temperatures; for example, activity has been reported in the Himalayan midge at −16°C (Kohshima 1984). Currently the lowest reported temperature for growth and reproduction of microorganisms is −12°C (Breezee et al. 2004). Because reproduction is difficult to measure directly in the environment, current studies on reproduction at low temperatures rely on laboratory cultures of isolates and standard measurement techniques such as plate counts or turbidity measurements. Unfortunately, even

Table 2.2 Activity of microorganisms at subzero temperatures

T (°C)	Activity	Method	Environment	Reference
−9	CO_2/CH_4 production	Correlation of cell counts with CO_2, CH_4 and clay grains	Glacial ice	Tung et al. (2006)
−10	Cell division	Plate counts, turbidity measurement	Laboratory culture of permafrost isolate	Bakermans et al. (2003)
−10	Metabolism	Incorporation of ^{14}C-labeled acetate into lipids	Arctic permafrost	Rivkina et al. (2000)
−11	CH_4 production	Correlation of cell counts with CH_4	Glacial ice	Tung et al. (2005)
−12	Cell division	Turbidity measurement	Laboratory culture of sea ice isolate	Breezee et al. (2004)
−15	DNA/Protein synthesis	Uptake of ^{3}H-thymidine and ^{3}H-leucine	Laboratory cultures of glacial ice isolates	Christner (2002)
−15	Metabolism	Incorporation of ^{14}C-labeled glucose	Arctic permafrost	Gilichinsky et al. (2003)
−16.5	CH_4 production	CH_4 production from ^{14}C-labeled bicarbonate or acetate	Arctic permafrost	Rivkina et al. (2002)
−18	Photosynthesis	CO_2 exchange	Polar lichens	Kappen et al. (1996)
−18	Metabolism	Incorporation of $^{14}CO_2$, release of CO_2	Laboratory cultures of permafrost isolates	Panikov and Sizova (2007)
−20	Respiration	CTC reduction	Sea ice	Junge et al. (2004)
−20	Respiration	Resazurin reduction	Laboratory cultures of permafrost isolates	Jakosky et al. (2003)
−20	Metabolism	Oxidation of ^{14}C-labeled glucose	Permafrost soil columns	Panikov et al. (2006)
−20	Protein synthesis	Uptake of ^{3}H-leucine	Laboratory culture of sea ice isolate	Junge et al. (2006)

under "ideal" growth conditions in the laboratory, reproduction rates at low temperatures quickly approach long time spans with generation times of several years predicted for growth at −20°C (Jakosky et al. 2003). Hence, measurements of microbial activity in low-temperature environments can provide valuable information on low-temperature capabilities of microorganisms.

Microbial activity has been demonstrated at temperatures from −9 to −20°C via a variety of techniques. Unfortunately these techniques cannot readily distinguish between growth, maintenance, or survival metabolism of microorganisms (without knowing if low rates correspond to maintenance and survival or just long generation

times). In addition, individual enzymes can have temperature optima well outside the growth temperature range of their parent organism. Hence, techniques that measure metabolic processes which require the coordinated activity of many enzymes and processes will provide more substantial evidence for active metabolism at low temperatures. Microbial activity at −20°C has been convincingly demonstrated by several laboratories using different techniques that are measures of coordinated metabolism. However, the ability of microorganisms to actively metabolize at temperatures below −20°C remains uncertain. Several intriguing studies describing activity of microorganisms at temperatures below −20°C have recently been published (Christner 2002; Junge et al. 2006; Panikov et al. 2006; Panikov and Sizova 2007).

Panikov et al. (2006) sequentially incubated columns containing permafrost soils that had been thawed to +1°C for the addition of ^{14}C-glucose at temperatures from 0 to −33°C and measured the $^{14}CO_2$ released into air flowing through the columns. Permafrost soil at all temperatures produced two orders more $^{14}CO_2$ than autoclaved controls. However, incubation at individual temperatures was brief (2–7 days) and, at temperatures below −20°C, $^{14}CO_2$ release decreased with time. Interestingly, the ratio of $^{14}CO_2$ to total CO_2 released decreased as temperature decreased. In addition, permafrost soil was shown to release CO_2 at temperatures down to −39°C (negative controls were not shown). To control for the release of trapped CO_2, samples were preincubated for 2–7 days at −18°C with CO_2-absorbing soda lime. The total CO_2 released in incubations exceeded the amount of inorganic CO_2 present in samples.

In another study from the same laboratory, permafrost isolates were grown on ethanol mineral medium with microcrystalline cellulose at temperatures from −8 to −35°C and monitored for both CO_2 production and $^{14}CO_2$ uptake (released by ignition) for two months (Panikov and Sizova 2007). At all temperatures, cultures produced CO_2 at levels well above the autoclaved controls; however, no sustained activity was evident at temperatures below −18°C after 3 weeks. Interestingly, the "early" activity at all temperatures occurred at the same rate for temperatures from −16 to −35°C. This temperature-independent early activity may be indicative of some physical chemical process (rather than metabolic activity) or of a burst of metabolic activity that prepared cells for dormancy.

Junge et al. (2006) examined the uptake of ^{3}H-leucine into protein at temperatures of +13 to −196°C using laboratory cultures of *Colwellia psychroerythrea*, a sea ice isolate. Uptake of ^{3}H-leucine into the protein fraction of cells was seen at all temperatures and enhanced by the addition of exopolysaccharides (EPS). No significant activity was seen in TCA (trichloroacetic acid)-killed or heat-killed controls, samples with chloramphenicol or sodium azide, *Escherichia coli*, samples with only EPS, or time zero controls (which included the quick-freeze and thawing steps). Vitrification, as mediated by EPS and flash-freezing in liquid nitrogen, may be essential to maintaining enzymatic activity at such extreme low temperatures. Interestingly, saturation of enzyme-mediated uptake into TCA-insoluble material occurred at 8 h for samples at −80 and −196°C (saturation was reached in 2 h at −1°C and 12 h or less at −20°C). As in Panikov and Sizova (2007), rates were temperature-independent below −15°C.

In contrast, Daniel et al. (1998) demonstrated that the activity of a glutamate dehydrogenase did not deviate from Arrhenius behavior at temperatures from +77 to −83°C and Christner (2002) reported no significant activity at −70°C when examining ^{3}H-thymidine and ^{3}H-leucine incorporation at −15 and −70°C into strains of *Psychrobacter* and *Arthrobacter* that were isolated from Antarctica. Incorporation of ^{3}H-thymidine and ^{3}H-leucine into the TCA-insoluble material occurred only at −15°C and not in TCA-killed controls, at −70°C, or with antibiotics. The maximum incorporation of leucine or thymidine at 15°C was evident at ~50 days of incubation.

These studies present tantalizing data that may suggest microbial activity at temperatures below −20°C and await replication and verification by other researchers. Certainly, it has long been recognized that extreme low temperatures are not lethal to organisms; cells are routinely preserved with cryoprotectants at −196°C in liquid nitrogen. More research is needed to understand what is occurring in these systems, what factors enhance low-temperature activity, and how to interpret the temperature-independence of activity below −15°C.

2.4 Conclusions

The functional limits of biomolecules and active metabolism at low temperatures remain ill-defined and understudied. Individually, biomolecules appear capable of function at extreme low temperatures. However, the functional limits of biomolecules within complex systems may vary drastically. Currently, the functional low-temperature limits of cold-adapted microorganisms are −12°C for reproduction and −20°C for metabolism. The availability of liquid water appears to be the major growth-limiting factor at subzero temperatures.

The low-temperature adaptations and capabilities evolved on Earth may or may not represent the true functional low-temperature limits of terrestrial life, because these adaptations are highly dependent on the particular conditions of the environment and selection pressures experienced. For example, cold adaptation may be limited by time due to slow rates and/or the persistence and pervasiveness of low-temperature environments on Earth. Many modern low temperature environments are young (on a geologic or evolutionary time scale): Siberian permafrost began forming only 3 million years ago (Mya), while the permanent Antarctic ice sheet developed only 15 Mya (Gilichinsky et al. 1992; Barrett 2003). Just 55 Mya the global climate was warm enough for polar sea surface temperature to reach 23°C (Sluijs et al. 2006). While other low-temperature epochs are evident in the geologic record (34, 150, 300, 450, 600, and 730 Mya), low-temperature adaptations that evolved during glacial periods may or may not be preserved during warm periods.

Growth at extreme subzero temperatures happens slowly (relative to human life times and attention spans). Arctic lichens may be 3,700 to 9,000 years old, while Antarctic cryptoendoliths and microbes trapped in glacial ice have carbon turnover times of ~10,000 and ~100,000 years, respectively (Denton and Karlen 1973; Friedmann et al. 1993; Tung et al. 2005). Hence, new measurement techniques

must be devised and data carefully interpreted. A geology-based approach, using stable isotope techniques (Sowers 2001) or concentration gradients of substrates and metabolic products (D'Hondt et al. 2004), may be needed to measure very slow rates and long-term productivity at subzero temperatures. Reliable indicators of historical *in situ* temperature conditions will also be needed. Quantifiable definitions of growth, maintenance, and survival are required that can account for low temperatures, low nutrient concentrations, low diffusion rates, and low reaction rates. Microbial metabolism (whether continuous or sporadic) needs to be active enough to counter chemical degradation (such as amino acid racemization) and other damage (such as from background radiation). However, reproduction may not be necessary if adaptation and evolution are still possible; after all, why produce a competitor when nutrients and energy are scarce? The continued exploration of low-temperature environments and cold-adaptation will answer many of these questions and substantially increase understanding of microbial activity and life at subzero temperatures.

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Part II
Microbial Diversity in Cold Ecosystems

Chapter 3
Bacteria in Snow and Glacier Ice

Vanya Miteva

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3.1 Introduction

By definition, the cryosphere is the portion of the Earth where water is in solid form as snow or ice. It includes vast areas of sea ice, freshwater ice, glaciers, ice sheets, snow cover and permafrost. Because of the extremely harsh climatic conditions, these frozen environments had been considered for a long time to be devoid of life or serving merely as repositories for wind-transported microorganisms trapped in the ice (Cowan and Tow 2004). However, the increasing number of recent studies on the microbial ecology and diversity of natural ice samples have changed this view. Although still limited, they have shown that permanently frozen environments harbor abundant, live and diverse microorganisms that may be detected and recovered by cultivation. Priscu and Christner (2004) calculated the total number of bacterial

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cells in the Antarctic and Greenland ice sheets to be 9.61×10^{25}, which corresponds to a significant carbon pool of 2.65×10^{-3} Pg (1 Pg = 10^{15} g) and represents a considerable reservoir of microbial diversity. At present, the cryosphere is important not only as an integral part of the global climate system but as one of the major habitable ecosystems of Earth's biosphere and as the best analogue for the search of extraterrestrial life. This chapter presents the current knowledge about the detection, diversity, survival and activity of bacteria in snow and glacier ice. Because this topic has been previously reviewed (Priscu and Christner 2004; Cowan and Tow 2004; Priscu et al. 2007), the focus here will be to outline major earlier findings and to present the most recent advances in glacier ice and snow microbiology.

3.2 Characteristics of snow and glacier ice as microbial habitats

Snow and glacier ice are interrelated ecosystems because glacier ice is formed from snow as a result of gradual compression and burial for hundreds of thousands of years. Snow by itself is a significant climatic and ecological system that may cover 35% of the Earth's surface permanently or for varying times during the year. Dust particles, containing bacteria, viruses, fungi, small protists, pollen grains, seeds and plant and insect fragments, originating from different places around the globe, are carried by winds and deposited with snow on the surface. This eolian material varies in content and quantity depending on climate and creates different local snow ecological systems, characterized as dynamic nutrient and microbial reservoirs (Jones 1999). Microorganisms inhabiting snow ecosystems are usually exposed to high light and UV irradiation and seasonal temperature fluctuations.

Glacier ice is a unique ecosystem preserving microbial life and past climate changes chronologically for hundreds of thousands of years. Most of the glacier ice on Earth is represented by the ice sheets of Greenland and Antarctica corresponding to about 10% of Earth's terrestrial surface and containing 77% of the fresh water on the planet (Paterson 1994). Together, polar plus temperate glaciers on other continents cover an area of 15,861,766 km^2 (Priscu et al 2007). In China, there are more than 46,000 glaciers (Zhu et al. 2003) and most glaciers on the Tibetan plateau are at altitudes higher than 5,000 m. Glacier ice depths range from few hundred meters to 3–4 km with a gradual increase of temperature with depth. For example, at the South Pole it ranges from about −50°C on the surface to −6°C to −10°C in the deepest layers (Price et al. 2002).

Many studies have concentrated on the formation, structure, geochemistry and dating of ice sheets and glaciers, which are beyond the focus of this review. However, two studies should be mentioned as an attempt to view the physicochemical characteristics and structure of the polycrystalline glacier ice as a microbial habitat. Price (2000) and recently Mader et al. (2006) calculated vein sizes and chemistry and showed that the total molarities in the liquid veins at ice grain interjunctions are in the range typically used in laboratory media and contain variable

substrates that can facilitate bacterial metabolism and survival. Price (2007) distinguished two types of habitats in glacier ice: the liquid veins and the thin liquid film on the surfaces of mineral grains.

Although microbial cell numbers in clear glacier ice are generally low (10^2 to 10^4 ml^{-1}), they have been found to fluctuate with depth, with a major role in these fluctuations attributed to the insoluble mineral particles, which are considered the main carriers of microbial cells (Abyzov et al. 1998a). In contrast, the basal ice at the bottom of terrestrial glaciers and ice sheets represents a specific glacial environment containing much higher concentrations of microorganisms, mineral grains, gases, organic and inorganic ions (Price 2007). Sheridan et al. (2003) detected 10^7cells ml^{-1} in the basal Greenland ice (GISP2) that could have originated either from eolian deposition as in other glaciers or from the underlying bedrock. This layer of ice between 3,040 and 3,053 mbs (meters below surface) has a high load of mineral grains that were suggested to provide the nutrients and conditions for metabolic activity of the numerous microbial cells attached to their surfaces (up to 10^9 ml^{-1}) (Tung et al. 2006). Similarly, the basal ice of a high Arctic glacier had 100 µM dissolved organic carbon (DOC), compared with 24 µM in the glacier ice, as well as other distinct chemical and physical characteristics (Skidmore et al.2000; Bhatia et al. 2006). DOC in the clear Vostok ice ranges from 4×10^{-7} to 7×10^{-7} M (Priscu et al. 2007).

Another specific microbial habitat existing on the surface of glacier ice are the cryoconite holes, named by the Swedish explorer A.E. Nordenskjöld during his 1870 Greenland expedition ("cryo" meaning ice and "conite" meaning dust). Cryoconite holes are vertical cylindrical melt holes on the ice surface which have a thin layer of dark colored material at the bottom and are filled with water. The formation and structure of cryoconite holes have been reviewed in detail (Wharton et al. 1985; Cowan and Tow 2004). Briefly, they are formed by small particles of debris that absorb more solar radiation than the surrounding ice, and melt downwards at a faster rate. They have been found on the surface of polar and non-polar glacier ice in many parts of the world. Most cryoconite holes are open systems to the surrounding environment. In contrast, cryconite holes in Antarctic Dry Valley glaciers are covered by an ice lid, up to 30 cm thick, which isolates them from the environment and from the atmosphere (Porazinska et al. 2004). Cryoconite holes have been suggested to play an important role in glacier ecosystems because they contain abundant populations of active living organisms (Wharton et al. 1985). Since each one of these mini-environments is usually spatially separated, cryoconite holes are drawing significant attention as model systems for microbial activity and adaptation to cold.

All mentioned ecosystems are characterized by variable environmental factors that control the survival and persistence of microbial organisms and, hence, their diversity and metabolic activity. The growing interest in studying glacier ice and snow as microbial habitats may lead to exciting new discoveries and provide valuable information on the microbial diversity, the mechanisms for long term survival and activity of microbial cells at subzero temperatures, and the origin, evolution and limits of life on Earth and possibly on other planets.

3.3 History of detection of microorganisms in glacier ice and snow

Although microorganisms had been found in glacier ice and other frozen environments long ago (McLean 1918; Darling and Siples 1941; Straka and Stokes 1960), it was not until the pioneering works of Abyzov on the deep ice over lake Vostok in Antarctica in the late 1980s that the interest in ice microbiology was renewed. These authors studied ice core samples from the surface to 3,000 m depth (over 240,000 years old) using microscopy, cultivation and ^{14}C labeled substrates and found viable cells of different sizes and shapes at relatively low concentrations (10^2 to 10^4 cells ml^{-1}) (Abyzov et al. 1982, 1993, 1998a, 1998b). Interestingly, the fluctuations in the cell numbers were related for the first time to mineral particles content and climate changes with higher number of dust particles and cells occurring during colder periods. Later, a comprehensive study of glacier ice core samples from different geographic locations, ranging in age from 5 to 20,000 years, performed by Christner et al. (2000, 2002), showed that diverse bacteria can be successfully recovered. Furthermore, the same authors obtained isolates from 420,000-year-old Vostok accretion ice (Christner et al. 2001) and from a 750,000-year-old ice core (the most ancient to date) from the Guliya ice cap in Tibet (Christner et al. 2003b). These results point to the important issue of long-term microbial survival that is pertinent to previous terrestrial glaciation periods such as snowball Earth.

3.4 Trapped and dormant or actively metabolizing?

One question has drawn significant recent interest to the unique and vast glacial environment: Do microbial cells simply survive trapped frozen in glacial ice for hundreds of thousands of years or are they metabolically active and responsible for certain natural processes? Although this question is still unresolved, and probably varies for different sites and organisms, it is now assumed that at least some cells have survived due to their ability to carry on metabolic activity with or without reproduction or persist during long periods of dormancy by maintaining low levels of activity to support macromolecular damage repair.

It was first proposed by Price (2000) that thin liquid veins exist between ice crystals in ancient polar ice that can provide water, energy and carbon to microbial cells. Accordingly, the supply of organic carbon in the veins was calculated to be sufficient to maintain a small population (10–100 cells ml^{-1}) for up to 400,000 years. Additional data have accumulated in support of this hypothesis including microscopic observation of cells partitioned in veins and demonstrations of microbial activity at different subfreezing temperatures down to −39°C (Christner 2002b; Bakermans et al. 2003; Junge et al. 2003, 2004, 2006; Panikov et al. 2006). These findings raise another important question about the lowest temperature limits for life. Price and Sowers (2004) analyzed the temperature dependence of metabolic rates for microbial growth, maintenance and survival and found no evidence for a minimal temperature threshold for metabolism.

3.5 Methods for microbial analysis of snow and glacier ice

3.5.1 Sampling and decontamination methods

Studies of snow and glacier ice are challenging for several reasons. First, samples of snow and ice from non-polar mountainous regions are collected from remote, logistically difficult places. Sampling polar ice is even more difficult because the drilling process is slow and requires expensive equipment. In addition, the ice cores from Antarctica and Greenland, available for microbiological studies, were drilled long ago without taking precautions to prevent contamination. Given the recent increased interest in studying microbial populations immured in glacier ice, it is important to demonstrate that the microorganisms are truly indigenous to the corresponding ice sample. In order to obtain reliable and authentic results, different decontamination procedures have been developed, including physical removal of the outer surface and aseptic subcoring, and washing with sodium hypochlorite, ethanol, HCl, NaOH or H_2O_2, followed by gradual melting. Recently, Christner et al. (2005a) reviewed the existing decontamination methods and pointed out the importance of developing strict criteria for assessment of the authenticity of microbiological results obtained from ice core material on Earth and the implication for future studies with samples from Mars and other planets. Similar issues were discussed by other authors (Rogers et al. 2004, 2005; Willersev et al. 2004b) and at the workshop "Life in Ancient ice" in 2001. It has been strongly recommended that any future deep ice core drilling should be performed with the participation of microbiologists, who can use new chemical and fluorescent tracing techniques (Haldeman et al. 1995; Smith et al. 2000; Juck et al. 2005) to test the degree to which external microbes may penetrate the interior of the core during the drilling process and post-core relaxation. Finally, attention was drawn to the importance of the fluids used for drilling that may penetrate the ice potentially causing contamination and eventually changing the composition of the microbial population (Jepsen et al. 2006).

3.5.2 Methods for enumeration and morphological characterization

Microscopic studies using both fluorescent and scanning electron microscopy were the first to show the presence of microbial cells of varying size and shape in the deep Vostok ice (Abyzov 1993; Karl et al. 1999). They also helped to estimate the relatively low abundance of cells in clear glacier ice. These initial observations are impressive, since these cells had been preserved in the ice for hundreds of thousands of years. More recently, novel electron microscopic approaches combined with image analysis, as well as environmental scanning electron microscopy, atomic force microscopy and spectrofluorometry, have been used to study the size, shape and chemical nature of the ice mineral particles, detect autofluorescence and differentiate live and dormant microbial cells (Bay et al. 2005; Royston-Bishop et al.

2005; Vorobyova et al. 2005; Tung et al. 2006). Flow cytometry, combined with specific cell staining, has certain advantages and future potential for enumerating cells and determining cell size distribution in glacier ice and snow (Miteva et al. 2004; Miteva and Brenchley 2005; Liu et al. 2006; Priscu et al. 2007).

Viability tests are particularly important when studying frozen environments because the cells may be dead, or in dormant, viable but not culturable state, or partially damaged, although still retaining viability characteristics such as active membrane potential, cellular integrity and capacity for metabolic activity. Because very few microorganisms from natural samples can be cultivated, alternative methods for determining viability have been developed. They include various approaches for assessing cellular integrity and activity, such as specific cytochemical staining procedures, various molecular analyses of DNA, mRNA and detection of respiration (Keer and Birch 2003). Nevertheless, it is also possible that cells may be recalcitrant to the test method. Despite the availability of a range of cellular and molecular methods for assessment of bacterial viability, no one is universally appropriate and the application of several methods has been recommended.

3.5.3 Culture independent methods

It is well established that less than one percent of the microbial organisms present in most ecosystems can be cultivated (Amann et al. 1995). Thus, the composition of microbial communities cannot be accurately described without the use of culture independent methods, such as sequence analysis of 16S rRNA gene clone libraries, different *rrn* operon and whole genome fingerprint techniques, FISH and dot-blot hybridization, DNA microarray and real-time PCR of functional genes, and most recently metagenomic approaches. This significant array of molecular methods is increasing and creating new ways to study the microbial physiology, while linking phylogenetic diversity with potential metabolic functions within an ecosystem.

Molecular analyses of snow and glacier ice populations have been hampered by the availability of relatively small volume samples and the difficulties of lysing cells and obtaining DNA. This is particularly valid for deep glacier ice cores. In addition, the estimated extremely low number of microbial cells in most glacier ice cores, ranging from 10^2 to 10^4 ml^{-1}, limits the sufficient quantity and quality of extracted DNA for further cloning experiments. Our experience, using Vostok and Greenland ice core samples with low biomass, showed that it was difficult to obtain DNA yields that could be detected by conventional methods (unpublished). Such variable extraction efficiencies affect downstream experiments leading either to lack of PCR amplification or to PCR biases. These problems may be overcome using new technologies such as whole genome amplification. It is worth noting that using molecular methods based on PCR has the advantage of detecting DNA from live, dead, dormant or damaged

cells, thus giving a better representation of diversity. In addition, the constant low temperature in permanently frozen environments is believed to provide the ideal conditions for long-term preservation of nucleic acids due to significant reduction of the rate of molecular degradation (Willersev et al. 2004b; Willersev and Cooper 2005).

3.5.4 Cultivation methods

Considering the difficulty of cultivating organisms from environmental samples, it is not surprising that obtaining isolates by direct plating from frozen samples has been especially challenging. This very low culturability has been attributed to the extreme conditions in these environments, where cells may be dormant, damaged or simply not capable of growth on the media used. The big challenge is to elaborate new non-traditional cultivation methods. One rule of thumb when performing cultivation experiments has been to consider and mimic the in situ conditions, such as ambient temperature, water, nutrient and oxygen availability and possible geochemical gradients. Known cultivation strategies include extensive dilutions, use of natural substrates, miniaturization of the culturing process and consideration of slow growth.

Reports of successful recovery of isolates from frozen environments indicate that most researchers used typically low nutrient media such as R2A or diluted rich media, as well as several months long incubations at relatively low temperatures (Christner 2002a; Christner et al. 2003b; Zhang et al. 2002; Miteva et al. 2004; Xiang et al. 2005). Most authors observed much faster subsequent growth of the isolates suggesting that cells needed long periods for initial recovery of their growth abilities. It has been shown that initial liquid media incubations significantly increased the number and diversity of recovered isolates (Miteva et al. 2004). The anaerobic incubation of these liquid cultures was particularly efficient, which possibly permits dormant or damaged cells to avoid oxidative stress while recovering their ability to form colonies. Another approach, applied to glacier ice samples, is amendment of samples with medium, which possibly provides some unique compounds or microelements not present in the cultivation media (Skidmore et al. 2000; Zhang et al. 2002).

The selective fractionation of the microorganisms in a sample before cultivation may change the complexity of the recovered population. For example, eliminating larger cells by filtration may result not only in enrichment for small cells but possibly permit growth of cells otherwise inhibited by some metabolic products of larger cells. Such a filtration–cultivation strategy, applied to a GISP2 ice core sample, significantly increased the total number of cultured isolates, with higher diversity in filtered cultures dominated by Proteobacteria (Miteva and Brenchley 2005).

It is clear that no single approach can provide a complete unbiased picture of the diversity of microorganisms inhabiting a particular environment. By applying a polyphasic strategy in assessing diversity, the objectivity of a study may be significantly increased.

3.6 Diversity of bacteria in glacier ice

Results from studying glacier ice bacteria using a variety of methodological approaches have provided different yet complementary data on their morphological, phylogenetic, physiological and functional diversity.

3.6.1 Morphological diversity and size of glacier ice bacteria

The distribution of cells by size and shape is part of the structural diversity of natural ecosystems. Size and shape are important characteristics because bacterial cells depend on diffusion mediated nutrient import. Adaptation to changes in nutrient fluxes has led to a variety of morphological changes that may improve the surface to volume ratio, attachment, or motility. The issue of bacterial size and shape is relevant to ice microbiology because of the extremity of environmental factors imposed on the cells during their transport, deposition and long term persistence in frozen matrices. A significant morphological diversity is usually observed in glacier ice samples (Fig. 3.1). Abyzov et al. (2005) searched for common morphological features of microorganisms in Vostok ice. They found that morphological diversity in horizons with lowest cell concentration and low levels of organic carbon was restricted to micrococci and very short rods. In addition, some typical morphological features of the microorganisms were related to their taxonomy, indicating the presence of cyanobacteria, actinomycetes, fungi, *Caulobacter*-like and *Cytophaga*-like organisms. In this connection, a recent comprehensive review (Young 2006) discusses the selective value of bacterial shape and comes to the conclusion that cell morphology is a meaningful selectable trait that can even be charted in evolutionary scale. Different factors control cell size and shape by preventing/favoring evolution to a smaller or a larger size or to different shape variations.

After the postulation of the existence of liquid veins among ice crystals by Price (2000), the question of whether these veins could be occupied by bacteria was explored with aspect to cell size. Based on calculations and microscopic image analyses, Mader et al. (2006) demonstrated that the partitioning of bacterial cells in the narrow water-filled veins between ice grain boundaries depends strongly on their size. Only cells smaller than 2 µm would be predominantly excluded to the vein and possibly become metabolically active, while larger cells would stay trapped within ice crystals. These results are consistent with previous findings of very small bacterial cells in ice samples. Karl et al. (1999) found small coccoid (0.1–0.4 µm) and thin rod shaped cells (0.5–3 µm). Priscu et al. (1999) observed similar small cells in Vostok ice. Studies of a 3,043-m-deep Greenland ice core sample also revealed that the majority of the cells were smaller than 1 µm in diameter (Sheridan et al. 2003). The successful application of a filtration–cultivation procedure for recovery of these ultra-small cells from the same ice core samples resulted in obtaining phylogenetically diverse isolates that passed through 0.4 µm

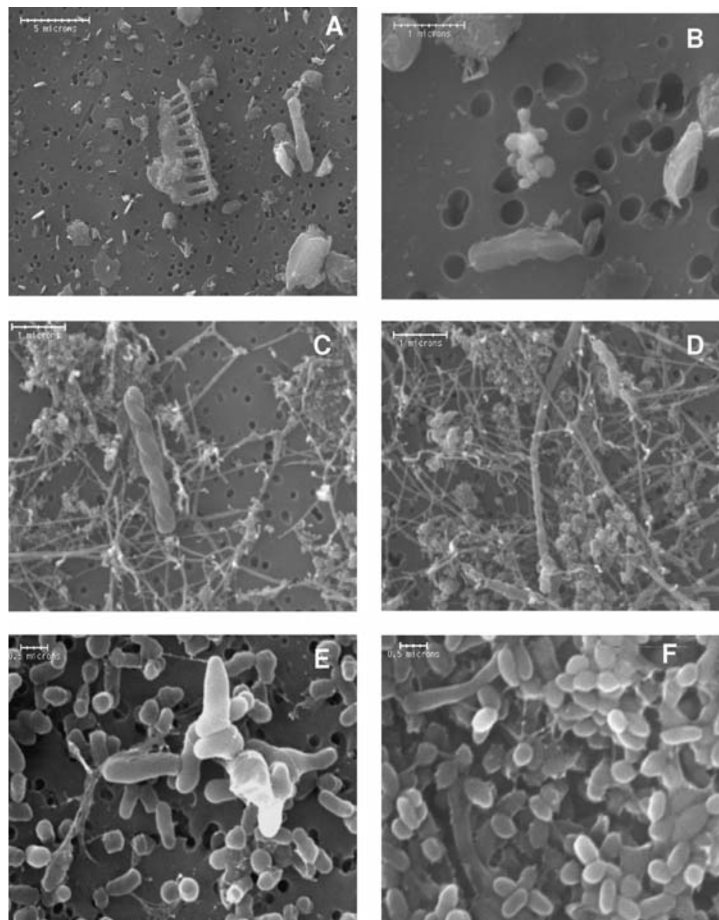

Fig. 3.1 Scanning electron microscopic (SEM) images of microbial populations, present in a 3,043-m-deep Greenland ice core sample: **A,B** small sized cells of different morphologies and a diatom fragment with smaller cells attached to its surface in the melted ice; **C,D** thin filamentous and small coccoid cells in a 50-day-old liquid medium culture along with thick filaments and cells with unusual structure; **E** glacier ice isolate (*Cryobacterium*) with pleomorphic cell shapes; **F** glacier ice isolate (*Sphingomonas*) embedded in extracellular material. (Photo credit: V. Miteva)

and 0.2 µm filters and retained their small cell sizes after re-cultivation (Miteva and Brenchley 2005). Many were phylogenetically novel and belonged to the class of ultramicrobacteria. Free-living ultramicrobacteria have been found to be dominant in many natural habitats and are considered to be better adapted to oligotrophic conditions and occupying micro-environments, e.g., the liquid veins in ice. The isolation of novel ultramicrobacteria may be useful for studying their unique physiological and genetic properties and help understand the ecological role of this distinct class of microorganisms, how they contribute to total biomass and geochemical cycling. Defining the theoretical limits of cell size is as important as

defining the lowest temperature limits of life with regard to the origin of life on Earth and the search for life elsewhere.

3.6.2 Bacterial diversity detected by culture independent methods

Because of the methodological constraints, mentioned earlier, very few studies have succeeded in characterizing the bacterial diversity directly in melted ice samples. Christner (2002a) constructed clone libraries using bacterial and universal 16S rRNA gene primers to PCR-amplify DNA extracted from several glacier samples from different geographic locations including a 500,000-year-old ice from Guliya, China, and found 16S rRNA gene sequences related predominantly to Proteobacteria. Similar dominance of proteobacterial sequences, followed in number by Cytophaga–Flavobacterium–Bacteroides (CFB), was detected in three 16S rRNA gene clone libraries constructed from different depths of the Malan ice core, Tibetan plateau (Xiang et al. 2004). Four of the sequences were suggested to represent new genera of Proteobacteria.

Clone libraries of 16S rRNA genes have been constructed not only directly from glacial ice samples but also from liquid cultures. Sequences related to Proteobacteria together with members of the *Thermus, Bacteriodes*, *Eubacterium* and *Clostridium* genera were found in anaerobically incubated cultures from the deepest ice from GISP2 (Sheridan et al. 2003). Among those were four possible representatives of new species, seven of novel genera and one possibly of a new order. Another molecular method, PCR of 16S–23S rRNA gene spacers, found useful application in this study for monitoring dynamic population changes in different enrichment cultures.

Denaturing gradient gel electrophoresis of 16S rRNA gene PCR products was used to assess the vertical profiles of bacterial population DNA structure in the Puruogangri ice core, Tibet (Zhang et al. 2006c). The high bacterial diversity, estimated by image cluster analysis and calculations of Shannon indices, was positively correlated to Ca^{2+} concentration, which is a good proxy of dust. Similar to the earlier observation of Abyzov et al. (1998a), these authors concluded that more bacterial species are deposited during cold periods, when there is intensified atmospheric circulation and increased airborne dust particles. Such conditions are suggested to decrease desiccation and increase the likelihood of bacterial survival.

3.6.3 Recovery and characteristics of bacterial isolates from glacier ice

Though highly challenging, the process of recovery of bacterial isolates from glacier ice has been fairly successful with a growing number of reports from new polar and non-polar sites. One common observation deserves special attention: Interestingly, different research groups have obtained isolates from geographically

diverse glacier samples following different decontamination and cultivation procedures yet, nevertheless, the dominant bacterial genera currently reported are remarkably similar, including representatives of high and low G+C Gram positives, Proteobacteria and CFB.

One of the first comparative studies of the bacterial diversity of six glaciers of different age and from different geographic locations, performed with the same sampling and analytical approach, led to several important conclusions:

(i) ice cores from low-latitude, high altitude glaciers contained more recoverable bacteria than polar ice cores presumably because of their close proximity to terrestrial biological sources of airborne material;
(ii) the number of recoverable isolates did not correlate with the age of the ice (5–20,000 years), consistent with episodic deposition of bacteria;
(iii) more isolates were related to sporeforming and non-sporulating Gram-positives than to Proteobacteria and CFB;
(iv) the relatedness of some isolates to species, previously isolated from other cold environments, suggested common features facilitating their survival (Christner 2002a; Christner et al. 2000, 2002).

In one way or another most subsequent reports on obtaining isolates from glacier ice have presented similar conclusions. Members of the Proteobacteria, high and low G+C Gram-positive bacteria and the CFB group were predominantly isolated from the deep Greenland ice and from several glaciers from the Tibetan plateau (Christner 2003b, 2005b; Miteva et al. 2004; Xiang et al. 2004, 2005).

At present, there are more than 700 16S rDNA sequences retrieved from glacier ice in the GenBank database, including nearly 400 from isolates. Although these isolates were obtained from different glacier ice core samples using diverse cultivation methods, their tentative grouping in the major bacterial phylogenetic groups shows similar distribution (Fig. 3.2) with dominance of high and low G+C Gram positives, followed by Proteobacteria and CFB. The complex cell wall of Gram-positive bacteria could

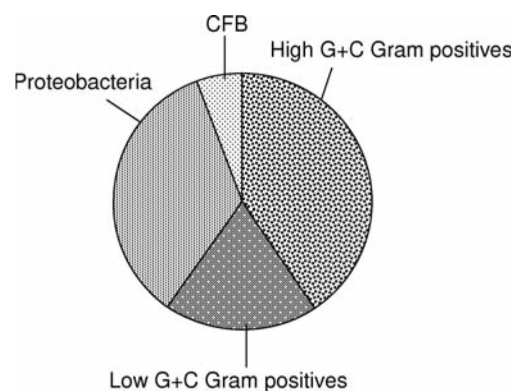

Fig. 3.2 Summary chart grouping 350 16S rDNA sequences of isolates from polar and non-polar glacier ice samples, available in the GenBank database

explain resistance and ability to survive. The most commonly identified genera are *Arthrobacter, Frigoribacterium, Rhodococcus, Exiguobacterium, Sphingomonas, Methylobacterium*, *Acinetobacter*, *Janthinobacterium, Chryseobacterium*.

The consistency of obtaining similar isolates suggests that these species may have features conferring resistance to freezing and extended survival under frozen conditions (Christner et al. 2000). Certainly, sporeforming bacteria may be expected to survive for thousands of years. Abyzov et al. (1998a) detected sporeforming bacteria microscopically but did not observe spores and even suggested that vegetative cells of sporeformers might be more resistant to long term low temperatures exposure. Bacilli were the most numerous isolates from all ice core samples from the Malan glacier, Tibet (Yao et al. 2003). Interestingly, they were recovered from Greenland basal ice only after a selective filtration and 18 months incubation, which was explained by possible elimination of some inhibitory substances or effect of other factors allowing spores to germinate (Miteva and Brenchley 2005). Finally, a recent study of the long term persistence of bacterial DNA in permanently frozen environment suggested that bacterial sporeformers may not be the most durable types of bacteria compared with non-sporeforming Actinobacteria (Willersev et al. 2004a).

Attempts were made to analyze the spatial distribution of culturable bacteria in the Himalayan East Rongbuk glacier and relate it to seasonal atmospheric circulations (Zhang et al. 2006b). Comparisons of four samples representing the four seasons showed highest number of culturable bacteria (7 cfu ml^{-1}) in the sample deposited during the premonsoon season, a period consistent with the highest atmospheric dust load. At the same time, although lower in number, the isolates from the sample corresponding to the monsoon season were more diverse, as explained with both marine and continental origin of the air masses.

The important conclusions from these studies based on 16S rDNA sequence analysis of glacier isolates are also valid for other terrestrial and marine frozen environments. The relatedness of isolates obtained from different in age and geographic location ice samples has been explained with a ubiquitous distribution of certain bacterial species in cold environments and similar adaptation strategies allowing them to survive in the cold. However, these could just be the species most easily cultivated. More studies are needed to understand the exact adaptation mechanisms that help them survive the extreme stress of low temperature, low water, oxygen and nutrient availability, and high pressure for extended time periods. Because 80% of Earth is cold with temperature below 5°C, it is reasonable to assume that adaptation to cold environments was evolutionarily necessary.

3.7 How different are bacteria in glacier ice and cryoconite holes?

Cryoconite holes are considered as eutrophic (nutrient-rich) habitats in the generally oligotrophic habitat of ice and melt waters (Margesin et al. 2002). One difference from glacier ice is that the microbial communities present in cryoconite holes are

more abundant and more diverse, containing a wide range of bacteria, algae, diatoms and metazoa, organized in relatively complex food webs. Another specificity is that photosynthetic cyanobacteria are the most active part of the communities, especially during the summer, and their activity is the driving force of cryoconite hole processes (Sawstrom et al. 2002; Stibal et al. 2006).

Cyanobacterial photosynthesis is an important source of organic carbon needed for the heterotrophic processes. Based on calculations of the photosynthetic rates (0.63–157 µg C l^{-1} h^{-1}), the abundance of organic debris and mineral material and the significant diversity of the cryoconite communities, it has been concluded that considerable carbon fixation and nutrient cycling occur (Sawstrom et al. 2002).

Previous studies have highlighted the physicochemical characteristics, structure and formation of cryoconite hole ecosystems. More recent studies relate microbial diversity with primary productivity and biogeochemistry and expand the studied sites to different geographic locations in Arctic, Antarctic and Alpine glaciers (Sawstrom et al. 2002; Margesin et al. 2002; Porazinska et al. 2004; Stibal et al. 2006). In these studies, microbial abundance and diversity is detected by microscopy and direct plate counts. The first molecular characterization of a cryoconite hole community was performed by Christner et al. (2003a) using Bacteria- and Eukarya-specific 16S/18S rRNA gene primers to analyze isolates and to construct clone libraries from total DNA extracted from an Antarctic cryoconite hole. Results showed the abundance of bacterial isolates and more representative sequences of cyanobacteria than algae in the clone libraries. Interestingly, metazoan sequences confirmed previous observations for metazoan life (nematode, tardigrade and rotifer species) present in cryoconite holes in Antarctica. Another advance in studying cryoconite hole communities was the description of a new species, *Pedobacter cryoconitis,* based on an detailed survey of heterotrophic microorganisms and their activity in an alpine glacier cryoconite (Margesin et al. 2003).

3.8 Diversity of bacteria in snow

Red-colored snow, mentioned in the writings of Aristotle, and by early Arctic explorers, appeared to be caused by the snow algae *Chlamidomonas nivalis*. During the twentieth century, numerous ecological studies of snow from high latitude and altitude sites have been published, mostly focused on snow algae. The interactions between snow as a hydrological reservoir and a source of nutrients and chemicals with microorganisms, plants and animals mediating nutrient cycling, have been reviewed previously (Jones 1999; Hoham and Duval 2001). More recently, the application of new molecular and cultivation methods to study bacterial diversity of snow has provided new data on the diversity of seasonally and permanently snow-covered ecosystems (Segawa et al. 2005; Amato et al. 2007).

The microbial content of mountainous and polar region snow is in the range 10^3 to 10^5 ml^{-1} (Carpenter et al. 2000; Skidmore et al. 2000; Sattler et al. 2001; Bauer et al. 2002; Uetake et al. 2006; Amato et al. 2007). Analysis of 16S rDNA

sequences from isolates and clone libraries from snow samples have shown significant prokaryotic diversity represented by all major bacterial phylogenetic groups, including cyanobacteria, with many related to known psychrophilic and psychrotolerant microorganisms. (Segawa et al. 2005; Liu et al. 2006; Amato et al. 2007). Eukaryotic ribosomal RNA sequences were also found. Seasonal variations in bacterial snow biomass have been explained by rapid bacterial growth during the summer (Segawa et al. 2005). Liu et al. (2006) also observed seasonal variations of the microbial communities and differentiated season-specific species in the snow of East Rongbuk glacier. Real time PCR was used to demonstrate dynamic quantitative changes of three typical psychrophilic isolates, *Cryobacterium psychrophilum, Janthinobacterium lividum* and *Variovorax paradoxus*, in mountain snow samples from Japan (Segawa et al. 2005). *V. paradoxus* appeared to be related to sequences previously detected in Greenland and Antarctic ice core samples and *J. lividum* was isolated from an Alaskan glacier, suggesting that these bacterial species are specialized members of the snow and biota in different parts of the world (Segawa et al. 2005). Interestingly, Carpenter et al. (2000) found 10–20% of all rRNA gene sequences obtained from Antarctic snow samples to be related to *Thermus–Deinococcus*-like organisms, known for their high radiation and desiccation resistance. These authors also reported evidence for low rates of bacterial DNA and protein synthesis in South Pole snow, which indicates metabolic activity at ambient subzero temperature.

Recently, bacterial communities from solid snow and snow meltwater from the high Arctic John Evans glacier were compared with basal ice and subglacial communities of the same glacier by T-RFLP of 16S rDNA products (Bhatia et al. 2006). Unexpectedly, very few common profiles were detected and distinct bacterial communities were found in each one of these physically and chemically different environments. While the potential exists for common bacterial types to be distributed throughout the glacial system, certain differences were suggested to develop under the selective pressure of the different physical and chemical conditions.

3.9 Novel bacterial isolates from glacier ice and snow

All authors found novel isolates and detected sequences from glacier ice and snow that are distantly related to known cultivated organisms. Phylogenetically novel sequences at species, genus and higher level were detected in 16S rDNA clone libraries from Malan ice core samples in Tibet (Xiang et al. 2004), a deep Greenland ice core (Sheridan et al. 2003), and snow in Japan and Tibet (Segawa et al. 2005; Liu et al. 2006). Moreover, novel isolates have been recovered from practically all studied glacier ice and snow samples and some of them have important physiological and biotechnological characteristics. Surprisingly, only a few of these novel isolates from glacier ice and snow have been officially described and validated (Table 3.1) compared, for example, to sea ice isolates. This inadequacy should be corrected because genetic and physiological studies of novel isolates, representing

Table 3.1 Novel bacterial species, isolated from glacier ice, snow and cryoconite holes

Organism	Origin	Taxonomic group	Type strain	16S rDNA sequence accession No.	Reference
Nocardiopsis antarcticus sp.nov.	Ice sheet Antarctica	Actinobacteria Actinomycetales	DSMZ 43884	X97885	Abyzov et al. (1983)
Flavobacterium xinjiangense sp.nov.	China No1 glacier	Bacteriodetes Flavobacteria	ZF-6	AF433173	Zhu et al. (2003)
Flavobacterium omnivorum sp.nov.	China No1 glacier	Bacteriodetes Flavobacteria	ZF-8	AF433174	Zhu et al. (2003)
Pedobacter cryoconitis sp.nov.	Alpine glacier cryoconite	Bacteriodetes Sphingobacteria	DSMZ 14825	AJ438170	Margesin et al. (2003)
Pedobacter himalayensis sp.nov.	Hamta glacier India	Bacteriodetes Sphingobacteria	HHS22	AJ583425	Shivaji et al. (2005)
Dyadobacter hamtensis sp.nov.	Hamta glacier India	Bacteriodetes Sphingobacteria	HHS11	AJ619978	Chaturvedi et al. (2005)
Flavobacterium glaciei sp.nov.	China No1 glacier	Bacteriodetes Flavobacteria	DSMZ 0499	DQ515962	Zhang et al. (2006)
Exiguobacterium indicum sp.nov.	Hamta glacier India	Firmicutes Bacillaceae	HHS31	AJ846291	Chaturvedi and Shivaji (2006)

previously uncultured clades, are important for understanding the role and function of these specific organisms in their original environment.

3.10 Functional diversity and microbial activity in glacier ice and snow

Functional diversity includes the ecological interactions among species (e.g., competition, predation, parasitism, mutualism, etc.), as well as ecological processes such as nutrient retention and recycling.

On one hand, the functional diversity of bacteria in glacier ice and snow may be characterized on the basis of the physiological characteristics of isolates. Most of them are psychrophilic and psychrotolerant, many are oligotrophic and pigmented. The majority are related to species known for their versatile metabolic properties and high resistance to stress associated with their environment, such as long-term freezing, freeze-thaw cycles, desiccation, solar radiation, and occupation of microniches. Nevertheless, it should be noted that the physiological characteristics

of cultured isolates do not necessarily reflect their performance in situ. At the same time, the possible role of microorganisms in ice biogeochemistry as a result of in situ metabolic activity is one of the most intriguing questions.

The supraglacial cryoconite holes provide a good example of glacial ice-related environments supporting active prokaryotic and lower eukaryotic communities performing real nutrient cycling, which can alter water chemistry and impact the geochemical weathering processes in the hole. Similar, more intense nutrient cycling is observed in some subglacier environments (see Chap. 4).

In the case of deep glacier ice, individual cells or small cell clusters rather than complex microbial communities might exist in the narrow liquid veins. The spatial limitations, along with other local factors, may restrict metabolic activity mainly to survival and life maintenance. The importance of deep glacier ice populations is that they provide a long-term reservoir of microbial diversity and organic carbon with a metabolic potential under suitable environmental conditions (Barker et al. 2006).

Because glacier ice records the history of past climates and preserves microbial cells chronologically, microbiological studies of glacier ice at different depths may provide important comparisons. As mentioned before, the early works of Abyzov (1993) found a correlation between the quantity of dust particles, microbial cells and cold climates. Later, similar correlations between high bacterial input, via atmospheric transport and cold climate conditions, revealed by $\delta^{18}O$ measurements were observed in Tibetan ice cores (Yao et al. 2003, 2006). These studies were based on the idea that the quantity and variability of microbial populations in different ice core layers most likely reflect prevalent climate, wind directions and other environmental events or human activities at the time of deposition. Estimates of the microbial concentrations in a 102-m-deep ice core from the Malan glacier, corresponding to four different temperature phases, were found to be negatively correlated to temperature and positively correlated with mineral particle concentrations. More organisms were deposited during colder climate with stronger and more frequent dust events, and fewer microorganisms were related to warmer climate with weaker and less frequent dust storms (Yao et al. 2006). This shows a possible future trend of linking detailed glacier ice microbiological records to the reconstruction of past climate changes.

At present, elemental and isotopic measurements of trace gases trapped in polar ice cores are the primary means of reconstructing past atmospheric compositions and climate changes. Studies of stable isotope fractionation also give insight into the possible role of microorganisms in ice geochemistry. For example, N_2O isotopic analyses of Sowers (2001) of 130,000 to 160,000-year-old Vostok ice core samples showed abnormally high N_2O values, characterized by high $\delta^{15}N$ and low $\delta^{18}O$ of N_2O that coincided with high dust and bacterial concentrations. These anomalies were suggested to be the result of in situ microbial N_2O production. If microbial metabolism occurs within ice, then interpretations of trace gas records would need to be reevaluated. Clearly, interdisciplinary studies of the functional diversity, metabolic activity and trace gas measurements are needed to provide a better understanding of microbial life in glacier ice and its role in biogeochemistry.

3.11 Conclusions

Many of the advancements and challenges to study microbial diversity in cold environments are similar to those of other habitats. Molecular techniques provide glimpses of what may be there, but we remain limited in our ability to cultivate and characterize the enormous diversity of prokaryotes and analyze their interactions and geochemical activity. Future investigations of glacier ice and snow habitats and new methods are needed to detect the microbial diversity in these low biomass habitats, study their potential microbial activity at subzero temperatures, determine the strategies allowing cell survival for hundreds of thousands of years, and cultivate and examine new organisms for the production of useful compounds.

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Chapter 4
Bacteria in Subglacial Environments

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4.1 Introduction

Glaciers exist where the annual temperature remains cold enough to allow snowfall to accumulate for an extended period of time and where conditions allow subsequent metamorphosis to ice. Glacial ice forms expansive continental ice sheets in the polar regions, (e.g., in Antarctica and Greenland), and at lower latitudes, ice fields (valley or alpine glaciers) and ice caps (if a volcano or mountain

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range is completely glaciated) exist globally at high altitude. Temperate glaciers comprise <4% of the glacial ice on the planet, but are important freshwater reservoirs and are often the sources for major rivers vital for irrigation, industry, and providing millions of people with drinking water. The Greenland and Antarctic ice sheets currently cover ~10% of the terrestrial surface (>1.5×10^7 km^2) and contain ~75% of the freshwater on Earth (Paterson 1994). The Antarctic ice sheet alone contains ~90% of the planet's ice and, if melted, would result in a sea level rise of ~65 m (The National Snow and Ice Data Center; http://nsidc.org/).

Evidence for liquid water in the basal zones of polar ice sheets (e.g., Ueda and Garfield 1970), the discovery of more than 140 subglacial lakes in Antarctica (Siegert et al. 2005), and expanding perspectives on the tenacity of life under cold conditions (e.g., Priscu and Christner 2004) have motivated research to determine if subglacial environments harbor viable microbial ecosystems. The presence of viable microorganisms has been documented in deep glacier ice (Abyzov et al. 1998; Christner et al. 2000, 2003, 2006; Miteva and Brenchley 2005), subglacial waters (Sharp et al. 1999; Mikucki et al. 2004), basal ice (Skidmore et al. 2000; Sheridan et al. 2003; Miteva et al. 2004; Foght et al. 2004), subglacial sediments (Skidmore et al. 2005), and subglacial lakes and accreted ice (Karl et al. 1999; Priscu et al. 1999; Christner et al. 2001, 2006; Gaidos et al. 2004). Here, we discuss current information on the diversity of environments for life at the base of glaciers and review what is known about subglacial ecosystems, which are inhabited by microbial assemblages that permanently exist at temperatures near the freezing point of water.

4.2 Liquid water in subglacial environments

4.2.1 Water and life

Water is required as a solvent in biochemical reactions, for mass transfer (i.e., the physical transport of molecules), and to establish electrochemical gradients (e.g., proton motive force). Nutrient-rich surface melt waters enter the basal zone of warm and polythermal alpine glaciers, and studies of subglacial outflow and sediments have provided key information on the biogeochemistry and microbial diversity of these environments (e.g., Sharp et al. 1999; Foght et al. 2004; Tranter et al. 2005). The occurrence of subglacial water under polar ice sheets was first discovered in Antarctica (Ueda and Garfield 1970) and was recently documented in Greenland (Anderson et al. 2004). If liquid water and chemical (inorganic or organic) energy sources are available, subglacial microbial habitats may harbor species adapted to one of the most extreme environments in the biosphere.

4.2.2 Liquid water in Arctic and Alpine subglacial environments

Ice masses may be classified into three categories based on the temperature regime of the ice: cold, polythermal, and temperate (Paterson 1994). Cold-based ice masses consist of ice with temperatures below freezing throughout, and liquid water is only present in the veins between ice crystals (Nye 1992), with no significant water layer at the glacier bed. Alpine glaciers in the McMurdo Dry Valleys of Antarctica and smaller glaciers in the Canadian High Arctic are examples of cold based ice masses. Polythermal ice masses are largely found at latitudes above the Arctic Circle, whereas temperate ice masses (i.e., ice is at the melting point from the surface to the base) are located at low and mid-latitudes typically as valley glaciers. Sections of polythermal ice masses are frozen to the bed especially beneath the thinner margins and termini. However, the bed is temperate beneath the thicker, inner zones of the ice mass. Where the basal ice is at the melting point, melting occurs due to geothermal heating and from friction, and there is a layer of liquid water between the basal ice and the substrate (Alley et al. 1997). Temperate-based ice masses have ice at the bed which is at the pressure-melting point throughout and thus the entire glacier bed is wet based. The surface of the ice remains snow covered during the winter and, during the summer ablation season, surface meltwater is delivered to the glacier bed via crevasses and moulins from surface snow and ice melt in both polythermal (Skidmore and Sharp 1999) and temperate ice masses (Nienow et al. 1998).

A number of different subglacial drainage system configurations are possible where the glacier bed is temperate, e.g., linked cavity systems, canals, sheetflow and channelized drainage (for reviews of temperate glacier hydrology and subglacial processes, see Hubbard and Nienow 1997; Fountain and Walder 1998; Clarke 2005). Where basal water is present, microbes have been documented in the subglacial environment at both temperate glaciers (Sharp et al. 1999; Foght et al. 2004; Skidmore et al. 2005) and polythermal glaciers (Skidmore et al. 2000; Wadham et al. 2004; Bhatia et al. 2006). Tranter et al. (2005) review how subglacial hydrological flowpaths control the connectivity of chemical weathering environments, access to atmospheric oxygen, and the redox potential (Eh) of the environments that microbes colonize.

4.2.3 Discovery of subglacial water beneath Antarctica's ice sheets

The Antarctic polar plateau has a mean annual surface temperature of −37°C and was first explored in an expedition led by Robert Falcon Scott in 1902. During the 1950s, geothermal heat flow models were developed that predicted an increase in temperature with depth in the ice, implying that, if the ice sheet is thick enough, then the basal ice in contact with the bedrock is at the melting point of water. Theoretical predictions, together with seismic measurements of the ice sheet thickness,

supported the idea that liquid water existed at the base of the ice sheet in central portions of the Antarctic continent (Zotikov 2006). More than a decade passed before the existence of water beneath Antarctica's ice sheets was confirmed.

The first deep drilling operation in Antarctica was initiated in 1966 at Byrd Station. An ice core was successfully recovered at Byrd Station and bedrock was reached at a depth of 2,164 m below the ice surface in January 1968. The drilling team did not expect to encounter liquid water at the ice-bedrock interface. However, water entered the borehole and raised the drilling fluid level 55 m. Due to the resulting "hydrostatic unbalance" and movement of the aqueous basal fluid into colder portions of the borehole, further coring and collection of subglacial materials were suspended to prevent loss of the drill by freezing (Ueda and Garfield 1970; Zotikov 2006). While an ice core was retrieved that yielded important information on the climate history of West Antarctica and the nature of ice in deep portions of the ice sheet, the implications of this finding for life in Antarctic subglacial environments was not fully realized for another 30 years.

4.2.4 Liquid water under the Greenland ice sheet

Several ice core drilling projects have drilled to bedrock in Greenland since the mid 1960s: Camp Century, the Greenland Ice Sheet Project (GISP and GISP2), the Greenland Ice Core Project (GRIP), and the North Greenland Ice Core Project (NGRIP). In the deepest portion of these ice cores, referred to as "silty ice", the ice contains numerous organic and inorganic inclusions, which are thought to originate from the subglacial environment rather than from aeolian deposition in snowfall (Gow and Meese 1996). Anderson et al. (2004) encountered pink-colored basal water when drilling at NGRIP in 2003, which entered the borehole and raised the fluid level 45 m. Even though ice sounding radar provided no evidence for liquid water at the base of the ice sheet, the borehole temperature profile indicated that the basal ice is near the pressure melting point of water under the NGRIP site. Importantly, the discovery of basal water at NGRIP revealed that liquid water also exists at certain locations at the bed of the Greenland ice sheet and wet-based conditions may be more prevalent than previously thought.

4.3 Subglacial lakes

4.3.1 Antarctic subglacial lakes

In the late 1950s, pilots with the Soviet Antarctic Expedition noted flat depressions on the polar plateau, which they used for navigation and referred to as "lakes" (Zotikov 2006). Oswald and Robin (1973) used radio echo sounding (RES) to survey portions of East Antarctica and provided evidence for the widespread existence of water under the ice sheet by identifying 17 subglacial lakes. Robin et al. (1977) conducted many

flights in the vicinity of Vostok Station and discovered that a very large lake (Subglacial Lake Vostok) exists beneath the Russian base, which was further supported by satellite radar altimetry (Ridley et al. 1993) and seismic data (Kapista et al. 1996).

In the last ten years, RES data have been used to identify 141 subglacial lakes in East Antarctica and 4 in West Antarctica (Siegert et al. 2005). Most (60%) of the subglacial lakes identified are <200 km from an ice divide and exist under 2,500–4,000 m of ice (Dowdeswell and Siegert 2002). The rapid transport of large amounts of water (1.8 km^3 of water over 16 months) beneath the Antarctic ice sheet through subglacial channels has also been documented (Wingham et al. 2006), providing evidence for hydrological connectivity between subglacial lakes. Thus, subglacial lakes may not be isolated systems and it is possible that periodic floods could transfer microorganisms, carbon, and nutrients between lakes. Estimates indicate that the volume of known Antarctic subglacial lakes is approximately 10,000 km^3 (Dowdeswell and Siegert 2002). This volume represents ~15% of all freshwater on non-glaciated continents, enough to cover the whole Antarctic continent with a uniform water layer thickness of ~1 m.

4.3.2 Subglacial Lake Vostok

Of all the subglacial Antarctic lakes identified to date, Subglacial Lake Vostok (hereafter referred to as Lake Vostok) is by far the largest with a surface area >14,000 km^2, volume of 5,400 ± 1,600 km^3, and maximum depth of ~800 m (Kapista et al. 1996; Studinger et al. 2004). The lake consists of a northern basin (water depth of ~500 m) and a larger southern basin (~800 m), which are separated by a bedrock sill (Studinger et al. 2004). The variation in ice sheet thickness between the north (~4,200 m) and south portion (~3,900 m) of the lake produces a 0.3°C difference in the pressure melting point of water. This gradient results in glacial ice melting into the lake in the north, and refreezing (i.e., accretion) to the bottom of the ice sheet in the south, which has important repercussions for circulation and vertical mixing within the lake (Siegert et al. 2001).

The lake water of Lake Vostok has not been directly sampled. However, molecular, microbiological, and biogeochemical analysis of the basal portion (3,539–3,623 m) of an ice core drilled at Vostok Station has provided valuable data to predict limnological conditions within surface waters of the lake (Karl et al. 1999; Priscu et al. 1999; Christner et al. 2001, 2006; Bulat et al. 2004). Based on ice sheet flow (Bell et al. 2002) and the concentration of particle inclusions within the accretion ice (Souchez et al. 2002; Royston-Bishop et al. 2005; Christner et al. 2006), ice cores recovered between 3,539 and 3,609 m (referred to as Type I accretion ice) are inferred to have formed from lake water that accreted in a shallow embayment in the southwestern portion of the lake, whereas accretion ice between 3,610 and 3,623 m (Type II accretion ice) is much cleaner and probably formed over the deep central portion of the lake's southern basin (de Angelis et al. 2004). Thus, the accretion ice profile represents a transect of surface waters from the shallow depths in the east to that over deep waters in the vicinity of Vostok Station.

Heterotrophic activity has been reported within melted samples of the accretion ice (Karl et al. 1999; Christner et al. 2006) and amplification and sequencing of small subunit (16S) rRNA genes from extracted DNA and isolated cultures imply the lake is inhabited by bacteria related to the Proteobacteria (alpha, beta, gamma and delta subdivisions), Firmicutes, Actinobacteria, and Bacteroidetes (Priscu et al. 1999; Christner et al. 2001, 2006; Bulat et al. 2004). Using a quantitative decontamination protocol (Christner et al. 2005; Fig. 4.1), Christner et al. (2006) examined 20 depths in the Type I and II accretion ice (between 3,540 and 3,623 m) and predicted that the average concentration of organic carbon, prokaryotic cells, and total dissolved solids in surface waters of the shallow embayment and open lake are 86 and 160 µM, 150 and 460 cells ml^{-1}, and 1.5 and 34 mM, respectively. The input of organic carbon from the ice sheet has been estimated to be insufficient to support reproductive growth of the entire lake community, and a sustained ecosystem would likely require a supplemental chemical energy source (Christner et al. 2006). Supplemental energy needed to support a sustained chemolithotrophic-based ecosystem (Fig. 4.2) may originate from sulfide and iron mineral substrates in subglacial debris (e.g., Bottrell and Tranter 2002; Tranter et al. 2002) entering the lake (Christner et al. 2006) and perhaps by geothermal input from deep faults within the bottom of the lake (Bulat et al. 2004).

4.3.3 Sampling Antarctic subglacial lakes

The exploration and direct sampling of Antarctic subglacial lake environments will require a substantial logistical effort (e.g., Priscu et al. 2003; Inman 2005; Siegert et al. 2007) and the implementation of protocols that introduce minimal chemical and microbial contamination to the pristine environment. The deployment of in situ observatories to measure the physical and chemical properties of the lake environment over spatial and temporal scales has been suggested as a prudent first step in the exploration of subglacial lake environments (Priscu et al. 2003). However, to study the physiology and diversity of microbial life in subglacial lake environments, it will be necessary to retrieve lake water column samples and sediments and return them to the surface while maintaining in situ pressures and temperature. The technological and logistical issues, together with concerns for environmental protection, make subglacial lake environments exigent systems for scientific study (National Research Council 2007). Despite these challenges, several projects are currently underway to drill into and sample subglacial lakes located beneath the ice sheet in East and West Antarctica.

The deepest ice core borehole at Vostok Station (designated borehole 5G) was drilled to a depth of 3,623 m in 1998 by a coordinated Russian, French, and American effort. Owing to concerns of contaminating the lake environment, drilling was terminated in a zone of accretion ice ~120 m above the water–ice interface. A solely Russian drilling effort began again in 2006 with

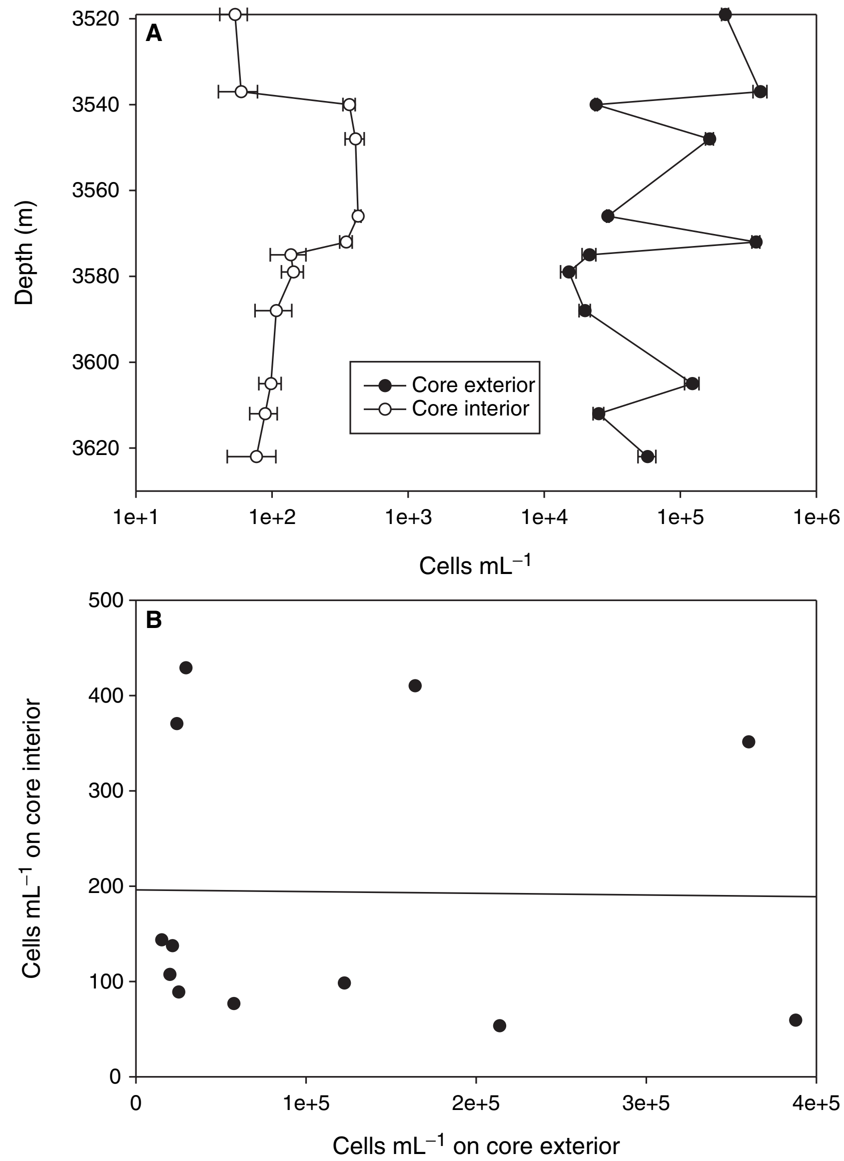

Fig. 4.1 **A** Concentration of cells on the exterior and interior of ice cores from the bottom ~100 m (i.e., accretion ice) of the Vostok 5G ice core. **B** Cell densities on the inside versus the outside of the ice core are statistically different ($r = 0.016$) and the data do not co-vary with depth (paired *t*-test, $p < 0.050$). The line is a regression plot of the data points. These data are not consistent with cells penetrating the ice core as a result of drilling (i.e. through microfractures in the ice) or via the drilling fluid, supporting the notion that bona fide cell concentrations were detected within the ice core interior. For extensive detail on the decontamination protocol and the cell enumeration method, see Christner et al. (2005, 2006)

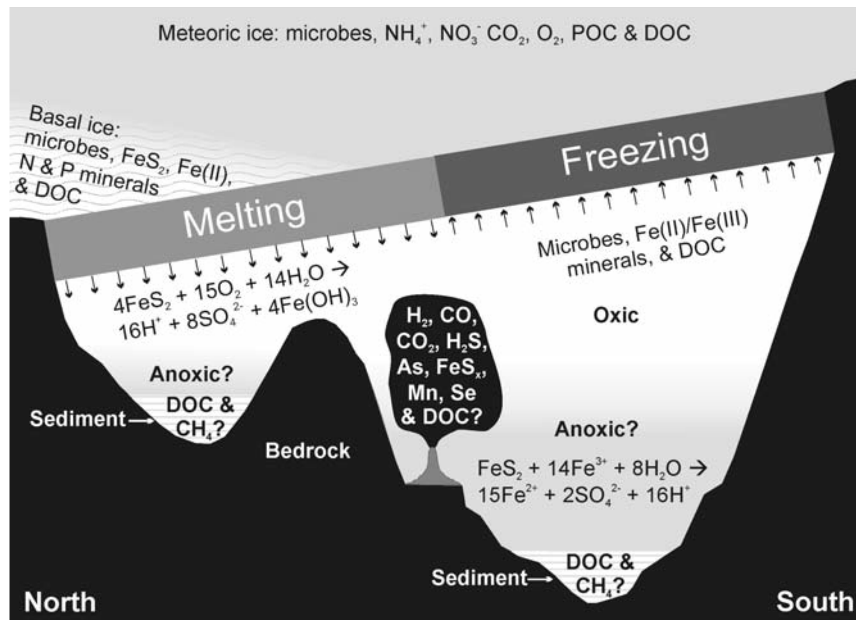

Fig. 4.2 Hypothetical scenario for chemically-driven biogeochemical reactions that could be used for bioenergetics in Lake Vostok. Inputs to the system (northern portion of the lake; see text) are through the melting of basal ice, which contains crushed sulfide and iron minerals and organic material from the bedrock, and glacial ice, which provides a constant supply of oxidants (O_2 and NO_3^-), nutrients, and organic material. Microbes, minerals, and organic carbon are removed from the lake via the accretion ice (southern portion of the lake). Shown are oxic and anoxic chemolithotrophic reactions (i.e., metal sulfide oxidation) that have been documented in Alpine and Arctic subglacial environments (Bottrell and Tranter 2002; Tranter et al. 2002; Wadham et al. 2004). Fault vents may be present in the shallow embayment of the lake (Bulat et al. 2004), which could introduce significant amounts of thermal energy, geochemical energy, and organic carbon to the lake. If biotic and/or abiotic oxygen sinks exist in the lake, then the deep waters and sediments would be expected to be anaerobic. POC = particulate organic carbon; DOC = dissolved organic carbon

the removal of an additional 27 m of ice core (3,624–3,651 m; V. Lukin, personal communication). The Russian Antarctic Expedition plans to mechanically drill another ~75 m, replace the existing fluid in the bottom of the borehole with an "ecologically clean drilling fluid", and then penetrate the lake with a thermal drill (Pomelov 2003). Lake water entering the borehole will eventually freeze and this material will be drilled and recovered for analysis in a subsequent field season.

An international collaboration of scientists is also preparing to drill into a subglacial lake in West Antarctica, named Ellsworth Subglacial Lake. The Lake Ellsworth Consortium (Siegert et al. 2007) have proposed to enter this lake by hot water drilling through the overlying 3,400 m of ice, lower an instrument probe to measure the biological, chemical and physical characteristics of the

lake water and sediments, and then return water and sediment samples to the surface for analysis. Hot water drilling (e.g., Fig. 4.3C–E) uses melted snow as the water source, which is heated to 80–90°C and pumped out of the drill tip. The surface snow naturally turns into firn and then glacier ice through burial and

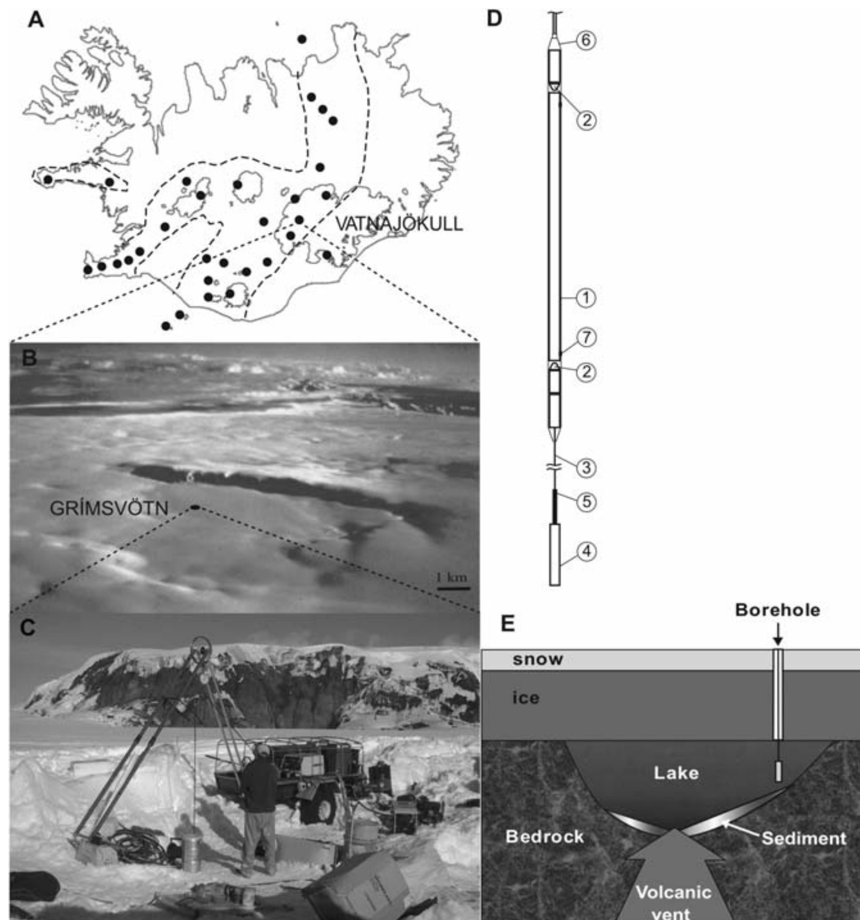

Fig. 4.3 **A** Location of zones of volcanic activity (*dotted lines*), volcanoes (*dots*), and major glaciers (including Vatnajökull) on Iceland. **B** Aerial view of the Grímsvötn caldera and ice shelf from the northwest. The approximate location of the drilling and sampling site. **C** Drilling operation on the Grímsvötn ice shelf. Modified from Gaidos et al. (2004). **D** Gas tight and water tight sampling device consists of a 400-ml sample chamber (*1*), open at both ends; a closure mechanism (*2*), comprising conical end-plugs driven by coil springs and a triggering mechanism consisting of a spring-loaded ratchet held closed by tension on a line (*3*) below which is suspended a weight (*4*) on a shock cord (*5*) and a load-bearing frame (*6*) which runs from the attachment point to the deployment cable to the triggering device beneath the sample bottle. The sample is retrieved using the sampling port (*7*). Modified from Gaidos et al. (2007). **E** Schematic cutaway diagram of the subglacial caldera lake, with borehole and water sampler. (Not to scale)

compaction and is eventually melted at the base of the ice into the lake. Thus, the hot water drilling is simply speeding up the natural process of transferring snow into the lake. Heating the water to >80°C in the drilling system likely causes macromolecular degradation, and when combined with inline filtering, the number of viable cells introduced into the lake via drilling can be minimized. Siegert et al. (2007) argue that Lake Ellsworth is a good initial candidate for subglacial lake exploration due to the lake's small size (10 km length × 10 km width × 250 m deep; lake volume ~25 km^3), comparable pressure and temperature to other subglacial lakes, accessibility to existing infrastructure (United Kingdom and United States field operations), and lower ice surface altitude (compared with the East Antarctic Ice Sheet), which reduces issues associated with human and mechanical performance at higher altitudes and colder temperatures. The sampling efforts described above are sure to make the next decade a very exciting time for subglacial lake research. Current investigations and techniques for sampling ice-entrapped lakes occurring elsewhere provide a training ground for testing sampling technology and an indication of the microbial species and metabolic lifestyles that may exist in the numerous lakes beneath the Antarctic ice sheet.

4.3.4 Subglacial caldera lakes

Smaller subglacial lakes have also been found in volcanic calderas beneath Icelandic ice caps. Hydrothermal vent activity results in melting of the overlying ice and filling of the subglacial caldera with water to form a subglacial lake (Fig. 4.3A). The overlying ice on Vatnajokull is ~250–300 m thick and water depths in the lakes vary from 20–140 m (Gaidos et al. 2004). These lakes typically drain catastrophically beneath the ice resulting in an outburst flood, which is termed a jokulhlaup. The draining and filling cycle for these subglacial lakes over the past four decades has ranged from one to less than ten years (T. Thorsteinsson, personal communication).

Two Icelandic subglacial lakes, one in the Grímsvötn Caldera (Fig. 4.3B) and one in the western Skaftárkatlar Cauldron, have been accessed via hot water drilling of a borehole into the lake from the ice surface. Analysis of the water column samples demonstrated that Grímsvötn Subglacial Lake contains a viable microbial assemblage (Gaidos et al. 2004). Molecular analysis of the Grímsvötn water samples indicates that the lake assemblage is distinct from the assemblages of organisms in the borehole water (before lake penetration) and overlying ice and snow. Sequencing of selected 16S rRNA gene fragments amplified from DNA extracted from the lake water revealed phylotypes with high identity to psychrophilic organisms (Gaidos et al. 2004). The uptake of ^{14}C-labeled bicarbonate in dark, low-temperature incubations of lake water samples indicates the presence of chemoautotrophs (Gaidos et al. 2004).

4.4 Adaptations, bioenergetics, and cosmopolitan genera in subglacial environments

4.4.1 Molecular adaptations for survival in icy environments

Microorganisms in subglacial environments are susceptible to the physical and osmotic stress associated with freezing and thawing during regelation. Ice crystals initially form in the extracellular phase and solute exclusion draws water from the cell, damaging the integrity of the cell membrane. Many plants, animals, and microbes adapted to freezing conditions therefore produce compatible solutes (e.g., proline, betaine, glycine, and trehalose) which reduce the shock of an osmotic imbalance (Tanghe et al. 2003). Thermal hysteresis antifreeze proteins may also be important to survival in subglacial environments, as these cold-induced proteins function to prevent damage initiated by intra- and extra-cellular ice crystal formation.

It is also possible that microorganisms entrapped within the solid basal ice of a glacier may remain metabolically active. This is an important transient phase in the subglacial environment due to melting and refreezing of water at the glacier bed.

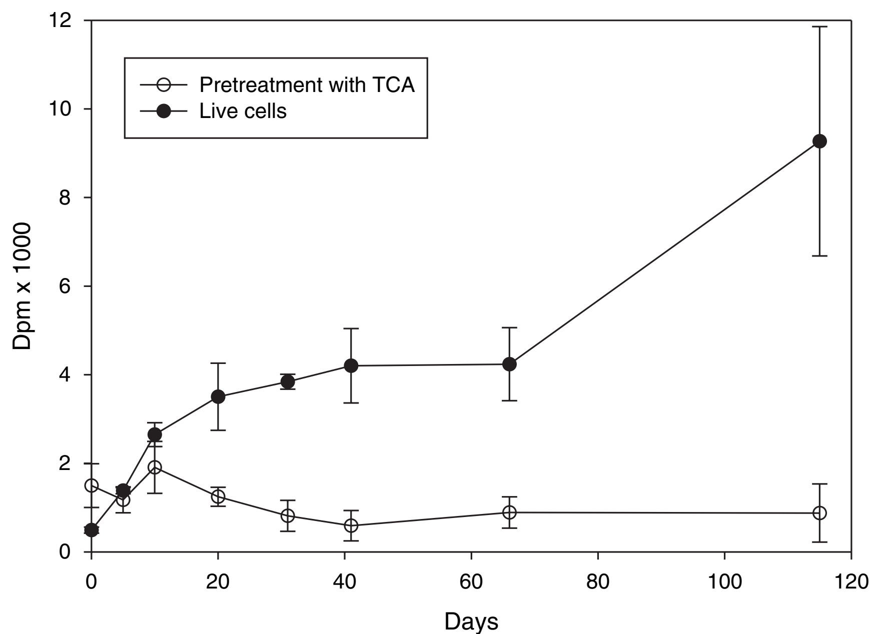

Fig. 4.4 Incorporation of [^{3}H]thymidine into TCA-precipitable material by frozen suspensions of *Sporosarcina* species TGTB5-5B at −15°C. The experiment was conducted for 115 days in a media with a total dissolved solid concentration of ~10^{-4} M. Freezing conditions and experimental details were as described by Christner (2002)

During freezing, cells are excluded into the interstitial liquid veins that exist between three grain boundaries in polycrystalline ice (Mader et al. 2006), similar to chemical impurities in the ice (Nye 1992). Figure 4.4 shows the incorporation of thymidine under frozen conditions (−15°C) by a psychrophilic bacterium isolated from the basal ice of Taylor Glacier, Antarctica. Based on these results and because the in situ temperature of the basal ice at Taylor Glacier is −17°C, it is possible that bacteria entrapped in basal ice metabolize in the aqueous veins between grain boundaries (e.g., Price 2000).

Determining the molecular and physiological adaptations of microbial life in subglacial environments will yield information that is vital for understanding the evolution and ecology of microorganisms inhabiting the deep cold biosphere, and may well yield biotechnologically relevant information for the identification of enzymes with improved cold-active properties.

4.4.2 Resistance to high oxygen concentrations

The precursor to glacial ice is firn, which is composed of granularized and compacted snowflakes. As the overlying snowfall accumulates and applies pressure, firn crystals glide and bond to other crystal planes, effectively squeezing intervening air spaces together into ice-entrapped bubbles that comprise ~10% (by volume) of the ice (Paterson 1994). At increasing depth, the gas bubbles are compressed into clathrate hydrates, which consist of a cage of water molecules around a gas molecule and are only stable at high pressure and low temperature.

In Lake Vostok, glacial ice melts into the northern portion of the lake, continually introducing air clathrates to the system. The accretion ice is essentially gas-free relative to the overlying glacial ice and very high (2.51 kg^{-1} water) dissolved gas levels have been estimated to exist in Lake Vostok and the dissolved oxygen concentration has been predicted to be ~50 times higher than air-equilibrated water (McKay et al. 2003). Metabolic activity at high oxygen concentrations inevitably results in the formation of hydrogen peroxide and superoxide by O_2-utilizing enzymes, which damages proteins, lipids, and DNA. Microorganisms capable of tolerating oxygen possess detoxifying enzymes such as catalase and superoxide dismutase. Resistance to oxidative stress is important for surviving freezing (Tanghe et al. 2003), and this may also be a vital trait for survival at the high oxygen concentrations predicted in Lake Vostok.

4.4.3 Survival under oligotrophic conditions

The low organic carbon concentration in the subglacial environment may select for heterotrophic species capable of surviving in oligotrophic conditions and extended periods of no growth. Low concentrations of dissolved organic carbon have been

reported in Lake Vostok (17–250 µM, estimated from the accretion ice: Priscu et al. 1999; Karl et al. 1999; Christner et al. 2006) and in subglacial meltwaters and basal ice in the high Arctic (8–100 µM: Skidmore et al. 2000; Barker et al. 2006) and glacial meltwaters of the Canadian Rocky Mountains (13–64 µM: Lafreniere and Sharp 2004). Miteva and Brenchley (2005) report that cells <0.1 μm^3 dominated the populations of cells in the GISP2 ice core and they also cultivated a number of bacterial isolates that are close phylogenetic relatives of known "ultramicrobacteria". *Sphingopyxis alaskensis* (formerly *Sphingomonas alaskensis*) is the most extensively studied ultramicrobacterium (e.g., Eguchi et al. 2001) and this microorganism is highly resistant to environmental stress (i.e., heat, hydrogen peroxide, and ethanol), is capable of growth at low organic carbon concentrations (~65 µM dissolved organic carbon), and maintains a small cell size (0.03–0.07 um^3) even when it is exposed to rich culture media.

Particle analysis, using flow cytometry, of two glacial and one accretion ice section from the Vostok core, revealed a pattern that contrasts the data of Miteva and Brenchley (2005). Total particles densities in the Vostok ice samples ranged from 4.4×10^4 to 1.9×10^5 ml^{-1} in glacial ice from 2,334 and 1,686 m, and 1.2×10^4 ml^{-1} in accretion ice from 3,612 m (Table 4.1). These data corroborate published data (Christner et al. 2006) that the accretion ice has lower total particle densities than the overlying glacial ice. The mean and median sizes of the biotic particles were 3.8, 3.4 and 4.6 µm for these same cores. The large biotic particles detected may, however, be illusory, representing cells that have aggregated or are attached to larger abiotic particles. We have observed cellular aggregation when viewing SYBR Gold stained ice core samples with epifluorescence microscopy and have shown with SEM that many of the bacteria in Vostok accretion ice are attached to particles of lithogenic origin (Fig 4.5; Priscu et al. 1999). A better representation of biotic particle size may be given by the mode. The modes for the ice cores from 1,686, 2,334 and 3,612 m were 0.7, 3.5 and 0.7 µm, respectively, which were 20%, 93%, and 15% of the biotic population means. The majority of the particles from the Vostok ice cores were abiotic with biotic counts ranging from only 2.5 to 19.2% of the total particles.

Table 4.1 Particle size distribution (mean, median, mode; µm) and particle density (particles ml^{-1} ice; percent of total particles) for the biotic and abiotic fractions at three depths in the Vostok ice core. The size distributions were determined on the raw data and from Weibull distributions (parentheses)

Depth (m)	Mean (µm)	Median (µm)	Mode (µm)	Particles ml^{-1}	Particles ml^{-1} (% of total)
1686					
Biotic	3.41 (2.45)	2.27 (2.04)	0.74 (1.02)	4,610	2.5
Abiotic	0.67 (0.60)	0.58 (0.59)	0.52 (0.56)	181,536	97.5
2334					
Biotic	3.81 (3.74)	3.43 (3.36)	3.54 (2.41)	8,512	19.2
Abiotic	2.65 (2.63)	2.26 (2.22)	0.51 (1.18)	35,804	80.8
3612					
Biotic	4.60 (4.60)	3.52 (3.51)	0.68 (0.72)	1,278	10.3
Abiotic	2.76 (1.24)	1.27 (1.03)	0.53 (0.52)	11,090	89.7

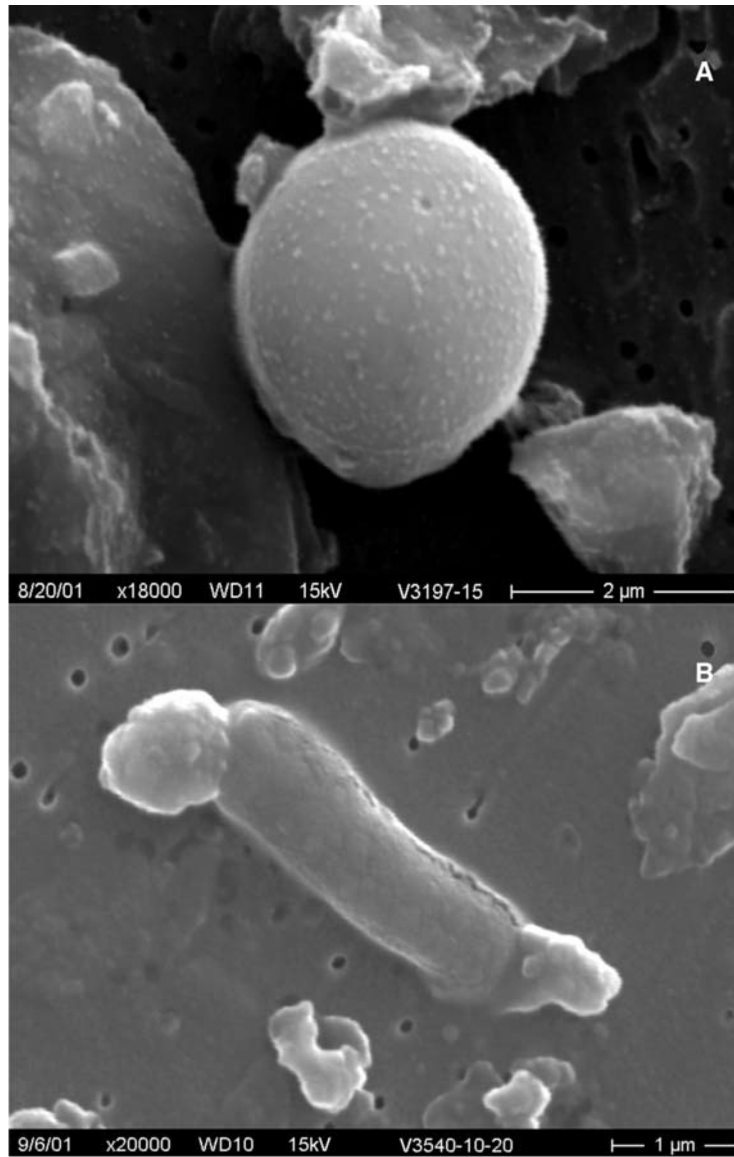

Fig. 4.5 SEM images showing bacterial cells aggregated with non-cellular material in Vostok cores from 3,197 m (**A**; glacial ice) and 3,540 m (**B**; accretion ice). Cryogenic SEM (JEOL-6100 SEM) and energy dispersive spectrometry (EDS) were used to image and analyze particles; EDS revealed that the non-cellular material was organic in origin

The ability to efficiently scavenge nutrients (i.e., small cells with high surface to volume ratios) and endure stress is likely to be valuable in terms of extended survival in all extreme environments, including those that exist deep beneath glacier ice. However, it is also possible that alternative energy sources and survival strategies are important in subglacial ecosystems.

4.4.4 Chemolithotrophy in subglacial environments

Glacier flow results in the comminution of mineral matrices in the underlying bedrock, releasing carbonate, sulfide, iron, and organic matter into the subglacial environment (Tranter et al. 2005). Geochemical evidence implies that the microbial oxidation of metal sulfides in glacial flour occurs in oxic and anoxic glacier bed environments (Bottrell and Tranter 2000; Tranter et al. 2002). Under oxic conditions, sulfide oxidation and heterotrophic activity will consume oxygen, eventually creating anoxia. Sulfide oxidation with Fe(III) as an oxidant can occur in the absence of oxygen, and sulfate reduction and methanogenesis are potential biogeochemical pathways for the anaerobic mineralization of organic matter. Thus, glacial physical processes may be sufficient to supply an energy source to microbes existing in subglacial environments.

Christner et al. (2006) suggest that sulfide and iron oxidation could serve as the basis for a chemolithotrophic food web in Lake Vostok (Fig. 4.2). Electron acceptors such as oxygen and nitrate are continually introduced into the lake through the melting of basal ice, and SO_4^{2-} is produced through the chemical weathering of sulfide minerals in the bedrock. There has been speculation regarding geothermal energy input from high-enthalpy mantle processes or seismotectonic activity (Bulat et al. 2004), which could introduce significant amounts of thermal energy and support an ecosystem similar to those found in deep-sea hydrothermal vents. However, since documented glaciological processes could supply subglacial lake ecosystems with nutrient and redox couples for microbial metabolism, the search for viable subglacial communities need not be exclusive to environments with geothermal input.

4.4.5 Do subglacial environments harbor endemic microbial species?

Microbiological investigations of glacier environments (i.e., cryoconite holes, subsurface glacial ice, accreted ice from Lake Vostok, subglacial sediment, and subglacial outflow and streams) in polar and non-polar locations indicate that strong phylogenetic relationships exist between bacteria from geographically distant environments (e.g., Priscu and Christner 2004). In plants and animals, allopatric speciation can occur when a geographically isolated population diverges from the parent population, resulting in the emergence of a genetically distinct species. Biogeographical relationships exist between cyanobacteria in hot springs (e.g., Papke and Ward 2004). However, the question remains as to whether geographically-separated glacier environments possess endemic or cosmopolitan species. .

Figure 4.6 shows the phylogenetic relationships (based on 16S rRNA gene analysis) between Alphaproteobacteria of the genera *Methylobacterim* and *Sphingomonas* isolated from glacial and subglacial environs in Antarctica (glacial ice, Lake Vostok accretion ice, cryoconite holes), Greenland (glacial and subglacial "silty" ice), China (glacial ice), and New Zealand (subglacial sediment). Also included in Fig. 4.6 are related strains and cloned sequences recovered globally from the deep sea, high mountain lakes, snow, sea ice, endolithic assemblages, cold

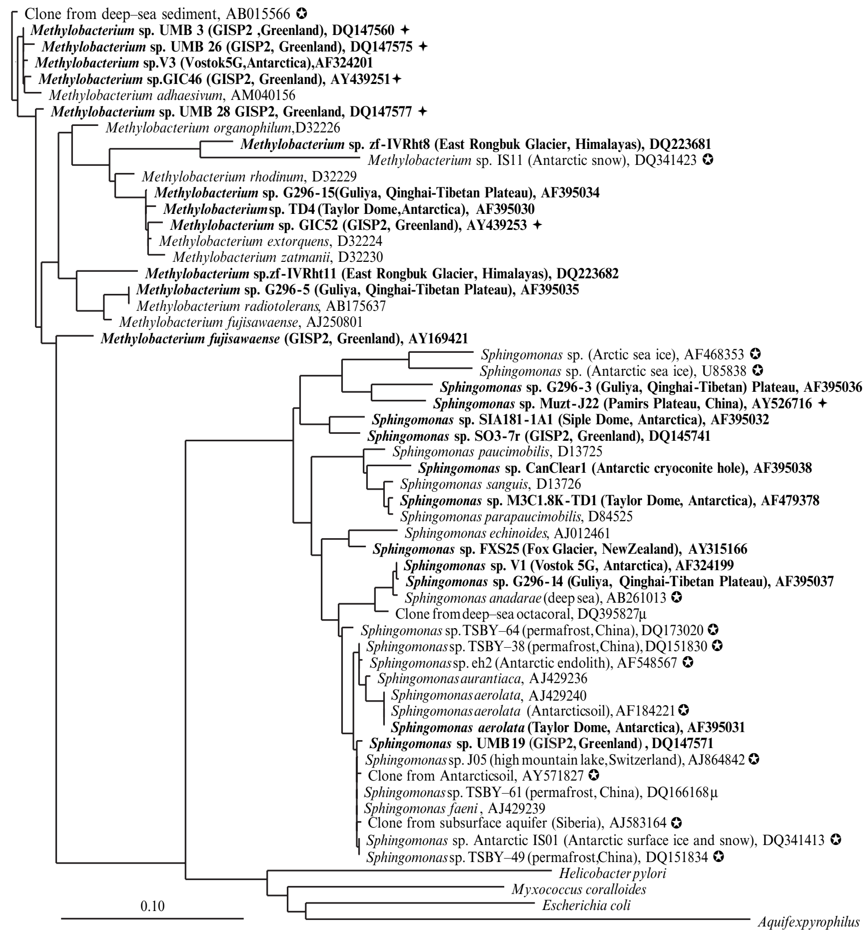

Fig. 4.6 Phylogenetic analysis of small subunit (16S) rRNA gene sequences from *Methylobacterium* and *Sphingomonas* species isolated from glacial and subglacial environments (in bold). The sequences were aligned on the basis of secondary structure and a 1,220-nucleotide mask (120-1,377, *Escherichia coli* 16S rRNA gene numbering) was used to generate the tree using the maximum likelihood method. The *scale bar* represents 0.1 fixed substitutions per nucleotide position. Branches labeled with a *white star* in a *black circle* are isolates and cloned sequences obtained from permanently cold and frozen environments; a *black star* designates lineages for which only short sequences (488–720 nucleotides) are available, and these sequences were added to the tree using the ARB parsimony insertion tool (http://arb-home.de/). The source environment is shown in parentheses, followed by the GenBank accession number

soil, and permafrost. The distribution of related bacteria in worldwide glacial and subglacial environments implies that some members of these genera evolved under cold circumstances and likely possess similar strategies to survive freezing and, possibly, to metabolize at low temperatures. While the phylogeny of a single gene (i.e., 16S rRNA; Fig. 4.6) is not sufficient to resolve fine scale evolutionary relationships, analysis of multiple loci and recent advances in genomic sequencing

technology make these type of experiments now feasible and cost-effective. Due to the isolated nature of subglacial environments, these systems may represent promising evolutionary models for investigating bacterial endemism and to test theory-based species concepts (e.g., Cohan 2002).

4.5 Conclusions

Despite the fact that >80% of the biosphere (by volume) is permanently below 5°C and most of the biomass is microbial (Priscu and Christner 2004), very little is known about the biology of microorganisms inhabiting permanently cold environments. Biologists have studied life on the margins and surfaces of glaciers for nearly a century, but until recently, the subglacial environment was thought to be inhospitable for life. The discovery of active microbial assemblages beneath glaciers and realization that large quantities of liquid water exist beneath polar ice sheets has resulted in a new paradigm in the study of life on Earth.

Knowledge of microbial life in subglacial ecosystems is limited due to sparse data and the technological, financial, and environmental challenges associated with sampling such cold and remote subsurface environments. Considerable progress has been made over the last 10 years in the exploration and study of subglacial environments, permitting a glimpse of the microbial life that exists under conditions of high pressure, cold temperature, low nutrient input, and no sunlight. Priscu et al. (2007) estimate that the number of cells and organic carbon content in Earth's glaciers and subglacial environs ($4 \times 10_{29}$ cells and 10 Pg C) exceeds that reported for the Earth's surface freshwater lakes and rivers ($1.3 \times 10_{26}$ cells and 0.5 Pg C) and is close to that for the open ocean. These tentative estimates imply that glaciated environments contain a considerable pool of cells and organic carbon, and the deep cold biosphere may represent a significant and previously unknown global source of CO_2 and CH_4 (Sharp et al. 1999). As such, biogeochemical cycling models assuming zero rates of microbial mineralization in glacially-overridden soils may underestimate the flux of CO_2 and CH_4 released to the atmosphere during glacial to interglacial transitions.

The study of ecosystems in the cold deep biosphere also has implications for the natural history and evolution of life on Earth, as well as on icy planets and moons in the solar system. Geological evidence indicates that a long period of low latitude pervasive global glaciation occurred during the late Proterozoic, referred to as a "Snowball Earth". Hoffman et al. (1998) argue that the planet was completely covered in ice for at least 10 million years, and liquid water only existed in the ocean under a thick ice cover. If this scenario is accurate, such a long period of global freeze would have had drastic consequences on ecosystems established prior to this event, and subglacial environments may have provided an important refuge for life during such an extended ice age. Polar ice caps composed of water ice exist on Mars, there is evidence for glaciers at lower latitudes during times of higher obliquity (Head et al. 2005), and the jovian moon Europa is thought to maintain a 50- to 100-km-deep liquid ocean under a 3- to 4-km-thick ice shell (Turtle and Pierazzo 2001).

Thus, the study of cold, dark, subglacial environments on Earth will provide insight as to the likelihood of microbial life surviving and persisting in icy extraterrestrial environments. Furthermore, the challenge of identifying appropriate extraterrestrial sites for exploration and developing technology to sample icy subsurface environs will directly benefit from the experience gained by studying earthly analogs.

Subglacial environments remain one of the last unexplored frontiers on our planet. While the study of microbial communities that function near the freezing point of water is inherently interesting, these ecosystems are also clearly relevant to determining the boundaries for life in the biosphere, biogeochemical cycling, the natural history of life on Earth, and astrobiology. We can therefore expect subglacial exploration to be at the forefront of cryospheric research in the future and the years to follow should prove to be an interesting time of discovery.

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Chapter 5
Bacteria in the Deep Sea: Psychropiezophiles

Yuichi Nogi

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5.1 Introduction

Despite the fact that up to 60% of the Earth's surface is covered by seas of depths exceeding 1,000 m, the study of microorganisms in the deep sea is very incomplete. The deep sea is regarded as an extreme environment with high hydrostatic pressures (up to 110 MPa), predominantly low temperatures (2–4°C), but with occasional regions of extremely high temperatures (up to 370°C) at hydrothermal vents, darkness, and low nutrient availability, although with sufficient dissolved oxygen (Fig. 5.1).

In 1949, a research project was started on the effects of hydrostatic pressure on microbial activities (ZoBell and Johnson 1949). The word "barophilic" was first used by them and is defined today as optimal growth at pressure greater than 0.1 MPa (= 1 atm) or by a requirement for increased pressure for growth. The term "piezophilic" has been proposed as a replacement for "barophile" as the Greek translations of the prefixes "baro" and "piezo" mean "weight" and "pressure," respectively (Yayanos 1995). Thus, the word piezophile is more suitable than barophile to describe bacteria that grow better at high pressure than at atmospheric

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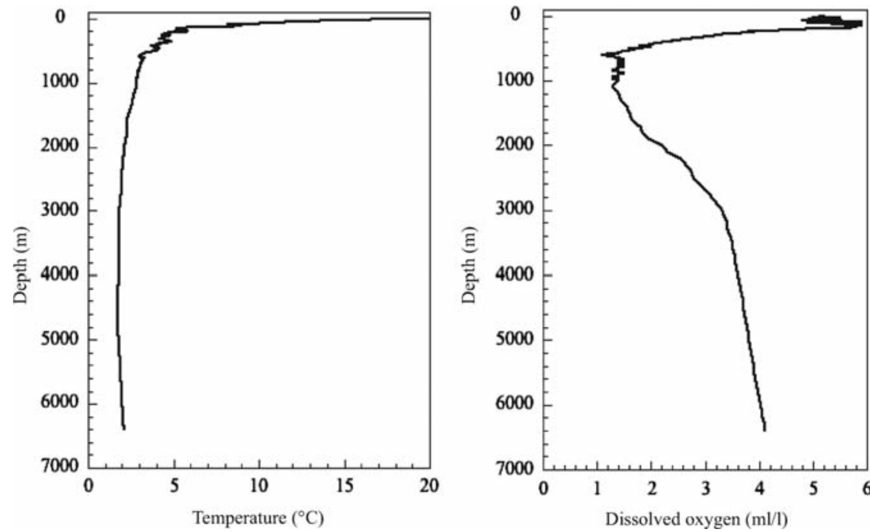

Fig. 5.1 Depth relation of temperature and dissolved oxygen in the Japan Trench (40°06′N, 144°11′E, in August 1995). Measurement by CTD/DO (Conductivity Temperature Depth/ Dissolved Oxygen profiler) of KAIKO systems operated by JAMSTEC

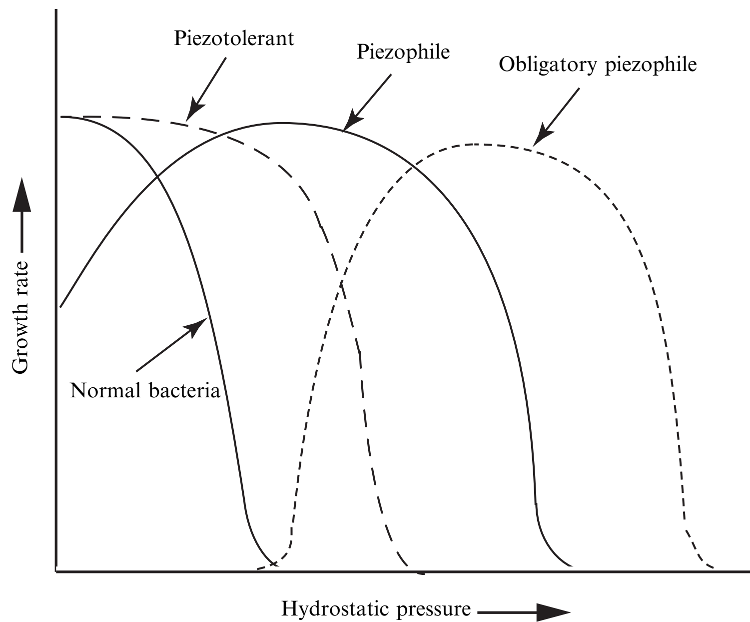

Fig. 5.2 Characterization of piezophilic growth properties (for details, see Sect. 5.1). This figure is a concept chart, which does not show specific pressure values on the x-axis

pressure. Therefore, authors have opted to use the term "piezophilic bacteria", meaning high-pressure-loving bacteria. Piezophiles display maximum growth at high pressure. They can either grow at atmospheric pressure or not; obligatory piezophiles cannot grow at atmospheric pressure. Piezotolerant bacteria grow best at atmospheric pressure, but can sustain high pressure (about 30–50 MPa), whereas piezosensitive bacteria totally stop growing at a pressure of about 30–50 MPa. The growth patterns of piezotolerant and piezophilic bacteria are shown in Fig. 5.2.

5.2 The deep-sea psychropiezophiles

Bacteria living in the deep sea have several unusual characteristics that allow them to grow in their extreme environment. In 1979, the first pure culture isolate of a piezophilic bacterium was reported (Yayanos et al. 1979). The spirillum-like bacterium strain CNPT-3 had a rapid doubling rate at 50 MPa, but did not grow at atmospheric pressure. However, no public culture collections are maintained and no name has been added to strain CNPT-3. We have isolated and characterized numerous piezophilic and piezotolerant bacteria from cold deep-sea sediments at depths ranging from 2,500 m to 11,000 m, using sterilized sediment samplers on the submersibles SHINKAI 6500 and KAIKO systems operated by JAMSTEC, the Japan Agency for Marine-Earth Science and Technology (Kato et al. 1995; Nogi and Kato 1999; Nogi et al. 2004). Most isolated strains are not only piezophilic but also psychrophilic (psychropiezophilic) and cannot be cultured at temperatures higher than 20°C.

5.3 Taxonomy of the psychropiezophiles

Numerous deep-sea piezophilic bacterial strains have been isolated and characterized in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants (Yayanos et al. 1979; Kato et al. 1998; Margesin and Nogi 2004). Thus far, all psychropiezophilic bacterial isolates fall into the gamma-subgroup of the Proteobacteria according to phylogenetic classifications based on 5S and 16S ribosomal RNA sequence information (DeLong et al. 1997; Kato 1999; Margesin and Nogi 2004). Prior to the reports by the JAMSTEC group, only two deep-sea piezophilic bacterial species have been described; they were named *Shewanella benthica* (Deming et al. 1984; MacDonell and Colwell 1985) and *Colwellia hadaliensis* (Deming et al. 1988). We have identified several novel piezophilic species within these genera based on the results of chromosomal DNA–DNA hybridization studies and several other taxonomic properties. Both previously described and novel species of bacteria have been identified among the piezophilic bacterial isolates. Nogi et al. (2002) reported that cultivated psychropiezophilic deep-sea bacteria were affiliated with one of five genera within the Gammaproteobacteria subgroup: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and *Psychromonas*. Figure 5.3 shows the phylogenetic relations between the taxonomically identified psychropiezophilic

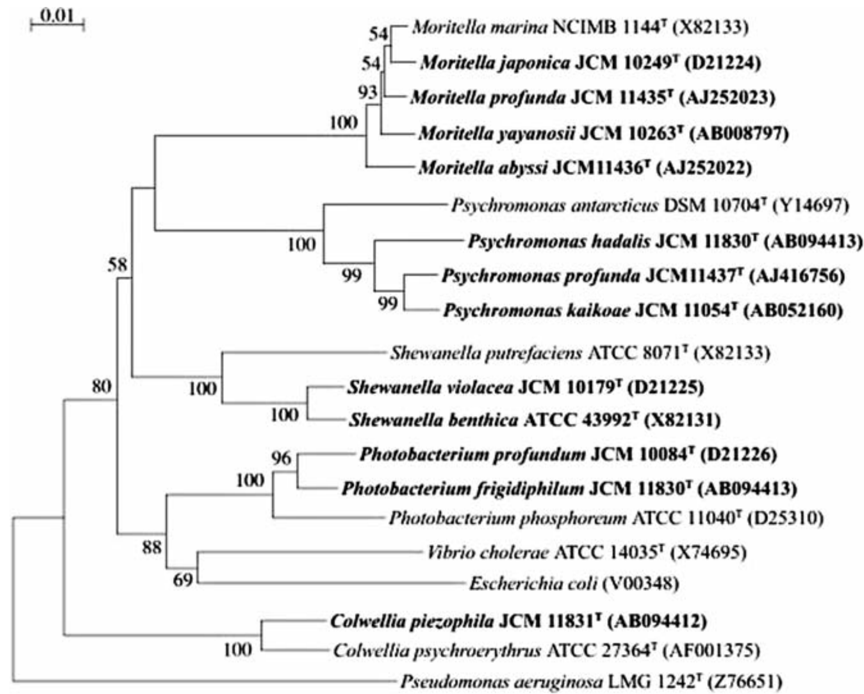

Fig. 5.3 Phylogenetic tree showing the relationships between isolated deep-sea piezophilic bacteria (*in bold*) within the Gammaproteobacteria subgroup determined by comparing 16S rDNA sequences using the neighbor-joining method (references for species description are indicated in the text). The scale represents the average number of nucleotide substitutions per site. Bootstrap values (%) are shown for frequencies above the threshold of 50%

species (shown in bold) and other bacteria within the Gammaproteobacteria subgroup. The taxonomic features of the psychropiezophilic genera were determined as described below.

5.3.1 The genus Shewanella

The genus *Shewanella* comprises Gram-negative, aerobic, and facultatively anaerobic Gammaproteobacteria (MacDonell and Colwell 1985) and is typical of deep-sea bacterial genera (DeLong et al. 1997). The genus includes psychrophilic and mesophilic species that are widely distributed in marine environments. The type species of this genus is *Shewanella putrefaciens*, a bacterium formerly known as *Pseudomonas putrefaciens* (MacDonell and Colwell 1985; Owen et al. 1978). About 45 *Shewanella* species have been isolated and described. The genus is divided into two subgenera on the basis of phylogenetic structure, growth properties in relation to pressure, and polyunsaturated fatty acid (PUFA) production. *Shewanella* group 1 members are characterized as cold-adapted species that grow at high pressure and

produce substantial amounts of eicosapentaenoic acid (EPA), whereas *Shewanella* group 2 members are characterized as mesophilic pressure-sensitive species (Kato and Nogi 2001). Some representatives of group 1, *S. hanedai* and *S. gelidimarina*, are not piezophilic but are rather piezotolerant. Thus, prior to the present report, *Shewanella benthica* and *Shewanella violacea* were the only known members of the genus showing psychropiezophilic growth properties (Nogi et al. 1998b). The psychrophilic and piezophilic *Shewanella* strains, including *S. violacea* and *S. benthica*, produce EPA, and thus the production of such long-chain PUFAs is a property shared by many deep-sea bacteria to maintain cell-membrane fluidity under conditions of extreme cold and high hydrostatic pressure (Fang et al. 2003). *S. violacea* strain DSS12 has been studied extensively, particularly with respect to its molecular mechanisms of adaptation to high pressure (Kato et al. 2000; Nakasone et al. 1998, 2002). As there are few differences in the growth characteristics of strain DSS12 under different pressure conditions, this strain is a very convenient deep-sea bacterium for the study of the mechanisms of adaptation to high-pressure environments. Therefore, genome analysis of strain DSS12 has been performed as a model deep-sea psychropiezophilic bacterium (Nakasone et al. 2003).

5.3.2 The genus **Photobacterium**

The genus *Photobacterium* was one of the earliest known bacterial taxa (Beijerinck 1889). The type species of this genus is *Photobacterium phosphoreum*. Phylogenetic analyses based on 16S rRNA gene sequences have shown that this genus falls within the Gammaproteobacteria and is, in particular, closely related to the genus *Vibrio* (Nogi et al. 1998c). *Photobacterium profundum*, a novel species, was identified through studies of the moderately psychropiezophilic strains DSJ4 and SS9 (Nogi et al. 1998c), and *P. frigidiphilum* was reported to be slightly piezophilic: its optimal pressure for growth is 10 MPa (Seo et al. 2005). About 15 *Photobacterium* species have been isolated, but *P. profundum* and *P. frigidiphilum* are the only species within this genus known to display piezophily and the only two known to produce the long-chain PUFA EPA. No other known species of *Photobacterium* produces EPA (Nogi et al. 1998c). *P. profundum* strain SS9 has been extensively studied with regard to the molecular mechanisms of pressure regulation (Bartlett 1999) and subsequently genome sequencing and expression analysis (Vezzi et al. 2005).

5.3.3 The genus **Colwellia**

Species of the genus *Colwellia* are defined as facultatively anaerobic and psychrophilic bacteria and the type species of this genus is *C. psychroerythrus* (Deming et al. 1988). This genus belongs to the Gammaproteobacteria. At the time of writing, *C. hadaliensis* and *Colwellia piezophila* were the only known members

of the genus showing psychropiezophilic growth properties (Deming et al. 1988; Nogi et al. 2004). However, *C. hadaliensis* and/or its 16S rRNA gene sequence information are not maintained in public culture collections. The other species, *C. piezophila*, has been isolated as an obligately psychropiezophilic strain from the sediment of the deep-sea fissure of the Japan Trench (Nogi et al. 2004). Bowman et al. (1998) reported that *Colwellia* species produce the long-chain PUFA docosahexaenoic acid (DHA). However, *C. piezophila* does not produce EPA or DHA in the membrane layer, whereas high levels of unsaturated fatty acids (16:1) are produced. This observation suggests that the production of long-chain PUFAs should not be a requirement for classification as a piezophilic bacterium, although the production of unsaturated fatty acids could be a common property of psychropiezophiles.

5.3.4 The genus **Moritella**

The type strain of the genus *Moritella* is *M. marina* (Urakawa et al. 1998), previously known as *Vibrio marinus* (Colwell and Morita 1964), and is one of the most common psychrophilic organisms isolated from marine environments. Many species of the genus are psychropiezophilic, but *M. marina* is not a piezophilic bacterium. Strain DSK1, a moderately psychropiezophilic bacterium isolated from the Japan Trench, was identified as *Moritella japonica* (Nogi et al. 1998a). This was the first piezophilic species identified in the genus. Production of the long-chain PUFA DHA is a characteristic property of the genus *Moritella*. The extremely piezophilic bacterial strain DB21MT-5 isolated from the world's deepest sea bottom, the Mariana Trench Challenger Deep, at a depth of 10,898 m was also identified as a *Moritella* species and designated *M. yayanosii* (Nogi and Kato 1999). The optimal pressure for the growth of *M. yayanosii* strain DB21MT-5 is 80 MPa; this strain is unable to grow at pressures of less than 50 Mpa, but grows well at pressures as high as 100 MPa (Kato et al. 1998). The fatty acid composition of psychropiezophilic strains changes as a function of pressure, and in general greater amounts of PUFAs are synthesized at higher growth pressures. Approximately 70% of the membrane lipids in *M. yayanosii* are unsaturated fatty acids, which is a finding consistent with its adaptation to very high pressures (Nogi and Kato 1999; Fang et al. 2000). Two other species of the genus, *M. abyssi* and *M. profunda,* were isolated from a depth of 2,815 m off the West African coast (Xu et al. 2003b); they are moderately piezophilic and their growth properties are similar to those of *M. japonica*.

5.3.5 The genus **Psychromonas**

The genus *Psychromonas* is composed of psychrophilic bacteria; it also belongs to the Gammaproteobacteria, and is closely related to the genera *Shewanella* and

Moritella on the basis of 16S rRNA gene sequence data. The type species of the genus, *P. antarctica,* was isolated as an aerotolerant anaerobic bacterium from a high-salinity pond in Antarctica (Mountfort et al. 1998). This strain did not display piezophilic properties. *Psychromonas kaikoae* is a novel obligatory psychropiezophilic bacterium (Nogi et al. 2002). This strain was isolated from sediment collected from the deepest cold-seep environment (an area of the ocean floor where hydrogen sulfide, methane and other hydrocarbon-rich fluid seepage occurs; sometimes called a cold vent) in the Japan Trench at a depth of 7,434 m, where chemoautotrophic animal communities were also found. The optimal temperature and pressure for the growth of *P. kaikoae* are 10°C and 50 MPa, respectively, and both EPA and DHA are produced in the membrane layer. In contrast, *P. antarctica* does not produce either EPA or DHA in its membrane layer. *Psychromonas profunda* strain CNPT-3 proved to be closely related to *P. kaikoae* based on 16S ribosomal RNA sequence information, and therefore it was assumed that strain CNPT-3 should be included in this genus. In addition, *Psychromonas profunda* is a moderately piezophilic bacterium isolated from deep Atlantic sediments at a depth of 2,770 m (Xu et al. 2003a). This strain is similar to the piezo-sensitive strain *Psychromonas marina*, which also produces small amounts of DHA. In this genus, only *P. kaikoae* produces both EPA and DHA.

5.4 The fatty acid composition of psychropiezophiles

The psychropiezophilic *Shewanella* and *Photobacterium* strains produce EPA (Nogi et al. 1998b, 1998c), *Moritella* strains produce DHA (Nogi et al. 1998a; Nogi and Kato 1999), and *P. kaikoae* produces both EPA and DHA (Nogi et al. 2002), but *C. piezophila* does not produce such PUFAs (Nogi et al. 2004). The fatty acid composition of these psychropiezophilic strains is dependent on the taxonomic affiliation (genus); commonly high levels of unsaturated fatty acids (about 50–70%), including EPA or DHA, are found in their membrane layer. The fatty acid composition of psychropiezophilic strains also changes as a function of pressure and, in general, greater amounts of PUFAs are synthesized under high-pressure conditions for growth (DeLong and Yayanos 1985, 1986). Psychropiezophilic bacteria were believed to produce one of the long-chain PUFAs, either EPA or DHA, but this does not appear to be obligatory. For example, Allen et al. (1999) reported that monounsaturated fatty acids, but not PUFAs, are required for the growth of the psychropiezophilic bacterium *P. profunda* SS9 based on the analysis of pressure-sensitive mutants. In their mutant experiment, the 18:1 fatty acid proved to be necessary for growth under low-temperature and/or high-pressure conditions. In the case of *C. piezophila*, the 18:1 fatty acid is absent, but the strain produces a large amount of the fatty acid 16:1 in the cell membrane. All psychropiezophilic bacteria analyzed so far have 16:1 fatty acid and, thus, this fatty acid appears to be one of the important components required for high-pressure growth.

5.5 Conclusions

Cultured deep-sea psychropiezophilic bacteria are affiliated with one of five genera within the Gammaproteobacteria subgroup: *Shewanella, Photobacterium, Colwellia, Moritella,* and *Psychromonas*. These psychropiezophiles are characterized to contain unsaturated fatty acids in their cell membrane layers but PUFAs, like EPA and DHA, are not obligatory for growth under high-pressure.

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Chapter 6
Bacteria in Permafrost

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6.1 Introduction

Significant numbers of viable ancient microorganisms are known to be present within the permafrost. They have been isolated in both polar regions from the cores up to 400 m deep and ground temperatures of −27°C. The age of the cells corresponds to the longevity of the permanently frozen state of the soils, with the oldest cells dating back to ~3 million years in the Arctic, and ~5 million years in the Antarctic. They are the only life forms known to have retained viability over geological time. Thawing of the permafrost renews their physiological activity and exposes ancient life to modern ecosystems. Thus, the permafrost represents a stable and unique physicochemical complex, which maintains life incomparably longer than any other known habitats. If we take into account the depth of the permafrost layers, it is easy to conclude that they contain a total microbial biomass many times higher than that of the soil cover. This great mass of viable matter is peculiar to permafrost only.

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The terrestrial cryosphere consists of two parts: glaciosphere (snow and ice) and frozen ground, which contains long-term and seasonal cryogenic formations with ancient and periodically frozen viable microorganisms, respectively (Table 6.1). Permanently frozen formations are a widespread, rich terrestrial depository of ancient viable cells and represent a significant part of the biosphere, the Cryobiosphere. These permanently frozen formations (ice and ground) maintain life during geological time.

Biota of Greenland and Antarctic Ice Sheets (120,000 and 400,000 years, respectively) have been widely studied up to depths of 3–4 km (Abyzov 1993; Kapitsa et al. 1996; Karl et al. 1999; Priscu et al. 1998; Petit et al. 1999; Skidmore et al. 2000; Miteva et al. 2004). The oldest, with more than 500,000 years, glacial ice (Thompson et al. 1997), as well as immured bacteria (Christner et al. 2003), were found at Guliya ice cap on Tibetan Plateau. Table 6.1 shows that the number of viable, mostly airborne, cells in snow and seasonal ice covers are in the same order of magnitude as within the ancient Ice Sheet cores. Such data could be interpreted as an absence of reduction of the microbial population once bacteria were immured in ice hundreds of thousand years ago. The studies have shown that the number of viable cells in these cores increases sharply with the presence of dust particles (Abyzov 1993) and the ultra small cells were dominating (Miteva and Brenchley 2005). The cell distribution along the Antarctic Ice Sheet borehole indicates that the abundance of viable cells in Antarctic Ice Sheet decreases with increasing age of the ice—most abundant are the upper (<12,000 years) layers in spite of extremely low temperatures, −50°C (Abyzov 1993). Studies of Greenland ice indicate a good preservation of the genomic DNA in relatively young, 2,000–4,000 years, cores (Willerslev et al. 1999), as well as of bacterial and plant viruses in samples from 500–100,000 years old (Castello et al. 2005). Unfortunately, this relates to human danger viruses too: in the Arctic, influenza A RNA is preserved in high concentrations in the seasonal ice

Table 6.1 Bacteria in terrestrial Cryosphere

<table>
<tr><td colspan="5">EARTH CRYOSPHERE</td></tr>
<tr><td colspan="2">Glaciosphere</td><td colspan="3">Cryolithosphere</td></tr>
<tr><td colspan="5">Seasonally cryogenic formations</td></tr>
<tr><td colspan="2" rowspan="2">Snow & ice Covers</td><td colspan="3">Cryopedosphere (frost-affected soils)</td></tr>
<tr><td colspan="2">Seasonally thawed soil (Permafrost-affected soil or Cryosol)</td><td>Seasonally frozen soil</td></tr>
<tr><td colspan="2">10^1–10^2 cells ml^{-1}</td><td colspan="3">10^7–10^9 cells g^{-1}</td></tr>
<tr><td colspan="5">Long-term cryogenic formations</td></tr>
<tr><td>Ice sheets Glaciers</td><td>Ice veins</td><td>Rocky permafrost (overcooled dry rocks)</td><td colspan="2">Frozen ground and Buried soils (fine dispersed icy sediments)</td></tr>
<tr><td colspan="2">10^1–10^2 cells ml^{-1}</td><td>no data</td><td colspan="2">10^3–10^7 cells g^{-1}</td></tr>
<tr><td colspan="5">CRYOBIOSPHERE</td></tr>
</table>

of lakes (Shoham 2005). Recently, the preservation of influenza A virus genes was reported in ice and water from Kolyma lowland lakes on the East Siberian sea coast that are visited by large numbers of migratory birds. This type of temporal gene flow might be a common feature of viruses that can survive entrapment in environmental ice and snow (Zhang et al. 2006).

Table 6.1 shows that the most colonized part of Cryosphere is represented by modern frost-affected soils and permafrost with cells adsorbed on organic or mineral particles. This is why, after brief description of the contemporary soil cover in high altitudes and latitudes, we focus on permafrost as a habitat, and its biodiversity. However, firstly we have to clarify the terminology and emphasize that the term permafrost designates the permanently frozen ground—soil or rock that remains at or below 0°C for at least two consecutive years (van Everdingen 1998). In the literature, the term "soil" is the synonym of fine dispersed sediments or deposits. So, in the above mentioned definition, the term "permafrost soil" is a synonym of "permafrost". Unfortunately, in recent years, some microbiologists used in presentations and papers the term permafrost soil as a synonym of modern soils in permafrost zone—seasonally (summer) thawed soils underlain by permafrost. Thereby, these authors ignore the principal differences between permanently and seasonally frozen grounds as microbial habitats and mislead readers about the microbial community which is investigated: ancient or modern. In the case of the soil cover in the permafrost zone, several terms could be used—seasonally thawed soils or active layer. More recent terms are permafrost-affected soils or cryosol.

6.2 Soil cover

The frost-affected soil cover consists of two main groups, which contain a similar number of viable cells (Table 6.1): (1) seasonally (summer) thawed soils with mean annual temperatures lower than 0°C, underlain by permafrost; and (2) seasonally (winter) frozen soils with mean annual temperatures higher than 0°C, underlain by non-frozen deposits. In the cold period, both groups are in the frozen state and melt during each summer. The leading factor in differentiation of soil horizons is temperature transition through 0°C, resulting in freeze-thawing processes, ice-water phase exchange, cryoturbation, soil heaving, shattering and continual renovation of soil profile. This is why it is so important to understand the influence of multi-time freeze-thawing stresses on soil microbial community.

Arctic tundra and north taiga soils in frozen state are consolidated by ice, and the depth of seasonal thawing varies between 0.3 and 2.0 m. The maximal number and biodiversity of microorganisms correlate with the upper soil horizon A and decrease with depth up to the surface beneath the seasonal thaw layer, called permafrost table. This table represents the physical barrier with the sharp accumulative peak of microorganisms (Fig. 6.1), which came down from the upper layers due to infiltration of melted water (Fyodorov-Davydov and Spirina 1998).

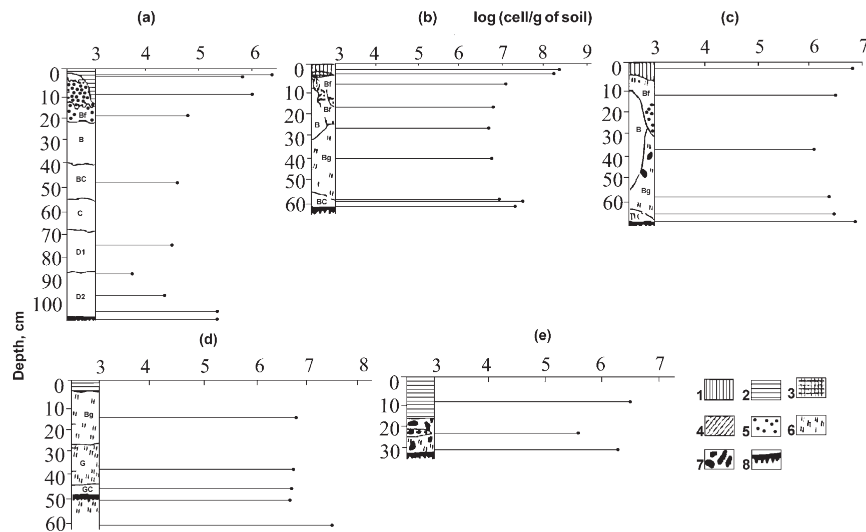

Fig. 6.1 Bimodal profile distribution of microorganisms in tundra and north taiga soil cover. *Key*: *1* forest litter; *2* peaty horizon; *3* mucky horizon; *4* soddy horizon; *5* iron-enriched horizon; *6* morphological features; *7* organic inclusions in mineral horizons; *8* permafrost table

The surface of Arctic tundra soil is under the influence of solar radiation. But covers of snow and vegetation decrease and minimize this impact, as well as temperature oscillations. The surface conditions in Antarctica (intensive solar radiation, absence of snow and vegetation covers and ultra-low subzero temperatures down to −60°C) differ from Arctic. This is why the upper 10–25 cm thick Antarctic Dry Valleys sandy "active" layer is dry and lacks ice-cement due to sublimation. The overcooled (frosty) layer with no water and therefore no ice may often be mobilized by storm wind. At elevations of 1500 m, there is no summer air temperature above freezing. However, the surface temperatures of soil or rock may exceed 0°C for several hours (Llano 1962; McKay et al. 1993, 1998), and for short periods even reach 10°C (Campbell et al. 1997). In such a situation, the upper ~2 cm layer of the surface often contains a low number of viable cells in comparison with the underlain horizons (Cameron et al. 1970; Horowitz et al. 1972), and, in some cases, these microorganisms cannot be isolated on agar plates. This correlates with the poor diversity of bacterial phylotypes, a low number of mycelial fungi strains, and a minimum of chlorophyll content. The occurrence and biodiversity of microorganisms is higher at depth (horizon C) than in top of the "active" layer (Gilichinsky et al. 2007). Such distribution is typical for cryptoendolithic microbial communities on and within Antarctic sandstone (Friedmann 1982; Meyer et al. 1988; Nienow and Friedmann 1993).

Microbiologists have carried out research of Arctic soil microbial communities by classical bacteriological methods for more than 60 years (Jensen 1951; McBee and McBee 1956; Boyd and Boyd 1962). Numerous studies have shown that the bacterial composition in the active layer of Arctic tundra include members of

Alpha-, Beta-, Gamma-Proteobacteria, Firmicutes, Actinobacteria (*Arthrobacter, Nocardia, Mycobacterium*), Cyanobacteria and members of the Cytophaga/Flexibacter/Bacteroides group (Nelson and Parkinson 1978; Parinkina 1989; Dobrovolskaya et al. 1996; Mannisto and Haggblom 2006).

Gram-negative bacteria, such as *Burkholderia* sp., *Collimonas* sp., *Pedobacter* sp., *Janthinobacter* sp., *Duganella* sp., *Dyella* sp., *Achromobacter* sp., *Pseudomonas* sp. and *Sphingomonas* sp., are typical components of the tundra soil microbial complex, while Gram-positive strains are often a minor component (Mannisto and Haggblom 2006; Belova et al. 2006). Since the processes of methane production and oxidation are common in Arctic polygonal tundra, methanogens and methanotrophs (*Methylocella tundrae*, *Methylocella palustris, Methylobacter psychrophilus*) are always present in the community structure (Berestovskaya et al. 2002, 2005; Dedysh et al. 2004).

However, determination of phylogenetic diversity of a bacterial community from soil DNA started by Zhou et al. (1997) has come only now to the active phase. In that study, no dominant clones were found; all 43 environmental clones were different with most of the phylotypes from Proteobacteria (60.5%), especially from Delta (25.6%), Alpha (20.9%), Beta (9.3%) and Gamma (4.7%) subdivisions, followed by *Fibrobacter* (16%), Gram-positive bacteria (11.6%) and members of the Cytophaga-Flexibacter-Bacteroides group (2.3%). However, due to the small size of the clone library, it was impossible to compare the microbial abundance and diversity of tundra soils with soils of other northern regions. Partly, this deficiency was filled up by Neufeld and Mohn (2005). Using the data of serial analysis of ribosomal sequence tags (SARST) and denaturing gradient gel electrophoresis (DGGE), they estimated and compared the bacterial biodiversity in Arctic tundra and boreal soils. Between 1,487 and 2,659 ribosomal sequence tags (RSTs) were obtained from each sample of three arctic tundra sites and three boreal forest locations. Rarefaction analysis, Chao1 estimates, and Shannon–Weiner diversity index consistently indicated that the undisturbed arctic tundra soil libraries possessed greater bacterial diversity than the boreal forest soil libraries. The taxonomic affiliations of RSTs demonstrated the dominance of Proteobacteria and substantial proportions of Actinobacteria, Acidobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia, and Cyanobacteria. All libraries contained a large proportion of RSTs (10–25%) with close affiliations to 16S rRNA gene sequences of unknown phylogenetic affiliation. This report and our studies demonstrate that the Arctic serves as an unrecognized reservoir of microbial diversity and thus of biochemical potential.

In our study, in order to get higher diversity of phylotypes, we extracted the total community genomic DNA from the original sample (T_0) and after aerobic (T_a) and anaerobic (T_{an}) enrichments (Fig. 6.2). A total of 243 environmental clones were selected and partial 16S rRNA gene sequences for each clone were obtained using the high throughput DNA sequencing approach, and the phylogenetic relatedness of the 16S rRNA gene sequences was studied. All variants yielded a high proportion of Proteobacteria and unclassified bacteria, while the proportion of all other bacterial groups varied depending on the conditions of enrichment or on the respective DNA isolation kit (Fig. 6.2). Therefore, we present here a summary of all clones spread over 15 phyla. Most of the clones (29.3%) belonged to unclassified

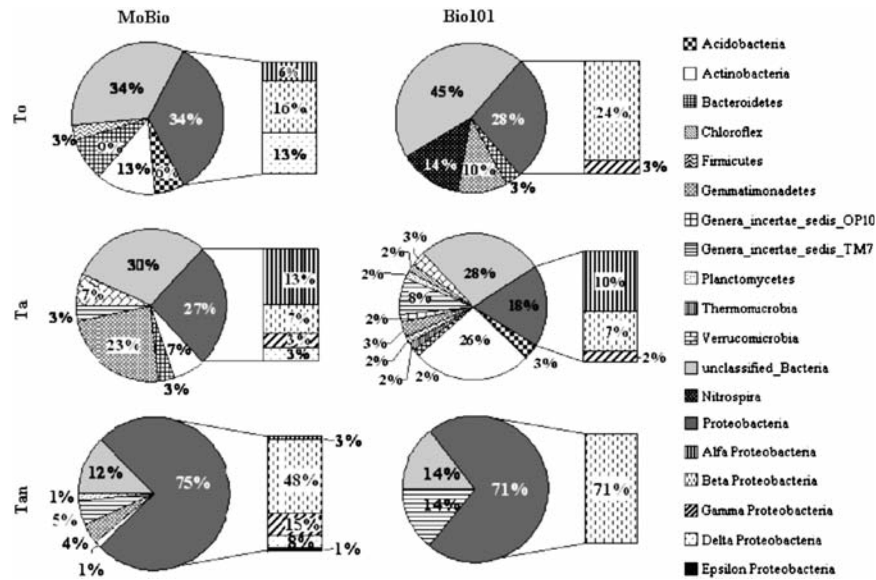

Fig. 6.2 Bacterial diversity in one sample of Arctic soil as obtained after aerobic (T_a) and anaerobic (T_{an}) enrichments in comparison to original community (T_0). The total community genomic DNA for each variant was isolated using MoBio and Bio101 kits. The proportion of different subdivisions of Proteobacteria is given on the right of each pie. Bacterial phyla which environmental clones were closely related to are shown

bacteria and bacteria of uncertain position, so the majority of the bacterial community of tundra soil appears to have never been isolated and the physiology and function of these presumably dominant organisms are unknown. The dominant bacterial group was represented by Proteobacteria (40.4%) with the majority of clones from the Beta (23.9%) subdivision, in comparison to the Alpha (5.7%), Gamma (5.7%), and Delta (4.5%) subdivisions. The distribution of other detected bacterial groups was as follows: Gram-positive bacteria consisted of Actinobacteria (9.5%) and Firmicutes (0.8%), then Gemmatimonadetes (7.8%), Nitrospira (3.3%), Cytophaga–Flexibacter–Bacteroides group (2.4%), Verrucomicrobia (2.4%), Acidobacteria (1.6%); other detected bacteria constituted less than 1%.

To date, two tundra soils and four permafrost samples, all of them of different composition and origin, were characterized in three independent studies based on culture-independent approaches (Zhou et al. 1997; Vishnivetskaya et al. 2006; Steven et al. 2007; and this review). Deeper permafrost layers contain microbial communities which have been formed in the surface ecosystems and then trapped and buried during sediment accumulation and freezing. However, because of the complex vertical structure of the soil/sediments and the physical and chemical differences between the horizons (Zvyagintsev 1994), it is obvious that the subsurface community structure differs from that of surface soils. In spite of the fact that a bacterial community structure depends on sample characteristics, we found similarities between upper soil layers and underlain permafrost sediments. While

the diversity of the genera detected in tundra soil was higher than within permafrost, Gram-positive bacteria with high and low G+C content, Alpha-, Beta-and Gamma-Proteobacteria, and Cytophaga–Flexibacter–Bacteroides group were detected in both soil and sediments. These bacterial groups were also detected in different textured tundra soil horizons by fluorescence in situ hybridization (FISH), a new approach for studying the composition of an active community in an environment (Kobabe et al. 2004). We found that Proteobacteria (Delta-, Alpha-, or Beta-) were predominant in tundra soil, while Gamma-Proteobacteria dominated within permafrost. However bacteria of the genus *Pseudomonas* and the family Xanthomonadaceae could be easily detected in tundra soil as well. The comparison of environmental clones and previously characterized isolates from tundra soil showed that *Arthrobacter, Nocardioides, Methylocystis, Janthinobacterium, Burkholderia*, and *Pseudomonas* could be detected by both culture-dependent and culture-independent methods.

6.3 Permafrost

The first data related to the existence of bacteria in permafrost appeared at the beginning of the 20th century, in relation to the discovery of mammoths and studies of soils in Siberia (Omelyansky 1911; Isachenko 1912). In the 1930s–70s, separately, unrecognized by each other, microbes were discovered in many Arctic regions (Kapterev 1936, 1938; Kriss 1940; James and Sutherland 1942; Kriss and Grave 1944; Kalyaev 1947; Becker and Volkmann 1961; Boyd and Boyd 1964; Kjoller and Odum 1971), and in Antarctic Dry Valleys (Cameron and Morelli 1974). As early as 1975, Pewe first emphasized the need for further research in this field and, 20 years later, the overview of these studies was published (Gilichinsky and Wagener 1995). In all these studies, the procedures and the application of drilling fluids did not guarantee the sterility of the cores. Because of these methodological and technical difficulties, the above mentioned reports were not considered with due attention and the permafrost was not studied as a living stratum. Nevertheless, the authors of these early studies first raised the question of the possible preservation of viable cells in the permafrost. The recent status of permafrost microbiology has been reviewed by Steven et al. (2006). This is why we focus below on some new aspects only.

6.3.1 Bacterial biodiversity

Abundance and diversity of microbes inhabiting permafrost are very high. The total cell number counted by epifluorescence microscopy was 105–106 cells g^{-1} dry mass in Antarctica (Gilichinsky et al. 2007) and 10^7–10^8 cells g^{-1} dry mass in Siberian (Vorobyova et al. 1997) permafrost. The number of bacterial cells that grow on nutrient

media was <0.1% (Antarctica) and 0.1–1.0% (Siberia) of the total amount counted by epifluorescence microscopy. Bacterial communities from both Siberian and Antarctic permafrost samples were precisely characterized by culture-dependent and culture-independent methods. Both methods revealed the presence of Gamma-Proteobacteria and Gram-positive bacteria with high and low G+C content in both ecosystems (Table 6.2). From Table 6.2, we can easily see that some of the bacterial genera, such as *Arthrobacter, Bacillus, Pseudomonas,* and Enterobacteriaceae, could be detected by both methods. Culture-independent approaches showed the dominance of Gamma-Proteobacteria, especially Xanthomonadaceae (75–84%), and Actinobacteria (39–57%) in Siberian permafrost (Petrova, unpublished data; Vishnivetskaya et al. 2006), and Gram-positives (up to 45%) and Proteobacteria (up to 25%) in Antarctic permafrost (Spirina et al. 2003). Numerous studies showed abundant viable bacteria in Siberian permafrost (Shi et al. 1997; Vorobyova et al. 1997; Vishnivetskaya et al. 2000), these bacteria were isolated with different isolation techniques and approaches. Table 6.2 shows that there were more environmental clones from Antarctic permafrost than from Siberian; this may be a consequence of the high resolution approach we used to access the total community biodiversity in Antarctic permafrost core samples. The high throughput DNA sequencing of environmental clones yielded over 2,000 partial 16S rRNA gene sequences, which were automatically aligned using SEQUENCE MATCH against closely related sequences in the Ribosomal Database Project (RDP) (Maidak et al. 2001). In comparison to 265 environmental clones from Siberian permafrost, which were grouped using amplified ribosomal 16S rRNA restriction analysis (ARDRA), only representatives of the major ARDRA clusters were sequenced. Thus, viable isolates from Siberian permafrost and environmental clones from Antarctica are well characterized; therefore the dissimilarities and similarities between them may suggest that (1) some genera are indigenous, and (2) similar genera inhabit distinct permafrost systems. We have also found that most of our isolates and clones are phylogenetically related to previously characterized strains or clones from different cold ecosystems (Vishnivetskaya et al. 2006; Gilichinsky et al. 2007).

6.3.2 Cyanobacteria

350 permafrost cores were screened for presence of viable cyanobacteria. 30 cyanobacteria strains were isolated from Siberian samples (Vishnevetskaya et al. 2001), while no cyanobacteria were found in Antarctic permafrost. However, a few cyanobacterial environmental clones were amplified from the total community genomic DNA isolated from Antarctic permafrost (Gilichinsky et al. 2007). To compare the environmental clones and isolates obtained from permafrost of both Polar Regions, phylogenetic analyses of 16S rRNA genes of cyanobacteria were performed, which placed them into three groups (Fig. 6.3). Three viable cyanobacterial strains from Siberian permafrost and four environmental clones from Antarctic permafrost have close relatives within the Nostocales family. However, these environmental clones

Table 6.2 Summary of the bacterial diversity in Siberian and Antarctic permafrost as characterized by culture-dependent and culture-independent methods

	Siberian		Antarctic	
Class	Isolates[a,b,c]	Clones[a]	Isolates[b,d]	Clones[e]
Actino-bacteria, Gram-positive, high G+C	*Arthrobacter, Micrococcus, Microbacterium, Rhodococcus, Mycobacteria, Cellulomonas, Streptomyces, Kocuria, Brevibacterium, Nocardioides, Propionibacterium*	*Arthrobacter, Micrococcus, Renibacterium, Clavibacter, Cryobacterium*	*Arthrobacter, Micrococcus, Rhodococcus, Cellulomonas, Promicromono-spora, Streptomyces*	*Arthrobacter, Acidimicrobium, Conexibacter, Kineosporia, Friedmanniella, Rubrobacter, Sporichthya, Nocardioides, Rhodococcus, Propionibacterium*
Firmicutes, Gram-positive, low G+C	*Bacillus, Sporosarcina, Paenibacillus, Planomicrobium, Planococcus, Exiguobacterium*	*Bacillus, Clostridium*	*Bacillus*	*Bacillus, Sporosarcina, Planomicrobium*
Gamma-Proteobacteria	*Xanthomonas, Pseudomonas, Escherichia, Aeromonas, Serratia, Stenotrophomonas, Acinetobacter Psychrobacter*	Xanthomonadaceae, *Lysobacter*, iron-oxidizing lithotroph ES1, *Pseudomonas,* Enterobacteriaceae, *Aeromonas, Serratia, Yersinia, Citrobacter*	*Pseudomonas,* Enterobacteriaceae, *Aeromonas, Azotobacter*	*Pseudomonas, Escherichia, Stenotrophomonas, Citrobacter*
Alpha-Proteobacteria	*Sphingomonas, Nitrobacter*			*Sphingomonas, Paracraurococcus, Rhizobium, Sphingobium, Sphingopyxis, Ochrobactrum, Sinorhizobium, Methylobacterium*
Beta-Proteobacteria[a]	*Alcaligenes, Nitrosomonas, Nitrosospira*			*Polaromonas, Rhodoferax*
Delta-Proteobacteria	*Myxococcus*			*Myxobactereales*
Bacteroidetes	*Flavobacterium, Sphingobacterium*			*Chitinophaga*
Others				*Acidobacterium, Vulcanithermus, Gemmatimonas, Nitrospira, Planctomyces, Thermomicrobium* incertae sedis

Data from following studies were used: [a]Vishnivetskaya et al. (2006); [b]Vorobyova et al. (1997); [c]Shi et al. (1997); [d]Gilichinsky et al. (2007); [e] Pirina et al. (2003)

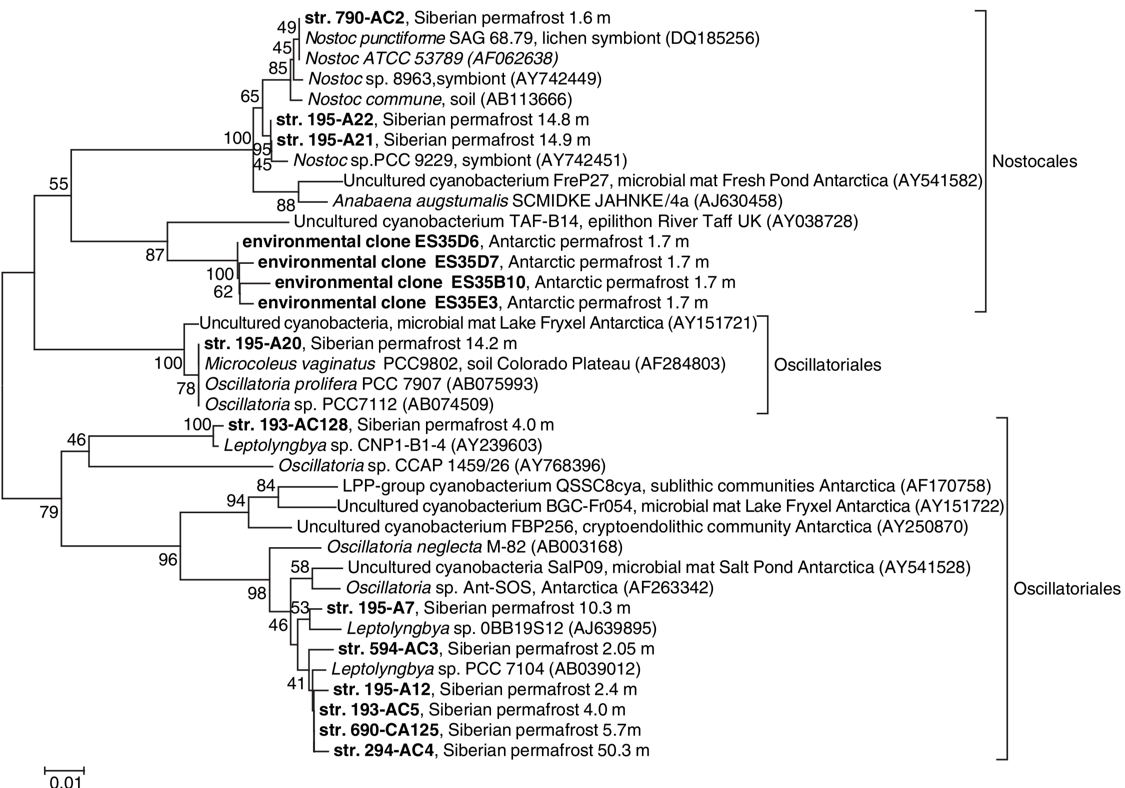

Fig. 6.3 Phylogenetic relationships of cyanobacterial isolates and environmental clones derived from Siberian and Antarctic permafrost. Tree was produced by the neighbor-joining method (Saitou and Nei 1987). Bootstrap values, expressed as percentages of 100 replications, higher than 40% are shown

were closely related to an uncultured cyanobacterium found in river epilithon. Viable *Nostoc*-like strains formed heterocysts in the absence of combined nitrogen source, and were characterized by different phycoerythrin/phycocyanin ratio depending on nitrogen source. Among eight strains of non-heterocystous filamentous cyanobacteria, we found seven that were close to each other and to *Leptolyngbya* (80–95.8% identity), and one which was closely related to *Microcoleus* (96.8%), both of them in the family Oscillatoriales. The phylogenetic analyses were confirmed by studying the morphological features of the isolates. We have found that viable cyanobacteria were dominated by non-heterocystous filamentous cyanobacteria of the family Oscillatoriales. Permafrost cyanobacteria were closely related to strains and mostly to uncultured cyanobacteria derived from microbial mat or cryptoendolithic communities in Antarctica.

6.3.3 Anaerobic bacteria

Permafrost contains both aerobic and anaerobic bacteria. In addition, the reducing conditions within the permafrost are more favorable for the preservation of anaerobic bacteria. Most-probable-number (MPN) incubations showed evidence of viable denitrifiers, acetoclastic methanogens, hydrogenotrophic methanogens, Fe(III) reducers, and sulfate reducers in some of the aged frozen soils (Rivkina et al. 1998). The denitrifiers and hydrogenotrophic methanogens were found in higher numbers and in the oldest layers. Acetoclastic methanogens and sulfate reducers were found in low numbers, and not in all samples. Iron-reducing bacteria were only found in samples of moderate age (from modern to 10,000 years). Sulfate-reducing bacteria were detected in half of the samples without a specific pattern. The number of some anaerobic groups of microorganisms growing at +15°C is presented in Table 6.3.

Table 6.3 Numbers of viable permafrost anaerobes (cells g^{-1} dry mass) growing at 15°C

Period (age, years)	Depth (m)	Methanogens (CO_2+H_2)	Denitrifying (NO_3+citrate)	Sulfate-reducers (SO_4+ lactate)
Q_{IY} $(5–10)\times10^3$	0.1	2.0×10^7	2.0×10^7	2.0×10^2
	1.2	1.2×10^7	1.2×10^5	0
Q_{III} $(1–4)\times10^4$	2.2	2.5×10^7	2.5×10^5	0
	4.4	2.5×10^7	2.5×10^6	0
	17.0	2.5×10^7	2.5×10^5	0
Q_{II} $(1–6)\times10^5$	30.0	2.3×10^7	2.3×10^3	2.3×10^2
	32.7	2.0×10^7	2.0×10^6	0
N_2–Q_I $(0.6–1.8)\times10^6$	37.2	2.0×10^7	2.0×10^6	2.0×10^2
	43.5	2.5×10^7	2.0×10^4	2.5×10^2
	48.8	2.0×10^7	2.5×10^3	2.0×10^2
	54.8	2.0×10^4	2.0×10^6	0
	64.3	2.5×10^7	2.5×10^7	2.0×10^2

Methane is also trapped in the permafrost and this is why, among viable anaerobic microorganisms, research was mainly oriented towards methane-producing Archaea. Using radiolabeled substrates, $NaH^{14}CO_3$ and $Na^{14}CO_2H_3$, it was shown that methane formation in frozen deposits may occur at subzero temperatures down to −16.5°C (Rivkina et al. 2004, 2007). Our specific goals were to isolate methane-producing Archaea and to investigate the effect of long-term preservation of the methane-producing community in the permafrost on its metabolic activity.

Active methanogenic enrichment cultures (40% of CH_4 in headspace) were obtained after 6 and 12 months of incubation, respectively, and only on H_2+CO_2 at 20°C, although trace amounts of methane were also detected on acetate. Three strains from Holocene and Pliocene age were isolated for the first time in pure cultures: JL01, M2 and MK4 (Fig. 6.4). Although CO_2+H_2 served as a favorable

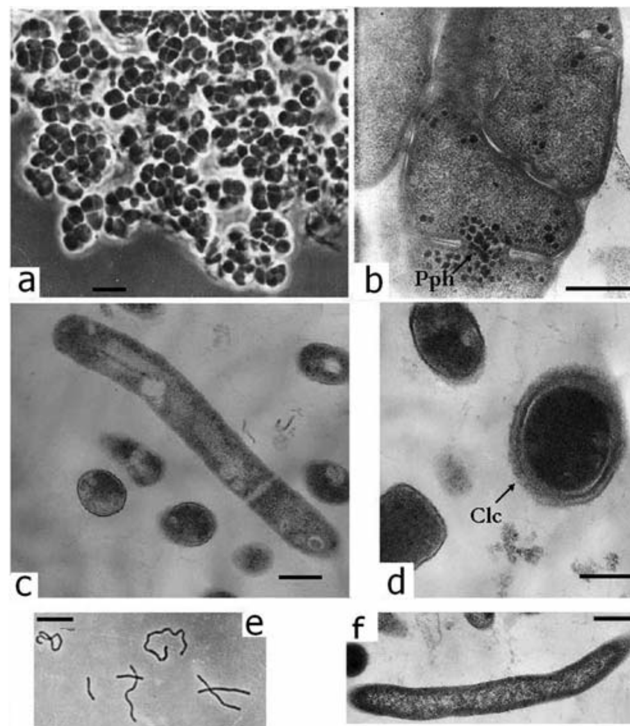

Fig. 6.4 Micrographs of methanogenic permafrost isolates. *Methanosarcina mazei* strain JL01: **a** phase contrast image, bar 10 mm; **b** ultrathin section, bar 0.5 mm. *Methanobacterium* sp. strain M2: **c** phase contrast image, bar 10 mm; **d** ultrathin section, bar 0.5 mm. *Methanobacterium* sp. strain MK4: **e** phase contrast image, bar 10 mm; **f** ultrathin section, bar 0.5 mm. *Pph*, polyphosphate inclusions; *Clc*, cyst-like cells (Photo of N. Suzina)

substrate for all enrichments, strain JL01 used only acetate, methanol, monomethylamine, dimethylamine and trimethylamine as carbon sources, while the other two strains grew exclusively on CO_2+H_2 (Rivkina et al. 2007). The presence of biogenic methane in permafrost includes original methane formation in sediments at temperatures above 0°C followed by its conservation during freezing. At the same time, one cannot exclude the possibility of methane formation within permafrost at subzero temperatures. This would depend on the ability of methanogens not only to survive and adapt in the permafrost but also to carry out metabolic reactions. Discovery of viable methanogens in ancient permafrost sediments provides significant evidence of the stability of these microbial populations through extremely long existence at subzero temperatures. The comparison of ancient isolates with modern methanogens provides a mean to understand their adaptation strategy, which is the goal of our future studies.

6.3.4 Resistance of permafrost bacteria to antibiotics and heavy metals

The occurrence of viable Cenozoic microorganisms within the permafrost is intriguing because an analysis of their features may provide a window into microbial life as it was before the impact of humans. It is often argued that the impact of industrial and urban pollution on bacterial communities results in the wide dissemination of various drug and heavy metal resistance genes carried by plasmids and transposons. The only environment on Earth which is a depository of unaltered microbial communities is permafrost. This is why the most straightforward way to check this idea is to obtain the data on the distribution of these genes among bacteria of the pre-industrial era, as well as to determine if the pre-industrial and modern microbial communities have different sensitivities to antibiotics and heavy metals. The first study was carried out in eastern Arctic, where microbial populations of modern tundra soil and ~3 million years old permafrost were tested for their resistance to antibiotics. The reduction in CFUs caused by these antibiotics on microbial populations recovered from modern tundra soils was loosely in agreement with the reduction expected for bacteria from arable temperate soils. At the same time, some of the ancient bacteria were more resistant to a number of antibiotics (novobiocin, carbenicillin, ampicillin, trimethoprim and bacitracin) than the modern populations, and the pattern of antibiotic sensitivity in permafrost was clearly very different from any that have been seen in a wide variety of modern soils studied (Tiedje et al. 1994).

Recently, strains resistant to the following antibiotics—chloramphenicol, streptomycin, kanamycin, gentamicin, tetracycline, spectinomycin, neomycin—were isolated from permafrost. The analyses of these strains indicate the presence of all types of mobile elements known among modern bacteria: plasmids, insertion sequence elements, transposons and, probably, integrons. For example, among streptomycin resistant bacteria from permafrost, strains that contain well studied and wide spread transposon Tn5393 with streptomycin-resistance genes were found

(Petrova et al., in press). This indicates that antibiotic resistance was common in microbial communities well before the commercial use of antibiotics. The cause of such enhanced antibiotic resistance is not clear, however it may be suggested that a generalized response of the community to in situ stresses, e.g., freezing and starvation, may also protect bacteria from some antibiotics.

Permafrost provides a unique possibility of direct molecular comparisons between "prehistoric" bacteria, which are perfectly free from industrial impact, and present-day bacteria, which experience anthropogenic stress. Mercury-resistant bacteria are an excellent subject for paleomicrobiological molecular studies. The "prehistoric" transposons closely related to mercury resistance transposons Tn*5041*, Tn*5042,* Tn*5053,* and Tn*5056,* which are widely distributed in present-day bacteria, were detected in mercury-resistant *Pseudomonas* strains isolated from permafrost (Mindlin et al. 2005). The number of mercury-resistant bacteria in permafrost varied significantly from 0.001 to 1.2–2.7% in sediments with high mercury concentrations (Petrova et al. 2002). The results testify that no drastic changes in distribution mode of the different types of mercury resistance transposons among environmental bacteria took place in the last 40,000 years. At the same time, the complex transposons of the Tn*21*-branch were not found in permafrost, but the transposon named Tn*5060*, nearly identical to the hypothetical mercury resistance transposon-precursor for wide family of complex transposons of Tn*21*-branch, was isolated (Kholodii et al. 2003). The results of the study of the ancient mercury resistance transposons allow to formulate that *mer* operons (mercury resistance transposons) have been widely distributed in environmental bacterial populations long before the beginning of the industrial era, and that the formation of integron-carrying transposons containing the determinants of multiple antibiotic resistance in addition to *mer* operons occurred much later, as a result of increasing antibiotic usage in men and animals.

6.3.5 Resistance of permafrost bacteria to radiation

Preserving bacterial cells during millions of years is a challenge since permafrost is not only characterized by stable cryogenic conditions inducing cryodesiccation of the cells, but these are, in addition, submitted to constant irradiation from native radio nuclides. The first estimation of ground radiation in Arctic permafrost has been made by McKay and Forman, using both elemental analysis of the radioactive elements in samples and direct in situ measurements in the boreholes. The dose of background radiation received by the permafrost bacteria depends on sediment type and is ~2–4 mGy year^{-1} (0.23 μGy h^{-1}) in sand and loams of alluvial origin on the Eurasian northeast, and ~1.3 mGy year^{-1} (0.15 μGy h^{-1}) in volcanic ash and scoria. Taking into account the age of bacteria, late Pliocene to late Pleistocene, the total dose received by cells would therefore range from 0.024 kGy in soils of 12,000 years old to 6 kGy in sediments over 3 million years in age (Gilichinsky 2002). Thus, bacterial cells within the permafrost should have some protecting mechanisms, allowing them to survive such a long time under constant irradiation conditions.

Experimental data demonstrate that bacteria entrapped in frozen soil have a much greater resistance to irradiation than bacteria in thawed soil. Firstly, the samples were irradiated by 22.8 Gy min^{-1} with Co^{60} γ source at temperatures above 0°C, and it was shown that the amount of water within the sample does not affect the radiation efficiency. Secondly, irradiation was performed in an especially designed cryostatic device at temperatures ranging from −20 to −25°C and the effect of irradiation differed for frozen and thawed samples. At equal levels of ionizing radiation, viable cell quantities and total radiation dose, this difference was about one order of magnitude for a dose of 1 kGy and is expected to increase for larger doses. Only 1% and 10% of the microbial population survived a dose of 1 kGy as calculated for unfrozen and frozen samples, respectively. Important indexes for estimation of irradiation stability of microbial population are LD_{50} and $LD_{99.9}$, i.e. doses of 50 and 99.9% lethality, respectively. These parameters differ by a factor of 3 for frozen and unfrozen samples.

From the biological point of view, subzero temperatures sharply decrease the microbial metabolic activity: the lower the rate of metabolic processes, the lower the radio lesions to biological objects. Subzero temperatures also induce the osmotic desiccation of the cells decreasing this way the effect of ionizing radiation. These facts indicate that: (1) the irradiation sensitivity of soil samples and furthermore for pure cultures at temperatures above 0°C differ from the sensitivity of microorganisms preserved in permafrost; (2) the frozen environment protects microbial cells from diffuse ground irradiation; and (3) permafrost is a unique environment where microorganisms display a high resistance over thousands and millions of years. Taking into account the natural radiation background of 1–2 mGy $year^{-1}$, the dose from radio nuclides diffused through the permafrost is far from sufficient for complete sterilization, i.e. it is not fatal to viable cells, but it is high enough to cause some selection effect and to destroy the DNA of ancient cells. The calculated data correlate with the number of viable cells in permafrost of different age and with experimental results: at 5 kGy, most of the cells in unfrozen samples died, while the number of surviving cells in frozen samples was still sufficiently large. The cell viability and growth on media implies a high capacity for DNA repair. On the basis of data concerning a metabolic activity at subzero temperatures (Gilichinsky et al. 1995; Rivkina et al. 2000, 2004; Carpenter et al. 2000; Price 2000; Price and Sowers 2004) we can conclude that DNA repair occurs in the frozen environment, i.e. at the stable rate of damage accumulation, while a comparable or lower rate of reparation also exists. Using the experimental data, some surviving forecasts for microbial complexes in native frozen ground, exposed to space radiation conditions could be done.

6.3.6 Resistance of permafrost bacteria to freezing-thawing stress

In nature, microorganisms inhabiting tundra soils show high resistance to annual temperature fluctuations, which cause the repetitive phase transition of water through the freezing point. But the question is: how would permafrost microorganisms

conduct itself in such a situation? Experiments have shown that microorganisms isolated from syngenetically frozen sediments, as well as soil microbial communities which have been exposed to the impact of multiple freeze-thaw stress, are resistant to sharp temperature transitions through 0°C and to freezing/thawing (12 h/12 h) stress. Such experiments simulate daily temperature fluctuations on the soil surface in spring and fall. In laboratory experiments, even after hundreds of repetitive freeze-thaw cycles, the number and diversity of viable cells did not change within the syngenetic permafrost samples, while samples from tropical soils often become sterile after a dozen of these cycles. Microorganisms from epigenetically frozen marine sediments are somewhat intermediate; they are resistant to the long-term impact of subzero temperatures, but do not experience the action of temperature fluctuations in their natural habitat and this is why they are sensitive to the phase exchange in surrounding environment. Similar repetitive freeze–thaw cycles led to an increase of microbial numbers by several orders. These results may be explained as follows. In the first stage, the frequent transitions through the freezing point may lead to massive cell death (Gilichinsky et al. 1993). In the following stage, the remaining cells stop dying and start to adapt to the new conditions.

Water formed during thaw contains sufficient nutritive materials, which initially are frozen and trapped in the ice. These nutritive solutes are expected to be sufficient for supporting the heterotrophic growth and prolongation of microbial communities. Certain group(s) of microorganisms (monoculture in most cases) become adapted to water phase transitions between the melted and frozen state, occupying these unique microhabitats created by the thin films of unfrozen water in the permafrost (Gilichinsky 2002). The same data were obtained with cyst-like resting forms of non-spore-forming permafrost bacterial strains of *Arthrobacter* sp. and *Micrococcus* sp. (Soina et al. 2004). The members of permafrost community, both prokaryotic (*Arthrobacter* sp., *Flavobacterium* sp.) and eukaryotic organisms (yeasts of the genus *Rhodotorula* sp., green algae of the species *Chlorella vulgaris, Chodatia tetrallantoidea*), also demonstrated resistance to freezing/thawing stresses after 3 months in frozen state at −5°C and complete darkness, modeling the annual soil freezing/thawing variations (Vishnivetskaya et al. 2003). After adaptation to the impact of prolonged subzero temperatures, the microbial communities within permafrost samples suddenly melted in the laboratory, subjected to stress of thawing, accompanied by exposure to oxygen, light, and temperatures above 0°C. This thawing stress induces all the other stresses; it is the most dangerous for permafrost organisms and known to inhibit the recovery of a fraction of the community. Improved strategies and techniques for recovery of bacteria from permafrost environments are only just beginning to be developed and one of them is the low-temperature cultivation.

Successive freeze-thaw cycles, which are characteristic of tundra soils, offer challenges and produce selective environments for cold adaptation of microbial communities. In order to characterize the freeze–thaw resistance of single-cell isolates, five species of the genus *Exiguobacterium* were subjected to 20 freeze-thaw cycles. Viable cell counts evidenced that bacteria grown in complex, structured environment (agar medium) better tolerated the freeze–thaw challenge than bacteria grown in mass-action environment (liquid medium) regardless of growth temperature.

However, growth temperature was a key factor of cryotolerance in mass-action (liquid) habitat. Bacteria grown at 4°C in liquid medium tolerate freezing/thawing much better than when grown at 24°C (Vishnivetskaya et al. 2007). From these experiments, we may conclude that microbes liberated in soil solution suffer more lethal effects from soil freeze–thaw than microbes sorbed on soil matrix.

6.4 Conclusions

Permafrost bacteria represent a unique material for research on microbial evolution and low temperature adaptation, and they may possess unique mechanisms that allow them to maintain viability for very long periods. Therefore, permafrost is of great significance for research in cryo- and microbiology, biotechnology, ecology, molecular biology, paleontology and the newly emerging field of Astrobiology.

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Chapter 7
Anaerobic Bacteria and Archaea in Cold Ecosystems

Kai Finster

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7.1 Introduction

Permanently cold environments are very common on Earth. For example, the average temperature in bottom waters of the largest fraction of the world oceans is 5°C or less and including terrestrial habitats about 80% of the Earth's biosphere is to be found in permanently cold habitats (Russell 1990). In addition to being cold, many of these environments are also oxygen-free, thus supporting exclusively facultative or obligately anaerobic microbial life. Anoxic permanently cold environments are very diverse and include the marine sea floor (Rysgaard et al. 1998; Sagemann et al. 1998; Bowman et al. 2003; Vandieken et al. 2006b), microbial mats (Mueller et al. 2005; Fernández-Valiente et al. 2007), endolithic communities in sandstones (Friedmann 1982), permafrost soils of the Arctic and Antarctic regions (Kobabe et al. 2004; Gilichinsky et al. 2005; Steven et al. 2007), and chilled food (Broda et al. 2000a, 2000b, 2002). It has also been demonstrated that these habitats harbor extensive microbial communities, including many microorganisms that are phylogenetically affiliated with obligately anaerobic organisms in culture (Ravenschlag et al. 1999; Purdy et al. 2003; Ganzert et al. 2007). Despite their ecological and economical significance very little is known about anaerobic bacteria and archaea

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that live in these environments and the mechanisms by which they thrive or survive under in situ conditions.

In this review paper, I have collected all accessible (to me) information on validly described obligately anaerobic psychrophilic bacteria and archaea. In addition, I have included information on selected psychrotolerant species. I have applied the definition of psychrophily introduced by Morita (1975) with modifications proposed by Scherer and Neuhaus (2006) for the definition of psychrotolerant microbes. Thus obligate psychrophiles are organisms with a minimal growth temperature $<0°C$, a temperature optimum for growth $\leq 15°C$ and a maximum temperature $\leq 20°C$. Psychrotolerant organisms have a minimal growth temperature $<7°C$, a temperature optimum for growth $\leq 20°C$ and a maximum temperature $\leq 35°C$. According to Scherer and Neuhaus (2006) the minimum temperature was set to $<7°C$ instead of $\leq 0°C$ to cope with the variation in reporting on the growth of microorganisms in chilled food.

7.2 Bacteria

7.2.1 The genus Clostridium

The genus *Clostridium*, which contains obligately anaerobic fermenters, accounts at present for a large number of validly described species of psychrophilic and psychrotolerant obligate anaerobes within a single genus. This may mainly be due to the fact that the genus is defined by rather broad morphological and physiological traits, such as a Gram-positive cell wall, spore formation and energy generation by fermentation. On the basis of these characters, strains are clustered into the same genus, which are, based on 16S rRNA phylogeny, more than 10% different.

7.2.1.1 Psychrophilic clostridia

Of the nine psychrophilic clostridia, five were isolated from microbial mats in Antarctica (*C. frigoris, C. lacusfryxellense, C. bowmannii, C. psychrophilum* (Spring et al. 2003) and *C. vincentii* (Mountford et al. 1997), two from chilled meat (*C. estertheticum subsp. estertheticum*; Collins et al. 1992; and *C. gasigense*; Broda et al. 1999), one from cattle manure (*C. estertheticum* subsp. *laramiense*; Kotsyurbenko et al. 1995a) and one from overcooled water brine of arctic permafrost (*C. algoriphilum*; Shcherbakova et al. 2005). With the exception of *C. algoriphilum,* the publications on the other psychrophilic or psychrotolerant clostridia compile information on the isolates without specific focus on their ability to grow at low temperature (for details see http://www.bio.au.dk/KaiFinster/tables/). Therefore, the following summary of observed responses to temperature among clostridia is exclusively based on the study of *C. algoriphilum*.

C. algoriphilum was isolated from overcooled water brines, the so-called cryopegs, a habitat type that can be found in permafrost soil of marine origin. Cryopegs

are characterized by high salt concentration, which allows water to be in the liquid state even at in situ temperatures of about −10°C (Gilichinsky et al. 2005). *C. algoriphilum* has the lowest documented temperature limit of growth (−5°C) of all validly described psychrophilic obligately anaerobic bacteria and archaea. A transformation of the temperature dependence of growth rates using the Ratkovsky model (Ratkovsky et al. 1983) results in a predicted lowest growth temperature of −43°C, which is the lowest theoretical growth temperature yet determined. This indicates that *C. algoriphilum* may not only survive in its habitat but may also be metabolically active and even proliferate. However, incubation of cells of *C. algoriphilum* in original cryopeg water induced sporulation not growth. The strain shows several interesting physiological responses depending on the temperature at which it was grown. For example, a change in the optimum growth rate as a function of NaCl concentration of the growth medium was observed when the culture was grown at +5°C or −5°C, respectively. At −5°C, the highest grow rate was obtained with 1.0% NaCl in the growth medium and growth was observed between 0 and 10% NaCl, while at 5°C the culture grew best at 0.5% NaCl and the highest NaCl that permitted growth was 5%. The growth temperature also affected the fermentation pattern of glucose as well as the patterns of substrate production. Gilichinsky et al. (2005) also reported that the incubation temperature not only had an effect on the velocity at which the organism was able to grow on the specific substrate but whether it could grow on a specific compound at all. While glucose, sucrose, and trehalose supported growth at 18, 5 and −2°C, growth on L-glutamate was only observed at −2°C. The observation suggests that combinations of substrates should be employed during enrichment and isolation rather then single compounds, as specific substrate may incidentally not be used at a particular incubation temperature and consequently the enrichment may fail.

The composition of lipids that constitute the cell membrane of *C. algoriphilum* show clear adaptations to low temperature by a significant prevalence of short chain fatty acids ($C_{14:0}$ = 33%) and a high content of unsaturated fatty acids (60%). Both types of compounds increase the fluidity of the cell membrane at low temperature. Unfortunately, Shcherbakova et al. (2004) determined the composition of the lipids only at one temperature and thus the effect of temperature on the lipid composition of the membrane cannot be evaluated.

7.2.1.2 Psychrotolerant clostridia

The website http://www.bio.au.dk/KaiFinster/tables/ compiles data on several psychrotolerant clostridia. The table is incomplete as it only contains strains that were selected according to the following criteria: (1) the title of the publication includes the terms psychrotolerant (*C. algidixylanolyticum, C. frigidicarnis*) or psychroactive (*C. fimetarium)*, or (2) the species was mentioned in the description of a psychrophilic/psychrotolerant *Clostridium* as a strain expressing temperature patterns that would classify it as psychrotolerant sensu Scherer and Neuhaus (2006). Despite the fact that *C. schirmacherense* expressed the highest growth efficiency in the

5–10°C temperature range and for that reason is described as a psychrophile by Alam et al. (2006), it is here grouped among the psychrotolerant strains, as its upper limit for growth is 35°C and thus by far exceeds the upper limit for psychrophiles sensu Morita (1975). Only *C. schirmacherense* and *C. fimetarium* have been subjected to studies that addressed the strains response to different temperatures. With *C. schirmacherense* the protease activity was measured as a function of temperature. Alam et al. (2005) demonstrated that a purified protease from *C. schirmacherense* expressed 5–8% of its activity measured at T_{opt} (37°C) at 0°C. The membrane composition of *C. fimetarium* was determined with cultures grown at 6 and 25°C, respectively. The content of unsaturated compounds was slightly higher in 6°C cultures (55.1%) than in 25°C cultures (48.6%), and the content of short chain fatty acids ($C1_{14:0}$) increased from 4% at 25°C to 16% at 6°C. Both adaptations are typically reported for psychrotolerant microorganisms (see Sect. 7.2.2 below). The growth rate curve was also typical for psychrotolerant microbes, showing an optimum between 20 and 25°C. The growth rate at 6°C, which is the in situ temperature of the manure the strain was isolated from, was $0.026\,h^{-1}$, a 70% reduction compared to the rate obtained at T_{opt}. Apart from general descriptions, surprisingly little work has been done on food-spoiling clostridia such as *C. algidixylanolyticum, C. frigidicarnis, C. estertheticum subsp. estertheticum* and *C. gasigense* at in situ temperatures. More detailed studies on the temperature dependence of their physiological properties could have significant practical implications for the treatment and protection of meat products against contamination and further food spoilage.

In general, more studies dedicated to the effect of temperature on metabolism and growth of clostridia are needed to either validate or extend the sparse information that is currently available. In addition, the biotechnological potential of psychrophilic and psychrolerant clostridia has hardly been explored as yet. I only came across one publication (Akila and Chandra 2003) which reports on low temperature active xylanase and cellulase activity from a cold tolerant clostridium, which had a maximal activity at 20°C. This is surprising as energy saving is an important aspect of future processing and production. Furthermore, psychrophilic clostridia or other fermenting microorganisms from anoxic permanently cold marine sediments have only been described sporadically so far (Finne and Matches 1974). They may be important players in the anaerobic food chain in marine sediments (Arnosti and Jørgensen 2003; Arnosti et al. 2005) and may provide sulfate reducers and methanogens with their substrates (Schmitz et al. 2006).

7.2.2 Sulfate-reducing bacteria

7.2.2.1 Psychrophilic sulfate-reducing bacteria

The first psychrophilic sulfate reducer was described 10 years ago by Isaksen and Jørgensen (1996) and Isaksen and Teske (1996; http://www.bio.au.dk/KaiFinster/tables/). The sediment-inhabiting organism, designated *Desulforhopalus vacuolatus*,

contains large gas-vacuoles of as yet unknown function. The strain was subjected to detailed investigations focusing on its response to different temperatures. The temperature optimum of growth was significantly lower than the temperature optimum of sulfate reduction, which was determined in a short-term experiment with radiolabled sulfate (Isaksen and Jørgensen 1996). Sulfate reduction in contrast to growth involves a limited set of enzymes, which may all be relatively insensitive to higher temperature. The relatively high temperature maximum of sulfate reduction (28°C) would group *D. vacuolatus* among the mesophilic bacteria sensu Morita (Morita 1975), while the temperature optimum of growth (19°C) places it among the psychrotolerant bacteria. However, the Arrhenius plots of growth and sulfate reduction rate data showed a linear relationship between T_{opt} and T_{min} (28°C to −1.8°C for sulfate reduction and 18°C to 0°C for growth). Isaksen and Jørgensen (1996) interpret the linear response as an adaptation of the entire enzymatic machinery to low temperature. The observed linearity over the entire temperature range places *D. vacuolatus* among the psychrophiles. Thus, a single organism fits into three different temperature categories depending on the criteria that were used, which exposes the difficulties involved in grouping organisms according to the currently used definitions. With respect to growth yield (g biomass mol^{-1} substrate), *D. vacuolatus* expressed the highest and almost constant growth yield between 15°C and 0°C while the growth yield decreased towards the T_{opt} for growth. Both Arrhenius plot data and the growth yield data demonstrate that *D. vacuolatus* is particularly well adapted to low temperature (Isaksen and Jørgensen 1996). It is a weakness of this study and all the other studies of obligately anaerobic prokaryotes that they were carried out in batch cultures at high substrate concentrations. It would be very interesting to obtain information on the growth characteristics at low, and thus ecologically more relevant, substrate concentrations in combination with low temperatures.

D. vacuolatus was the only validly described psychrophilic sulfate reducer until Knoblauch et al. (1999a) reported the isolation of 19 psychrophilic sulfate reducers from permanently cold sediment from the coast of Spitsbergen, and published a detailed physiological study of three new genera of psychrophilic sulfate reducers (Knoblauch et al. 1999b; http://www.bio.au.dk/KaiFinster/tables/). The description was accompanied by comprehensive biogeochemical and molecular ecological investigations of samples from the sampling site from which the isolates were obtained, substantiating that the isolates very likely were biogeochemical key players in the system (Knoblauch et al. 1999a; Knoblauch and Jørgensen 1999; Sahm et al. 1999). All the isolates were metabolically active at in situ temperatures of −1.8°C and 2.6°C, respectively, and their relative growth rates at 0°C were >25% of the rates measured at T_{opt}. The latter observation distinguishes them from the psychrotolerant sulfate reducers, which suffer from a much more pronounced growth rate reduction at 0°C compared to growth rates at optimal growth temperature (Table 7.1). The growth yields determined by Knoblauch and Jørgensen (1999) in the low temperature range were comparable to yields measured with mesophilic sulfate reducers on the same substrates. The efficiency of an organism to transform a substrate into biomass and finally proliferate is crucial for its competitiveness in

Table 7.1 Cold adaptation among psychrophilic and psychrotolerant sulfate reducers

Genus and Species	Substrates	Rel. growth rate at 0 or [a]4°C vs. T_{opt} [%]	E_a [kJ mol^{-1}] and (Q_{10})	Temp. range [°C] used for calculation of [b]E_a and (Q_{10})	Growth yield [g dry weight mol^{-1}] (at °C)	T_{opt} for SRR	Growth
Psychrophilic							
Desulfofaba gelida[1]	Propionate	41	n.r.	n.r.	3.8 (5) and 3.2 (−1.8–5)	n.r.	7
Desulfofrigus oceanense[1]	Acetate	24	59 (2.5)	−2–17	4.9 (7) and 3.5 (−1.8)	17	10
Desulfotalea psychrophila[1]	Lactate	33	54.3 (2.3)	−4–12	4.2 (−1.8–15)	10	10
Moderately psychrophilic							
Desulfofrigus fragile[1]	Lactate	31	58.2 (2.4)	−4–22	7.2 (4) and 4.8 (18)	27	18
Desulfotalea arctica[1]	Lactate	29	54 (2.3)	−2–22	5.8 (0–4) and 2.8 (18)	23	18
Desulforhopalus vacuolatus[1,5]	Lactate	2	86.1 (3.4)	10–20	9.5 (0–12) and 5 (19)	28	19
Psychrotolerant							
Desulfobacter psychrotolerans[2]	Acetate	5	130.2 (8.0) and 64.3 (2.6)	(−3.6–6.4) and (6.4–16.4)	4.4 (5–15) and 3.5 (20)	26	20
Desulfobacter hydrogenophilus[6]	Acetate	8[1]	187 (18.4) and 59.9 (2.4)	(0–10) and (8–18)	n.r. n.r.	n.r.	n.r.
Desulfobacterium autotrophicum[3]	Lactate	3	n.r.	n.r.		n.r.	n.r.
Desulfovibrio cuneatus[4]	Lactate	22–26[a]	40 (1.75)[b]	(10–20)	3.4 (20) and 2 (28)	n.r.	28
Desulfovibrio litoralis[4]	Lactate	36[a]	38 (1.70)[b]	(10–20)	4.2 (10) and 3.2 (28)	n.r.	28

References: [1]Knoblauch and Jørgensen (1999);[2]Tarpgaard et al. (2006); [3]Rabus et al. (2002); [4]Sass et al. (1998); [5]Isaksen and Jørgensen (1996), [6]Bak (1988)

[a]Calculated at 4°C

[b]Calculated from values depicted from Sass et al. (1998)

n.r., not reported

E_a, Activation energy

SRR, sulfate reduction rate

nature and thus growth yield data are more informative then growth rate data, when using laboratory data to estimate the organisms' competitiveness in nature.

7.2.2.2 Psychrotolerant sulfate-reducing bacteria

The psychrophilic sulfate reducers can be distinguished from the psychrotolerant sulfate reducers when comparing the Arrhenius plots of growth and sulfate reduction rates of the two groups (http://www.bio.au.dk/KaiFinster/tables/). Though the material is currently still very limited it seems that the Arrhenius plot data of sulfate reduction and growth rates obtained with psychrophilic sulfate reducers were fitted best by one line ranging from T_{opt} to T_{min}, while the data obtained with psychrotolerant sulfate reducers could only be fitted with two lines resulting in two very different E_a values (Table 7.1; Bak 1988; Rabus et al. 2002; Tarpgaard et al. 2006). The point of infliction of the two lines in the Arrhenius plots of the psychrotolerant sulfate reducers is called the critical temperature, which indicates that cells are well adapted to temperature changes above $T_{critical}$, but only poorly to changes in temperature between $T_{critical}$ and T_{min}. The biochemical background for the bimodality has not yet been conclusively elucidated. Membrane fluidity may be an important factor, as this would influence transport processes including the supply with energy sources. Könneke and Widdel (2003), studying the effect of growth temperature on the fatty acid composition of the membrane of sulfate-reducing bacteria, reported significant differences in the response to different temperatures when psychrophiles were compared to psychrotolerant or mesophilic sulfate reducers. They observed that the proportion of cis-unsaturated fatty acids was high in psychrophiles and there was no significant change in the proportion with decreasing temperature. The latter was the case when psychrotolerant sulfate reducers were grown at decreasing temperatures. They conclude that the fatty acid patterns in psychrophiles were optimized to function in a permanently cold environment. The response of psychrotolerant sulfate reducers reported by Könneke and Widdel (2003) was also found by Rabus et al. (2002) investigating the effect of temperature change on the composition of the cellular fatty acids of *Desulfobacterium autotrophicum* as well as by Tarpgaard et al. (2006) studying *Desulfobacter psychrotolerans. D. autotrophicum* increased the relative fraction of unsaturated fatty acids as well as the relative fraction of short chain fatty acids, while *D. psychrotolerans* only increased the fraction of unsaturated fatty acids. The latter was also reported by Könneke and Widdel (2003) with both the psychrotolerant strain *D. hydrogenophilus*, which is closely related to *D. psychrotolerans*, and other mesophilic *Desulfobacter* species. During their investigation, Könneke and Widdel (2003) observed that the proportion of the unsaturated fatty acid *cis* 16:1(9) ranged from about 38% during late exponential growth to about 12% during stationary phase when cultures were grown at 28°C. In cultures grown at 12°C, the fraction of 16:1(9) steadily increased during exponential growth and reached a constant level of nearly 50% during late exponential growth. This observation indicates that the composition of the cellular membrane is not affected by temperature alone and care has to taken when the results are interpreted. Whether

psychrophilic or psychrotolerant sulfate reducers have other physiological adaptations, such as low temperature active enzymes or transport proteins, is currently not known. Clues may be obtained from genome sequence data from psychrophiles. Recently, the genome of *Desulfotalea psychrophila* was fully sequenced (Rabus et al. 2004). In contrast to results obtained with psychrophilic methanogens, where the analysis of proteins revealed a high content of non-charged polar amino acids and a lower content of hydrophobic amino acids than found with mesophilic and hydrophilic archaea and which were interpreted as adaptations to low temperatures (Saunders et al. 2003), no such patterns were obtained from the genome analysis of *D. psychrophila*. The presence of genes encoding cold-shock proteins that are involved in regulation and DNA processing were identified. However, it cannot be concluded from these data that the cold-shock genes are particularly important for the strain's ability to grow at low or at high temperature. In addition, Rabus et al. (2004) reported the presences of at least 12 tRNA modifying enzymes, which might be important for cold adaptation of the translation process and they also identified a new transcriptional regulation mechanism, which may be relevant for growth at low temperatures. Finally, they reported on the presence of a type of helicases that had been shown to enable bacteria to survive cold shock and grow at low temperature (Lim et al. 2000). Overall at present, mining of the genome sequence data is far from being completed and thus more information may be extracted with respect to the strain's psychrophilic nature.

7.2.3 Sulfur- and iron-reducing bacteria

Recently, the first obligately anaerobic sulfur and iron reducers have been isolated that expressed cold adaptation (Holmes et al. 2004; Nevin et al. 2005; Vandieken et al. 2006a). In particular from an ecological point of view, the isolation of cold-adapted iron reducers is very interesting because iron reduction has been identified as an important process in carbon cycling in many cold marine and freshwater environments (Lovley et al. 2004; Vandieken et al. 2006b). *Geopsychrobacter electrodipilus* was isolated from the surface of an anaerobic electrode in a laboratory-incubated marine sediment fuel cell, used to extract electro-chemical energy from anoxic sediments (Holmes et al. 2004). With acetate and Fe(III) oxide as substrates the growth rates were determined between 4 ($\mu = 0.006\,h^{-1}$) and 30°C ($\mu = 0.004\,h^{-1}$). The strain grew optimally at 22°C ($\mu = 0.011\,h^{-1}$). Since the strain grew at all temperatures tested, the lower and upper limits for growth have not yet been determined. However, the preliminary documented temperature regime would group *G. electrodipilus* among the psychrotolerant microorganisms. Organisms like *G. electrodipilus* may have interesting technical applications due to their capacity to transfer chemical energy to electrodes. It would thus be interesting to explore the temperature dependence of this mechanism.

Nevin et al. (2005) reported on the isolation of three closely related strains that were grouped into the species *Geobacter psychrophilus*. The iron reduction rate at 4°C was

about one-third the rate measured at 30°C. No iron reduction could be observed at 37°C. Thus, the strain's lower limit for iron reduction has not been determined yet.

Two psychrophilic iron reducers, designated *Desulfuromonas svalbardiense* and *Desulfuromusa ferrireducens*, were isolated by Vandieken et al. (2006a) from permanently cold sediment close to Svalbard. The two species expressed psychropilic temperature adaptations and grew well at the in situ temperature of their natural environment (−2°C). Both grew at −2°C and had a temperature optimum for growth at round 15°C, but differed slightly in their respective maximum growth temperature. While *D. svalbardiense* did not grow above 20°C, the upper temperature limit of *D. ferrireducens* was 23°C.

7.2.4 Acetogenic bacteria

Studies by Conrad and coworkers indicate that homoacetogens are important hydrogen consumers in cold anoxic sediments (Conrad et al. 1989 Schulz and Conrad 1996; Kotsyurbenko et al. 2001) and several cold-adapted strains of acetogens have recently been obtained in pure culture (Kotsyurbenko et al. 1995b; Simankova et al. 2000; Paarup et al. 2006). The isolates were obtained from habitats as diverse as paper-mill wastewater (*Acetobacterium bakii*), fen sediment (*A. paludosum*), digested manure (*A. fimetarium*), tundra soil (*A. tundrae*) and fjord sediment (*A. carbinolicum* subsp. *kysingense*). A phylogeny of the strains based on 16S rRNA gene sequence comparison indicates that they are closely related.

Despite the fact that the Kotsyurbenko and the Simankova publications characterize the isolates as psychrophilic, none of the isolates fits the definition sensu Morita (1975). The four strains, designated *Acetobacterium bakii*, *A. paludosum*, *A. fimetarium* (Kotsyurbenko et al. 1995b) and *A. tundrae* (Simankova et al. 2000), grew between 1 and 30°C with temperature optima for growth at about 20°C. On the basis of their cardinal temperatures they should thus be grouped with the psychrotolerant bacteria. The growth curves of the Kotsyurbenko isolates differed considerably. *A. fimetarium* grew fastest at 30°C ($\mu = 0.15\,h^{-1}$). The growth rate decreased rapidly down to 15°C ($\mu = 0.035\,h^{-1}$), hereafter it decreased slightly towards 1°C ($\mu = 0.02\,h^{-1}$), which was the lowest temperature at which growth was measured. The growth curve of *A. paludosum* peaked at 20°C ($\mu = 0.2\,h^{-1}$) and decreased steadily to 1°C ($\mu = 0.04\,h^{-1}$). *A. bakii* like *A. paludosum* peaked at 20°C ($\mu = 0.11\,h^{-1}$) but the growth rate was only about half the rate of *A.paludosum*. While the rate decreased very rapidly towards T_{max} at 30°C, it decreased very slowly towards T_{min} and was still about two-thirds the rate determined at T_{opt}. Thus, all three strains expressed relatively high growth rates at the lowest temperature at which the rates were measured while the lower limit for growth had consequently not yet been determined. The different growth curves reflect interesting underlying physiologies, which await further elucidation.

In a detailed study on the competition between acetogens and methanogens for hydrogen at low temperature, Kotsyurbenko et al. (2001) measured H_2 consumption

kinetics (V_{max}, K_m and the hydrogen threshold) of *Acetobacterium bakii*, *A. paludosum*, *A. fimetarium* and *A. tundrae*. The hydrogen threshold decreased with decreasing temperature in cultures of *A. bakii* and *A. tundrae*, while in *A. paludosum* and *A. fimetarium* thresholds increased again below 10–15°C. This observation indicates that *A. baki* and *A. tundrae* would out-compete *A. paludosum* and *A. fimetarium* at low temperatures and low hydrogen partial pressure.

7.2.5 Anoxygenic phototrophic bacteria

Herbert and coworkers published first results on isolated anoxygenic phototrophic bacteria from Antarctica in the mid-1970s (Herbert 1976; Herbert and Tanner 1977). Herbert (1976) reported that the isolated strains grew slowly between 0 and 5°C and expressed optimal growth at 25°C, which fits well with a psychrotolerant temperature regime. The strains survived well repeated slow freezing and thawing and survived long periods (2 years) of permanent freezing. Herbert (1976) concluded that they were able to withstand long periods of darkness and cold, as it is the case during the Antarctic winter.

The first validly described psychrotolerant anoxygenic phototroph was published by Madigan et al. (2000) and given the name *Rhodoferax antarcticus. R. antarcticus,* a member of to the purple non-sulfur bacteria, grew at all temperatures it was tested at between 0°C (μ = 0.003 h^{-1}) and 25°C (μ = 0.011 h^{-1}) and had a temperature optimum for growth between 12 and 18°C (μ = 0.03 h^{-1}). Cells survived temperatures above 25°C for at least 1 week but were not able to proliferate. Between 5 and 24°C, the growth rate varied by a factor of 2 while it decreased by a factor of 3 when the temperature dropped from 5 to 3°C. The cause or causes for this dramatic response in growth rate to a minor change in temperature was not investigated. Madigan et al. (2000) may have succeeded in the isolation of phototrophs growing at lower temperatures then the strains isolated by Herbert (1976) because they kept the inoculum and the enrichment cultures at 5°C, which may have prevented psychrotolerant microbes from out-competing psychrophilic once (Harder and Veldkamp 1971).

7.2.6 Miscellaneous

A few studies can be found in the literature which report on results obtained with unidentified (named) anaerobic heterotrophs that can be grown at low temperature. Dyrset et al. (1984) reported on an anaerobic bacterium designated strain B6 that shared phenotypic traits with *Bacteroides*. The isolate had the highest growth rate at 15°C and did survive but not grow at temperatures above 21°C. A fermenting coil-shaped psychrophilic bacterium was isolated by Franzmann and Rohde (1991) from anoxic water samples from the Antarctic meromictic Ace Lake. Prior to isolation,

the strain was part of a coculture with a trimethylamine-utilizing methanogen (*Methanococcoides burtonii*, see below). The strain grew well at the in situ temperature of Ace Lake (1.7°C; $\mu = 0.013\,h^{-1}$), had a temperature optimum between 15–16°C ($\mu = 0.12\,h^{-1}$) and did not grow at 22°C. Morphologically similar cells were observed in the anoxic hypolimnion of nearby Burton Lake at considerable abundance (10^5 cells ml^{-1}; McGuire et al. 1987). However, Franzmann and Rohde (1991) did not demonstrate that the isolate and the cells in MPN cultures, apart from sharing a common morphology, were related. Franzmann and Rohde (1991) also reported on the isolation of a cell wall-less anaerobic bacterium from the hypolimnion of Ace Lake, which affiliated with the genus *Spirochaeta* (Franzmann and Dobson 1992, 1993). The strain was psychrophilic with an optimum temperature for growth between 12 and 13°C (growth rate not reported). The growth rate at in situ temperature (1.7°C) was $0.013\,h^{-1}$. The strain grew well under proxy in situ conditions (salinity, pH, temperature) in the laboratory. Recently, the isolation of the first psychrotolerant syntrophic bacterium was reported by Kendall et al. (2006) from marine sediment in Skan Bay, Alaska. *Algorimarina butyrica* oxidized butyrate syntrophically in defined coculture with a hydrogen using methanogen. The presence of butyrate oxidizing syntrophic bacteria in marine sediment is surprising, as one would expect them to being out-competed by sulfate reducers such as the psychrophilic sulfate reducer of the genus *Desulfofrigus* or *Desulfofaba* (Knoblauch et al. 1999b). *A. butyrica* grew extremely slowly and colonies were not observed before 6–7 months at a growth temperature of 15°C. *A. butyrica* did not grow above 25°C, while the methanogenic coculture grew well above that temperature. *A. butyrica* grew at 10°C but was not tested at lower temperatures. Growth rates were not determined.

7.3 Archaea

Studies on cold-adapted obligately anerobic archaea are restricted to methanogens. Methanogens play a quantitatively very important role as terminal consumers in anoxic permanently cold environments such as lake sediments (Nozhevnikova et al. 2001), tundra soil (Kobabe et al. 2004) or permafrost (Ganzert et al. 2007). Hitherto, five psychrophilic and psychrotolerant methanogens have been isolated: *Methanococcoides burtonii* (Franzmann et al. 1992), *Methanogenium frigidum* (Franzmann et al. 1997), *Methanosarcina lacustris* (Simankova et al. 2001), *Methanosarcina baltica* (von Klein et al. 2002), and *Methanococcoides alaskense* (Singh et al. 2005) (http://www.bio.au.dk/KaiFinster/tables/). In addition, Simankova et al. (2003) have reported on the isolation of cold-adapted methanogenic strains that were closely related to validly described psychrotolerant and mesophilic species. All isolates of the Simankova study grew at 1–5°C. However, a temperature optimum of 25–35°C classifies them as psychrotolerant.

Methanococcoides burtonii, isolated from cold anoxic button waters of Ace Lake, is the best studied of all the cold-adapted methanogens and has been subjected

to detailed biochemical/proteome (Nichols and Franzmann 1992; Thomas and Cavicchioli 1998; Goodchild et al. 2004a, 2004b) and genome analysis (Saunders et al. 2003). These studies focused on cold-adaptations in that strain. The cellular adaptations of this organism to cold are nicely summarized by Cavicchioli (2006). They include cold-related modification both on the structural as well as on the process level. Nichols and Franzmann (1992) demonstrated a large fraction of unsaturated diether lipids (57%) in membrane components of *M. burtonii*, which they interpret as an adaptation to low temperature. In comprehensive studies of the proteome of *M. burtonii*, Goodchild et al. (2004a, 2004b) investigated protein patterns from cultures grown at 4 and 23°C, respectively. Apart from a significant difference in the expression of a large number of proteins as a function of temperature also on the mRNA level, the authors report the interesting observation that heat shock protein DnaK was expressed at much higher levels at $T_{opt,}$ than at 4°C. This may indicate, according to Goodchild et al. (2004a), that life at optimal temperature was stressful to the organism, a feature that was also discussed by Feller and Gerday (2003). This observation challenges the view of psychrophiles being poorly adapted to the low temperature of their habitat, because T_{opt} is usually much higher than $T_{in\ situ}$. The study on *M. burtonii* demonstrates nicely how new technologies (genomics in combination with proteonomics) can be deployed to study the biochemistry/physiology of difficult to grow microorganisms and should be extended to other psychrophiles.

Studies on the other methanogens are more on a descriptive level and have only generated information on the temperature regime of the isolates (http://www.bio.au.dk/KaiFinster/tables/). Data on yields at different temperatures, as it was the case for the sulfate reducers have not been published yet.

7.4 Conclusions

First, despite the fact that permanently cold anoxic environments are widely distributed around the globe, our knowledge about obligately anaerobic psychrophilic bacteria and archaea is very sparse. This is very likely a consequence of the intrinsic difficulties in working with these kinds of microbes. They are both difficult to isolate and grow, and enzymatic studies of their metabolic pathways are notoriously tedious and technically demanding. A combination of different "omics" is very promising and could help overcome the methodological problems with culturing the organisms. Nevertheless, there is also an urgent need for the isolation of more obligately anaerobic psychrophilies to understand the mechanisms by which they have adapted to the cold. Results obtained from the studies may also contribute to "Search for Life missions" to Mars or Europa, both cryo-environments with a potential for active life forms.

Second, and interestingly, the world of psychrophilic obligately anaerobic archaea is still very little explored. Considering the large reservoirs of methane in the sea floor and the large potential for methane productions in permafrost soil,

there is an urgent need to gain more insight into the physiology of these organisms. As producers of a very strong green house gas these organisms may have a very important impact on the prevalence and geographic distribution of permanently cold environments in the future.

Third, concerning the publications on cold-adapted microbes, more comprehensive efforts are needed that elucidate the strategies of the different microbes to cope with the cold. Often, little information on cold adaptation of the specific isolates is provided including activation energies and Q_{10}, and growth yield data. It would also be helpful to include square root transformed growth rate data to obtain comparable theoretical T_{min} values. Also, the often-observed large discrepancy between T_{opt} and $T_{in\ situ}$ should be addressed in more detail and studies should include investigations at $T_{in\ situ}$. Quoting from Cavicchioli (2006): "In the view of the ecological data and a range of physiological indicators (for example enzyme secretion, macromolecular synthesis, membrane permeability, viability and growth yield), the molecular indicators clearly show that T_{opt} is a poor measure of cold adaptation". T_{opt} may not only be a poor but even a misleading indicator.

Last but not least, there is a strong need for a clear unifying definition of the terms "psychrophilic", "psychroactive", "psychrotrophic" and "psychrotolerant" and an agreement on which terms should be used. Currently it seems to be up to the individual scientist to decide whether a specific isolate fits into one regime or the other. This creates a lot of confusion and makes it difficult to compare different organisms.

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Chapter 8
Cyanobacteria in Cold Ecosystems

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8.1 Introduction

Perennially cold environments in which temperatures remain below 5°C are common throughout the biosphere (Margesin and Häggblom 2007). In these habitats, the persistent cold temperatures are often accompanied by freeze–thaw cycles, extreme fluctuations in irradiance (including ultraviolet radiation), and large variations in nutrient supply and salinity. As a result of these constraints, polar and alpine environments

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contain a reduced biodiversity, with prokaryotes contributing a major component of the total ecosystem biomass as well as species richness. Cyanobacteria are of particular interest because they often represent the predominant phototrophs in such ecosystems. Current research shows that a diverse range of cyanobacteria can be found in polar and alpine habitats, and that they show a remarkable ability to tolerate the abiotic stresses that prevail in these cold environments. Their presence was already observed during the early explorations of the polar regions at the end of the nineteenth century (Vincent 2007).

The widespread distribution in cold habitats of communities dominated by cyanobacteria, particularly in ice-based environments, makes them of great interest for the reconstruction of microbial life and diversification on early Earth (Vincent et al. 2004b). These ice-based habitats with their sustainable microbial communities are potential analogues for biotopes present during the major glaciation events of the Precambrian. The fossil record suggests that cyanobacteria would have been present throughout these Proterozoic events, and perhaps during earlier periods of global cooling (Schopf 2000). Polar microbes, including cyanobacteria, are also of interest to astrobiologists studying the prospect of life beyond our planet. Antarctica has been proposed as an analogue to an early stage of Mars where liquid water occurred and where life could have evolved at a similar time to the development of cyanobacteria on early Earth (Friedmann 1986).

In this chapter, we first introduce the taxonomical status and the general characteristics of cyanobacteria. We then examine cyanobacterial diversity in Antarctic, Arctic and alpine habitats, focusing on the molecular approaches. The ecophysiological traits of cyanobacteria that allow them to survive and often thrive in such cold environments are also presented. We conclude this review by consideration of the biogeographical distribution of polar cyanobacteria, an active topic of current research.

8.2 Taxonomy and diversity

Cyanobacteria are Gram-negative oxygenic photosynthetic bacteria that, according to the fossil record, achieved most of their present morphological diversity by two billion years ago (Schopf 2000). Cyanobacteria were initially described as algae in the eighteenth century and the first classification system was based on the International Code of Botanical Nomenclature as described by Oren (2004). In the botanical taxonomy, two major works can be noted. Firstly, Geitler (1932) produced a flora that compiled all European taxa, which already encompassed 150 genera and 1,500 species based on the morphology. Secondly, the recent revisions by Anagnostidis and Komárek (e.g., Komárek and Anagnostidis 2005) aimed to define more homogeneous genera, still based on the morphology. After the prokaryotic nature of cyanobacteria became more obvious on the basis of ultrastructural and molecular studies, it was proposed that their nomenclature should be governed by the International Code for Nomenclature of Bacteria (Stanier et al. 1978).

Currently, the phylum of Cyanobacteria encompasses 5 subsections (corresponding to the 5 orders in the botanical classification) in the Bergey's Manual of Systematic Bacteriology (Castenholz 2001):

I. Chroococcales (unicellular);
II. Pleurocapsales (large cells subdividing into smaller baeocysts);
III. Oscillatoriales (simple filamentous);
IV. Nostocales (filamentous, non-branching heterocyst-forming);
V. Stigonematales (filamentous, branching, heterocyst-forming).

To date, only a few names of cyanobacterial taxa have been validly published according to bacterial rules, reflecting not only technical difficulties but also the confusion due to the existence of two nomenclatural systems (Oren 2004). Current taxonomical studies on cyanobacteria are now adopting a polyphasic approach, which combines genotypic studies with morphological and phenotypic analyses.

Early studies on the diversity and biogeographical distribution of cyanobacteria were based on the identification of the organisms entirely on the basis of morphological criteria. Cyanobacteria often have quite simple morphologies and some of these characters exhibit plasticity with environmental parameters, so that their taxonomic usefulness can be limited. Moreover, a number of botanical taxa have been delimited based on minute morphological differences (e.g., sheath characteristics, slight deviations in cell dimensions or form), and many authors have shown that the genetic diversity does not always coincide with that based on morphology (e.g., Rajaniemi et al. 2005; Taton et al. 2006b). To address these problems, studies on environmental samples (natural mixed assemblages of microorganisms) are typically based at present on clone libraries or DGGE (Denaturating Gradient Gel Electrophoresis) using molecular taxonomic markers, most often the 16S rRNA gene. The obtained 16S rRNA sequences are compared and generally grouped into OTUs (Operational Taxonomic Units) or phylotypes on the basis of their similarity (e.g., 97.5% similarity for Taton et al. 2003, or 98% for de la Torre et al. 2003). With such similarity values, each OTU might correspond to one or more bacterial species but is clearly distinct from other OTUs at the species level (Stackebrandt and Göbel 1994). This therefore provides a conservative estimate of the diversity, following bacterial criteria.

8.3 General characteristics

Cyanobacteria possess photosystems I and II, which are located on thylakoid membranes (except in the genus *Gloeobacter*). The cells usually have a characteristic blue-green coloration due to the phycocyanin (blue), allophycocyanin (blue) pigments in addition to chlorophyll *a*, although some species may additionally contain phycoerythrin that colors the cells red. In a few taxa, other chlorophylls have been observed, including chlorophylls *b* and *d* (Miyashita et al. 1996; Castenholz 2001). Some cyanobacteria are also able to fix atmospheric nitrogen. Furthermore, cyanobacteria have various storage bodies for carbon, nitrogen, phosphate and the enzyme

ribulose 1,5-biphosphate carboxylase/oxygenase (RubisCO) (Castenholz 2001). Cyanobacteria are also known to produce a great variety of secondary metabolites with diverse activities. To date, 600 bioactive molecules have been described, most of which have been found in the Oscillatoriales and Nostocales orders (Welker and von Döhren 2006).

8.4 Antarctic habitats

Studies on the molecular cyanobacterial diversity in Antarctica, using a culture-independent approach, have focused on the following regions to date: the Prydz Bay region (Bowman et al. 2000; Smith et al. 2000; Taton et al. 2006a), the McMurdo Dry Valleys (Priscu et al. 1998; Gordon et al. 2000; Christner et al. 2003; de la Torre et al. 2003; Taton et al. 2003; Smith et al. 2006; de los Rios et al. 2007), the McMurdo Ice Shelf (Jungblut et al. 2005) and the Antarctic Peninsula region (Hughes and Lawley 2003; Hughes et al. 2004). Strains have been isolated from the same regions, as well as from the Dronning Maud Land (Rudi et al. 1997; Vincent et al. 2000; Smith et al. 2000; Billi et al. 2001; Nadeau et al. 2001; Casamatta et al. 2005; Taton et al. 2006b; Comte et al. 2007).

8.4.1 Ice-based habitats

Cyanobacteria dominate microbial consortia formed in ice-based habitats such as cryoconite holes and meltwater ponds. Cryoconite (literally "cold rock dust") gives rise to vertical, cylindrically-formed holes in the ice surface that contain a thin layer of sediment overlain by water. The formation of these habitats is initiated through the absorption of solar radiation by the sediment and the subsequent ablation of the surrounding ice (Wharton et al. 1985). Studies of these holes on the Canada Glacier, McMurdo Dry Valleys, show that they contain cyanobacteria as well as heterotrophic bacteria, eukaryotic micro-algae and colorless protists, and even metazoans such as rotifers, nematodes and tardigrades (Mueller et al. 2001).

An analysis of 16S rRNA sequences from the Canada Glacier cryoconite communities showed the presence of cyanobacteria phylogenetically related to *Chamaesiphon* (96.2% 16S rRNA sequence similarity) (Christner et al. 2003), a genus that is known to occur in the periphyton that forms over rocks in European mountain streams. Other polar sequences in this lineage (ca. 96% similarity) originated from the ice cover of Lake Bonney (Priscu et al. 1998) and a deglaciated glacier area in the Peruvian Andes (Nemergut et al. 2007). A second group of sequences is 99.2% similar to a clone from a meltwater in Livingston Island (Antarctic Peninsula) (unpublished data) and the third sequence group is 99.7% similar to clones from lake Fryxell, lakes in the Prydz Bay area and the Antarctic Peninsula. Notably, sequences of the third group are also 99.2% similar to one sequence from the Peruvian Andes (Nemergut et al. 2007).

Another important class of ice-based habitat is the meltwater ponds that form on ice shelves. These contain liquid water during the summer months, but completely freeze over in winter. The biota of these habitats must therefore contend with extreme temperature changes, freezing and desiccation stress, and high salinities. The ponds on the McMurdo Ice Shelf have low nutrient concentrations, especially nitrogen, due to the marine origin of the sediments (Hawes et al. 1993), and their characteristics are described by Wait et al. (2006). Thick benthic cyanobacterial mats comprise a diverse community of Nostocales and Oscillatoriales as well as other bacterial phyla and microeukaryotes (Howard-Williams et al. 1989; Nadeau et al. 2001; Jungblut et al. 2005). In one of these mats, the presence of microcystin was detected (Jungblut et al. 2006).

8.4.2 Soils and rock

Cyanobacteria are often the primary colonizers of permafrost soils in areas where meltwater flushes occur through snow melt or retreated glaciers. In the Dry Valleys of Antarctica, the soils are old, weathered and have low carbon and nutrient concentrations (Vincent 1988). Thus, the colonization by cyanobacteria increases soil stability and nutrient concentrations through, for example, nitrogen fixation. Terrestrial dark crusts are found throughout Antarctica and are commonly dominated by cyanobacteria (e.g., Broady 1996; Mataloni and Tell 2002; Adams et al. 2006).

Cyanobacteria are also often identified in biofilms below and within the rocks where the microclimate gives protection against environmental stresses such as high UV radiation, temperature extremes, desiccation and physical removal by wind. They can be found in depth below the rock surface depending on the optical characteristics of the rocks and the level of available photosynthetically active radiation (PAR). Depending on the spatial location of the communities, they are hypolithic (beneath rocks), endolithic (in pore spaces of rocks), chasmoendolithic (in cracks and fissures of rocks), or cryptoendolithic (in the pore space between mineral grains forming sedimentary rocks) (Vincent 1988; Hughes and Lawley 2003).

Molecular analysis of such communities revealed a few cryptoendolithic cyanobacterial sequences in beacon sandstone of the Dry Valleys (de la Torre et al. 2003), and in granite boulders of Discovery Bluff (de los Rios et al. 2007). Interestingly, in the latter study, one 16S rRNA sequence was related (98.9%) to a DGGE band from Swiss dolomite (Sigler et al. 2003) and the second sequence is identical to one hypolithic sequence from quartz rocks in the Vestfold Hills (Smith et al. 2000). This group of sequences also has affinities (93.5% similarity) to the chlorophyll *d* containing *Acaryochloris marina* (Miyashita et al. 1996) and de los Rios et al. (2007) hypothesized that some cryptoendoliths could possess this pigment and that its particular absorption spectrum would be beneficial in environments with little light. Another well-known cryptoendolithic cyanobacterium belongs to the genus *Chroococcidiopsis* and was found in sandstones of the Dry Valleys (Friedmann 1986). It is remarkably resistant to desiccation and has close relatives in hot deserts (Fewer et al. 2002).

8.4.3 Ponds, lakes, rivers and streams

Similar to their presence in ice-based habitats, cyanobacteria form large biomass accumulations in Antarctic ponds, lakes, rivers and streams (Vincent 1988). They often form thick, cohesive, highly pigmented mats that coat the benthic environments. A large variety of lake types are present in Antarctica and span a wide range of environmental conditions (Gibson et al. 2006). Many of them are covered with ice for most of the year or even have a perennial ice cover. Studies on perennially ice-covered Lake Hoare in the McMurdo Dry Valleys have shown that PAR irradiance exerts an overall control on microbial photosynthetic production, composition and mat structure (Vopel and Hawes 2006). Other characteristics such as nutrients and salinity also influence the cyanobacterial diversity (Gibson et al. 2006). The diversity and function of the microbial lake communities have been reviewed by Ellis-Evans (1996). At the molecular level, Taton et al. (2003, 2006a, 2006b) showed a large cyanobacterial diversity in the benthic mats from five different Antarctic lakes in two regions (see Sect. 8.7). Cyanobacteria also form biofilms and microbial mats in rivers and streams (Vincent 1988).

In addition to the benthic communities, cyanobacteria are also found in the water column of lakes, and picoplanktonic forms often dominate the plankton. The abundance of planktonic picocyanobacteria is dependent on nutrient availability and light (Vincent 2000), The 16S rRNA sequences of S*ynechococcus*-like picocyanobacteria were obtained from lakes in the Vestfold Hills (Lakes Ace, Pendant, Clear). They appeared to be related, but distinct from other *Synechococcus* genotypes such as *Synechococcus* PS840 from the Russian marine coast (Waleron et al. 2007).

8.4.4 Marine ecosystems

The abundance of picocyanobacteria decreases markedly from temperate latitudes to the polar regions (Marchant et al. 1987; Fouilland et al. 1999). This decrease is assumed to be due to temperature-limitation (Marchant et al. 1987) as well as continuous losses due to grazing, advection and mixing (Vincent 2000). The 16S rRNA sequences of picocyanobacteria retrieved at the Subantarctic Front (51°S) were closely related to temperate oceanic *Synechococcus*, as WH8103 and WH7803 (Wilmotte et al. 2002).

8.5 Arctic habitats

Most available studies have so far focused on the Canadian Arctic, whereas no information is yet available from the Russian Arctic.

8.5.1 Ice-based habitats

Similar to the South Polar Region, ice shelves as well as glaciers provide a variety of habitats for cyanobacteria in addition to other biota (Säwström et al. 2002; Mueller et al. 2005). However, the total area of ice shelves is lower than in Antarctica, where 40% of coastline is fringed by ice shelves. Furthermore, the recent break up of the Ward Hunt Ice Shelf (Mueller et al. 2003) signals the massive reduction of these habitats through global warming. Arctic ice-based habitats can be differentiated into cryoconite holes, meltwater ponds and sediment patches without continuous coverage by water. Microbial mats can be prolific (Vincent et al. 2004a), but are less developed than in Antarctica, and this may be due to the increased grazing pressure in the Arctic (Vincent 2000).

8.5.2 Lakes, streams and ponds

Cyanobacteria play an important role in Arctic lakes, ponds and streams and have been well studied in the Canadian High Arctic (Bonilla et al. 2005).The most common groups are Oscillatoriales and Nostocales, with some Chroococcales. The benthic microbial mats in lakes often have a cohesive layering, which is established through an extra polysaccharide matrix and often have a characteristic pigment stratification (Bonilla et al. 2005). Planktonic picocyanobacterial communities of these lakes comprise mainly *Synechococcus* (Vincent 2000) and can be separated into fresh and saline ecotypes (unpublished data). In these lakes, primary production is only nutrient limited in the planktonic communities, whereas the microenvironments of the benthic mats result in increased nutrient availability and sufficiency (Bonilla et al. 2005).

8.5.3 Soils and rock

Terrestrial cyanobacteria in the Arctic are also major primary colonizers of soils and can be found within soil crusts, symbiotic in lichens and within rocks. They are an important source of nitrogen for the nutrient limited soils of the Arctic (Zielke et al. 2005). Cryptoendolithic communities are common in sandstone outcrops of Eureka, Ellesmere Island, and consist of similar cyanobacterial morphotypes as in Antarctic rocks (Omelon et al. 2006). However, their diversity seems higher than in comparable habitats of the Dry Valleys. This may be due to higher average temperatures, higher humidity due to close spatial distance to open water, and longer periods with available liquid water relative to the McMurdo Dry Valleys. Hypolithic cyanobacteria are commonly observed under opaque rocks subjected to periglacial movements (Cockell and Stokes 2004).

8.5.4 Marine ecosystems

In the Arctic marine environment, similar to the Southern Ocean, picocyanobacteria are rare, in contrast with their abundance in temperate and tropical oceans. A study of their molecular diversity in the Beaufort Sea showed that the picocyanobacteria were affiliated with freshwater and brackish *Synechococcus* lineages, but not to the oceanic ones. Their origin, therefore, seemed allochthonous, as the Arctic Sea is much influenced by large riverine inputs (Waleron et al. 2007).

8.6 Alpine habitats

8.6.1 Streams and lakes

In alpine streams, water chemistry, geochemical conditions, hydraulic conditions and permanence of flow are the key factors defining taxonomic diversity. Cyanobacteria have been found as part of microbial mats, epiphytic on mosses and endosymbiotic in lichens in stream habitats of many alpine regions, but there are no specific studies on their molecular diversity or adaptations (McClintic et al. 2003; Rott et al. 2006). Nutrient concentrations show large variations during the year with peaks in late winter and autumn. PAR and UV radiation also range from low levels in presence of ice and snow cover to high levels during summer months, creating a need for protective mechanisms to survive. Rott et al. (2006) have described different colonization patterns for several cyanobacterial morphotypes in alpine streams.

Cyanobacteria in alpine freshwater lakes can be found as benthic and planktonic communities. Plankton communities are mostly comprised of *Synechococcus* morphotypes and their abundance is correlated to nutrient availability, particularly nitrogen and phosphorus. Benthic communities were studied by Mez et al. (1998), and Sommaruga and Garcia-Pichel (1999). Interestingly, the presence of cyanotoxins was demonstrated by Mez et al. (1998).

8.6.2 Rocks and soils

Cyanobacteria are also dominant components of alpine soil crusts, and rock-associated communities as described for endolithic communities of dolomite rocks in the Swiss Alps (Sigler et al. 2003) and soils from recently deglaciated areas in the Peruvian Andes (Nemergut et al. 2007). The 16S rRNA sequences obtained from the Alpine dolomite layers show high similarities with Antarctic cryptoendoliths (see Sect. 8.4.2), or up to 97.7% similarity with the Andean cyanobacteria (Nemergut et al. 2007), or appear novel (less than 93% similarity with database sequences). Other Andean soil sequences are related to the sequences of *Chamaesiphon* PCC7430 (ca. 96% sequence similarity), of diverse *Nostoc* strains

(a.o. 97.4–98.5 % with the Antarctic ANT.L52B.1), of the Antarctic *Leptolyngbya frigida* ANT.LH52.2 (98.5%) and ANT.LH52B.3 (99.6%), or are quite different from database sequences and thus represent a novel diversity.

8.7 Ecophysiology

8.7.1 Coping with the cold

In general, high latitude and high altitude cyanobacteria tend to be cold-tolerant (psychrotrophs), with suboptimal growth under low temperatures, rather than psychrophiles that grow optimally at low temperature (Tang and Vincent 1999). They have a variety of mechanisms that allows them to tolerate and continue to grow, albeit often at slow rates, in the cold and to tolerate freeze–thaw conditions (Vincent 2007). To maintain membrane fluidity at low temperatures, polyunsaturated fatty acids with decreased chain-lengths are incorporated into the membrane. In addition, the production of compatible solutes (e.g., trehalose) helps to reduce the freezing point of the intracellular fluid. This strategy also reduces cell desiccation as less water is needed to retain the osmotic equilibrium (Welsh 2000). Furthermore, extracellular compounds such as polymeric substances can reduce ice nucleation around the cells (Vincent 2007). Cyanobacteria must also withstand prolonged seasonal dormancy phases in frozen and liquid water. Freeze-dried cyanobacterial mats in Antarctica have been shown to resume photosynthesis within minutes to hours after rethawing (Vincent 2007).

8.7.2 Osmotic stress

Typical hypersaline environments are saline ponds and lakes and brine channels in the sea ice (Vincent 1988). Sudden increases in salt concentration are counterbalanced by a rapid accumulation of salts to maintain the osmotic equilibrium. Long-term survival strategies involve uptake of inorganic ions, to balance the extracellular ion concentrations, as well as the production of organic osmolytes (Oren 2000).

8.7.3 High and low irradiance

UV radiation and high energy PAR can induce photo-inhibition, phycobiliprotein degradation, chlorophyll-bleaching and DNA damage, or the production of reactive oxygen species, and the net damage may be exacerbated at low temperatures (Vincent 2007). Cyanobacteria have evolved a variety of DNA repair mechanisms, such as excision repair and photo-reactivation, to cope with UV induced DNA damage (Castenholz

and Garcia-Pichel 2000). However, these processes are reduced at lower temperatures. Furthermore, the cyanobacteria produce photoprotective screening and quenching pigments (gloeocapsin, scytonemin, mycosporine), and many Antarctic cyanobacteria seem to avoid radiation by migrating to deeper layers within the microbial mats (Castenholz and Garcia-Pichel 2000). High concentrations of scytonemin can lead to a black coloration in many cyanobacterial mats and soil crusts (Vincent 2007).

Conversely, phototrophs in polar and alpine regions must also contend with low irradiances caused by prolonged snow and ice cover. The cyanobacteria utilize highly efficient light capturing complexes, with photosynthetic quantum yields close to the theoretical maximum (Hawes and Schwartz 2001; Vincent 2007).

8.8 Biogeography

The question of endemism and distribution of cyanobacterial taxa is still a topic of much debate. A long-standing theory of microbial distribution is that "everything is everywhere, but the environment selects" and that local habitats select for specific microbial flora that is globally distributed (Baas-Becking 1934). Castenholz (1992) noted the slow rates of speciation in the cyanobacteria together with their large dispersal abilities, and this, in combination with the relatively young age of most polar ice-free environments, suggests that endemism is likely to be rare amongst polar cyanobacteria. Morphological identifications seemed to support this hypothesis. However, such characterization is limited due to morphological plasticity (see Sect. 8.2). In addition, Komárek (1999) noted that a number of identifications of Antarctic cyanobacteria had been made with flora written for temperate countries without taking into account their ecology, which could give the false impression that mostly cosmopolitan taxa were found on this continent. Indeed, by avoiding such 'force-fitting', Komárek (1999) has found about 60% of endemic species amongst the 68 morphospecies found in various microbiotopes of ice-free areas of King George Island.

Several features of Antarctica suggest that endemism may be possible there, although it has yet to be demonstrated convincingly (Vincent 2000): (1) Antarctica has been more isolated than other parts of the world for several million years; (2) dispersal processes which favour local species are more efficient than long-range dispersal processes; and (3) there has probably been strong environmental selection for adaptive strategies. As a step towards addressing this question, the molecular characterization of cultured and uncultured cyanobacterial diversity has been carried out in a number of Antarctic biotopes (see Sect. 8.4). The results from these studies suggest the presence of OTUs/phylotypes with cosmopolitan and bipolar distributions, but also the presence of some genotypes that seem to be restricted to specific Antarctic sites. However, there is never complete 16S rRNA sequence identity for bipolar or cosmopolitan organisms that are members of the same OTU. The use of ITS sequences, which

are more variable than the 16S rRNA, could increase the resolution of the distributional patterns (Taton et al. 2006a).

Taton et al. (2003, 2006a, 2006b) have analyzed the molecular diversity in Lake Fryxell (Dry Valleys), four coastal lakes in the Prydz Bay area (East Antarctica) and two meltwater samples from Livingston Island (Antarctic Peninsula). Using clone libraries based on 16S rRNA sequences, a total of 63 OTUs were detected, of which 44 were only found in Antarctica (70%). This suggests a high degree of endemism, even if a portion of this uniqueness could be due to geographic gaps in the database. A higher proportion of the cosmopolitan genotypes are found in several Antarctic regions (47% compared to 16% for the potentially endemic sequences). Thus, if they were able to disseminate and colonize habitats in different continents, this could be due to resistance capacities that are also helpful to spread to different Antarctic regions. Furthermore, there appears to be an on-going exchange between freshwater and terrestrial biotopes (Gordon et al. 2000), which could also explain why temperature flexibility (psychrotrophy, see Sect. 8.7.1) is more common than psychrophily in cyanobacteria. Another conclusion is that each new sample brings new genotypes and this suggests that much diversity still awaits discovery.

8.9 Conclusions

Cyanobacteria evolved under the harsh conditions of the Precambrian, and their modern representatives retain a remarkable ability to adapt to and survive within extreme conditions. They dominate terrestrial and freshwater cold ecosystems of the Arctic, Antarctic and alpine regions, even though they do not seem to be specifically adapted to optimal growth at low temperatures. They play a major ecological role as they often are primary colonizers of substrates and major primary producers in these ecosystems.

The application of molecular tools in combination with classic morphological techniques has begun to provide new insights into the real diversity of cyanobacteria and their biogeographical distribution in cold environments. Our survey of recent studies suggests complex distributional patterns of cyanobacteria, with cosmopolitan, endemic, and habitat-specific genotypes. This ongoing research will help to identify specific geographical areas that have unique microbial communities. However, many more studies are needed to unravel the enormous diversity of cyanobacteria and to better define their biogeographical patterns in cold environments. This is an urgent task in view of the climatic changes that will undoubtedly alter the structure and functioning of microbial communities in polar and alpine ecosystems.

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Chapter 9
Fungi in Cold Ecosystems

Jens C. Frisvad

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9.1 Introduction

Many habitats on the Earth have temperatures that are constantly or seasonally low. Approximately 80% of the biosphere will have a temperature of −3 to −7°C and 90% of the volume of marine habitats is below 5°C. The deep seas will have temperatures from −1°C to −4°C, glacial ice habitats will have temperatures around −5°C, while the Arctic and Antarctic areas have temperatures from −1°C to −35°C.

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The biodiversity of fungi in snow-covered tundra has been shown to be very high (Schadt et al. 2003), so many different fungi can be expected to be isolated from cold ecosystems (Latter and Heal 1971). Psychrotrophic fungi can grow below 10°C, and these fungi are especially common in alpine, Arctic and Antarctic environments (Widden and Parkinson 1979; Pugh and Allsopp 1982; Vincent 1988; Cabello 1989; Del Frate and Caretta 1990; Vishniac 1996; Zucconi et al. 1996; Azmi and Seppelt 1997; McRae and Seppelt 1999; Deming 2002; Gocheva et al. 2005; Ruisi et al. 2007), but also in man-made refrigerated environments (Kuehn and Gunderson 1962, 1963; Gounot 1986; Baublis et al. 1991; Smith 1993; Pitt and Hocking 1997). However, many temperate habitats will often have temperatures at or below 0°C part of the year (winter). Even some species from warm, temperate or subtropical regions, such as pathogenic *Penicillium italicum* on citrus fruits, can grow comparatively well at 5°C (Pitt and Hocking 1997). *Epicoccum purpurascens* was listed as a psychrophile from Arctic tundra (Kurek et al. 2007), but it has been reported to have a temperature minimum for growth at −3°C to −4°C, an optimum at 23–28°C and a maximum temperature for growth at 45°C (Domsch et al. 1980). *Phoma herbarum* has been isolated from tundra soil (Flanagan and Scarborough 1974) and Antarctica (Selbmann et al. 2002; Singh et al. 2006), but it is a mesophilic fungus found mostly in soils of the temperate and subtropical zones (Domsch et al. 1980). On the other hand, tropical fungi have never been found actively growing in cold habitats. For example, none of the species in the predominantly tropical genus *Talaromyces* and its anamorphic state *Penicillium* subgenus *Biverticillium* can grow at 5°C. There were only two species that could grow at 5°C, *P. clavigerum* and *P. vulpinum* (synonym *P. claviforme*) listed by Pitt (1979) in subgenus *Biverticillium*, but these two species have been transferred to *Penicillium* subgenus *Penicillium*, containing species that all grow well at 5°C in general (Frisvad and Samson 2004). Similarly, other genera seem to be absent in cold ecosystems, for example *Byssochlamys* and its anamorphic state *Paecilomyces. Aspergillus* species in general grow poorly below 12°C, and thus may have been recovered as spores in cold ecosystems (Gunde-Cimerman et al. 2003) because they are common as marine spores, transported by wind or birds or are carried around due to human activity.

Psychrophilic species have only been found in permanently cold habitats, such as Arctic and Antarctic areas, oceans, caves, alpine lakes and soil, glaciers, snow and ice fields. In general, psychrophilic, psychrotolerant and mesophilic fungi are found in cold ecosystems (Möller and Dreyfuss 1996), while thermophilic and heat-resistant fungi have not been isolated from these habitats.

Fungi are common in cold ecosystems (Ruisi et al. 2007). Basidiomycetes and few ascomycetes may produce mycorrhizae associated to cold-adapted plants, and therefore basidiocarps of *Boletus* and other genera are often found in less extreme Arctic and Alpine habitats (Chlebicki 2002). Most plants in extreme Arctic regions are non-mycorrhizal, however (Kytöviita 2005). Ascomycetous macrofungi such as morels can also be found in cold habitats at high elevations (McFarlane et al. 2005). Lichens are very common in cold ecosystems and are especially common on rocks and on soil in Antarctica and in Arctic areas (Longton 1988; Øvstedal and Lewis Smith 2001; Seymour et al. 2005; Weber et al. 2007; Gadd 2007). Yeasts such as

Cryptococcus albidus (Cameron et al. 1976) and *Leucosporidium scottii* (van Uden 1984), *Candida frigida*, *C. gelida* and *C. nivalis* (Di Menna 1966a, b) are also common. Less work has been done of filamentous fungi (moulds) in cold ecosystems, but they have been reported from vegetation (Babjeva and Reshetova 1998; Tosi et al. 2002), soil (Broady 1993; Nienow and Friedmann 1993; Frisvad et al. 2006), permafrost layers (Golubev 1998; Broady 1993; Nienow and Friedmann 1993; Vishniac 1993; Rivkina et al. 2004), snow (Abyzoz 1993), glacier ice (Abyzoz 1993; Ma et al. 1999; Gunde-Cimerman et al. 2003) and water (Broady and Weinstein 1998).

Growth in cold habitats often involves ability to grow at low water activity as liquid water is only partially available in ice (Petrovi et al. 2000; Gunde-Cimerman et al. 2003). For this reason, xerophily may also play a role in psychrophily or psychrotolerance.

Since especially Antarctica seems to have few endemic fungal species, many terrestrial fungi have been introduced via humans, animals or wind dispersal (Ellis-Evans and Walton 1990). The mycospora of the air in Antarctica indicates that several species are introduced via this vector (Czarnecki and Bialasiewicz 1987; Marshall 1997, 1998). Airborne filamentous fungal spores are also present in the air at Jan Mayen and Svalbard, but the fungal counts are not large (Johansen and Hafsten 1988; Johansen 1991). On Greenland and Svalbard, however, certain new species seems to be endemic to the Arctic areas (Gunde-Cimerman et al. 2003; Frisvad et al. 2006; Sonjak et al. 2007b). More surveys on the funga of Arctic, Antarctic and alpine areas need to be done, however, and this effort can be somewhat restricted by the many new species that have not yet been described, and thus making reliable identification difficult at present.

In this review, filamentous fungi thriving in cold ecosystems will be emphasized, while yeasts, lichens and basidiomycetes will be mentioned briefly.

9.2 Methods for recovering psychrotolerant and psychrophilic species

Some fungi such as basidiomycetes and lichens can be directly isolated and, in some cases, cultures can be obtained from such material, but in most cases fungi are isolated from soil, ice, water, plant material, animal material, etc., via direct plating or dilution technique.

Many isolation media are efficient for recovering psychrotolerant and psychrophilic fungi, including malt extract agar (Möller and Dreyfuss 1996; Singh et al. 2006), malt yeast peptone agar (MYP) (Carreiro and Koske 1992) or dichloran 18% glycerol agar (DG18) (Hocking and Pitt 1980) if incubated at a low temperature for an extended period of time. The isolation plates can for example be incubated at 0°C and examined during 4 weeks (Carreiro and Koske 1992), or incubated at 4°C and examined during 10 weeks (Petrini et al. 1992), or incubated at 2–5°C for 45 days (Singh et al. 2006). For penicillia, the medium DG18 incubated in darkness for up to 4 weeks at 15°C worked fine (Gunde-Cimerman et al. 2003; Frisvad et al. 2006).

Usually, fungi are isolated in pure culture and subsequently identified on standard media for the genus at hand (Pitt and Hocking 1997). Some new species of *Penicillium* hardly grow at 25°C, so it is now recommended to grow penicillia from cold ecosystems both at 15 and 25°C on all standard media for identification purposes (see Frisvad and Samson 2004). For other genera, 15°C also seems to be a good additional temperature to use, as all psychrotolerant and psychrophilic species grow well at this temperature.

Most fungi appear to be recoverable from their habitats, in contrast to bacteria and archaea, where molecular methods are needed to discover all species present (Pace 1997). However, cultural methods in combination with molecular methods will surely give a much better characterization of the funga of cold ecosystems.

9.3 Protection and survival of cold ecosystem fungi

Fungi have several ways of protecting themselves against the harsh conditions in Arctic, Antarctic and alpine ecosystems (Baross and Morita 1978; Herbert 1986; Tearle 1987; Russell 1990; Wynn-Williams 1990; Robinson et al. 1996, 2004; Robinson 2001; Schmidt and Bölter 2002; Tosi et al. 2005). Growth and survival strategies by cold-adapted fungi include cell membranes, nutrient transport and enzyme function (Vincent 2000). Some fungi produce melanin to protect the fungal thallus and spores against UV-B light and other challenges (the melanin can be present in chlamydospores, ascomata, sclerotia, mycelium or conidiospores) (Bell and Wheeler 1986; Arcangali et al. 1997; Smith and Reed 1997; Butler and Day 1998; Jumpponen and Trappe 1998; Arcangali and Cannistraro 2000; Robinson 2001; Zucconi et al. 2002; Kogej et al. 2004; Day et al. 2006). Other chemical means of protecting the fungus during freezing and thawing (Lyakh et al. 1984) or at constantly low temperature can be mycosporins (Gorbushina et al. 2003; Kogej et al. 2006), antifreeze proteins (Hishino et al. 2003), antioxidants and glycogen (Ratner and Fikhte 1982; Gocheva et al. 2006), and exopolysaccharides (Selbmann et al. 2002). Compared to a mesophilic *Penicillium* strain, both Antarctic strains and a temperate strain accumulated trehalose (Montiel 2000) and protective enzymes, including superoxide dismutase and catalase, at low temperatures (Gocheva et al. 2006). Sterols and fatty acids are also of a unique composition in psychrophilic and psychrotolerant species of *Mortierella* (Weete and Gandhi 1999), and lipids may also have a protective role (Finotti et al. 1993; Istokovics et al. 1998).

9.4 Different ecosystems

9.4.1 Fungi in soil and permafrost

The soil in cold ecosystems contains a number of bacteria including streptomycetes and microfungi (Flint and Stout 1960; Horowitz et al. 1972; Onofri et al. 2000, 2006). Much of the biodiversity of Arctic and alpine soils is still undescribed (Schadt et al.

2003). It appears that Antarctica has less biodiversity concerning soil-borne fungi than Arctic and alpine areas (Latter and Heal 1971; Schadt et al. 2003; Ruisi et al. 2007).

Many fungi have been isolated from permafrost, but it is difficult to control whether the fungus is a recent (present day) contamination or if it is the propagule of an organism that has survived for thousands of years (Lydolph et al. 2005). Gilichinsky et al. (2005) found several fungi in cryopegs in permafrost and there are many reports of filamentous fungi and yeasts from permafrost soil. DNA from fungi, bacteria, archaea and other organisms can also be isolated from permafrost, but again, severe controls need to be used in order not to regard present day contaminations as ancient species (Willerslev et al. 1999; Lydolph et al. 2005).

9.4.2 Fungi in caves

Many caves will have a constantly low temperature at around 3–7°C and some of the fungi isolated from those caves may be psychrotolerant or psychrophilic. *Penicillium cavernicola* has been found in cool caves (Frisvad and Samson 2004) and can be classified as psychrotolerant. *Microascus caviariformis*, also called the chicken sandwich cave fungus (Malloch and Hubart 1987) is also psychrotolerant. These caves are special in that they rarely have temperatures below 0°C.

9.4.3 Fungi on rocks

Some fungi are specialized in growing on rocks and stones, especially lichen-associated fungi (Longton 1988; Øvstedal and Lewis Smith 2001; Gorbushina et al. 2005; Ríos et al. 2005), but a series of endolithic and cryptoendolithic species can also grow there (Friedmann 1982, 1993; Wierzchos and Ascaso 2001; Hughes and Lawley 2003; Nienow and Friedmann 1993; Ríos et al. 2003; Selbmann et al. 2005). These species can grow both in deserts (Staley et al. 1982) and in cold habitats. Low nutrient availability, a constant change between low and high water activity, extreme temperatures and extreme temperature differences, and UV radiation make this rock habitat one of the most challenging known (Brown 1978; Hughes et al. 2003; Sterflinger 2005).

9.4.4 Fungi on dung

Some psychrophilic or psychrotolerant fungi are associated to dung, especially *Thelobolus*, for example the species *T. globosus, T. ellipsoideus* and *T. microsporus* (de Hoog et al. 2005). Coprophilic *Penicillium* species also grow well at 5°C (Frisvad and Samson 2004) and *P. vulpinum* (= *P. claviforme*) has been found in Alaska, for example (Flanagan and Scarborough 1974).

9.4.5 Fungi on plants, mosses and lichens

Fungal partners of lichen symbiosis are manifold and common in polar and alpine areas (Smith 1984). Filamentous microfungi are very common on these lichens (Hawksworth 1979; 1981; 1982; Petrini et al. 1990). Filamentous fungi have also been reported from lichens in Antarctica (Fletcher et al. 1985; Øvstedal and Hawksworth 1986; Pegler et al. 1980; Möller and Dreyfuss 1996), but have been less studied in Arctic and alpine areas. Plants in Antarctica such as *Deschampsia antarctica* and *Colobanthus quitensis* are abundant and many fungi could be isolated from these plants (Möller and Dreyfuss 1996). In contrast, Arctic and alpine areas have a very diverse flora and each plant will often host a series of filamentous fungi in the rhizosphere soil (Domsch et al. 1980). Many filamentous fungi can also be isolated from mosses and liverworts (Möller and Dreyfuss 1996). In the study of the latter authors, geographic sampling seemed to be a better indicator of the funga of mosses and lichens, whereas the funga was more clearly associated to the plants than to geography.

9.4.6 Fungi on grass: snow moulds

Typhula ishikariensis, T. incarnata and *T. idahoensis* can cause snow mould in grasses (Desjardin and Ward 1971; Hsiang et al. 1999). *Myrosclerotinia borealis* (Saito 1998) is also a grass pathogenic psychrophilic fungus producing polypeptides in the sclerotia and several polygalacturonases. This species and *Microdochium nivale* (= *Monographella nivalis* = *Fusarium nivale*) can also cause severe damage in natural and domesticated grasses (Saito 1998). The so-called low temperature basidiomycete, *Coprinus psychromorbidus* can also cause snow mould attack on grasses (Traquair et al. 1987). Other fungi may cause diseases of plants in cold areas, but in situations without stress they can often be regarded as endophytes including for example *Monodictys arctica* in *Saxifraga oppositifolia* (Day et al. 2006).

9.4.7 Fungi in glaciers, ice and freshwater

Ice in lakes in Antarctica contains many fungi and other microorganisms (Priscu et al. 1998) and so does glacier ice in Arctic areas (Abyzoz 1993; Gunde-Cimerman et al. 2003; Sonjak et al. 2006; 2007a,b). Freshwater habitats in Antarctica have also been examined for fungi (Sugiyama et al. 1967; Ellis-Evans 1985). In parallel with results from Antarctica (McRae et al. 1999), psychrotolerant to mesophilic species of *Penicillium* have been found in glacier ice, including *P. crustosum*, *P. solitum*, *P. nordicum*, and *P. italicum* (Gunde-Cimerman et al. 2003; Sonjak et al. 2006), all rather common species in foods, too (Frisvad and Samson 2004). The number of conidia found in both glaciers and cold waters on Svalbard indicated that

the fungi can actually grow there and that they are not just wind-blown contaminant spores (Gunde-Cimerman et al. 2003).

The psychrophilic aquatic oomycete *Leptomitus lacteus* can grow heavily in effluent from papermills and cause considerable pollution. This species was formerly regarded as a fungus (Gounot 1986), and is only found in cold river water.

9.4.8 Fungi in chilled and frozen foods

Kuehn and Gundersen (1962, 1963) found several psychrotrophic fungi in fruit-filled pastries and in chicken pies and other frozen foods, including *Acremonium strictum*, *Arthrinium sphaerospermum, Aureobasidium pullulans*, *Botryotricum*, *Cladosporium cladosporioides*, *C. herbarum*, *Geomyces pannorum*, *Umbelopsis ramaniana*, *Mucor fragilis*, *M. hiemalis*, *M. plumbeus*, *Penicillium* spp. and *Phoma glomerata*. According to Möller and Dreyfuss (1996), of all the fungi they isolated, only *Acremonium psychrophilum*, *Chaunopycnis ovalispora* and an unidentified species each of *Cylindrocarpon*, *Lichenoconium*, *Monocillium* and *Myceliophthora* were real psychrophiles from Antarctica, while they classified *Acremonium cerealis*, *Arthroderma* cf. *cuniculi*, a *Chamarosporium* sp., *Chalara constricta*, some *Cladosporium* spp., some *Fusarium* spp., *Geomyces pannorum*, a *Monascella* sp., *Phialophora hyalina*, a *Pycnostysanus* sp., *Thelebolus microsporus*, *Volucrispora graminea* and *Tricellula* cf. *aquatica* as psychrotolerant species. They classified all *Penicillium* isolates they found as mesophilic.

Penicillium and *Cladosporium* species is a special case, as they are found very often on refrigerated foods. Some species, such as *P. commune* and its domesticated form *P. camemberti*, and *P. palitans* and its domesticated form *P. caseifulvum*, are often found on moldy cheeses, whereas *Penicillium freii* and *P. cyclopium* can grow on refrigerated cereal products and *P. expansum* can grow on refrigerated pomaceous fruits (Frisvad and Samson 2004). *Cladosporium* species are often found on refrigerated meat (Brookes and Hansford 1923). Many species in these two genera are psychrotolerant to mesophilic.

9.5 Different fungal taxa from cold ecosystems

9.5.1 Fungal genera and cold ecosystems

Different fungal genera are often predominantly psychrotolerant, mesophilic or thermotolerant (Gounot 1986; Kerry 1990). For example, the genus *Eupenicillium* and its associated *Penicillium* anamorphs is predominantly psychrotolerant to mesophilic, while *Talaromyces* and its associated *Penicillium* subgenus *Biverticillium* anamorphs

are predominantly mesophilic to thermotolerant. The genus *Aspergillus* is also mesophilic to thermotolerant, yet some spores of *Aspergillus* and its associated teleomorphs are found in Arctic regions (Gunde-Cimerman et al. 2003). One genus, *Humicola*, interestingly contains one of the most psychrophilic species, *H. marvinii* (Weinstein et al. 1997) and one of the most thermophilic species, *H. lanuginosa* (Cooney and Emerson 1964). This was one of the reasons why *H. lanuginosa* was later transferred to *Thermomyces* as *T. lanuginosus*. In a parallel example, the zygomycete *Mucor strictus* has been described as a psychrophilic species (Schipper 1967) while *Mucor miehei* is listed as a thermophile (Cooney and Emerson 1964; Hammonds and Smith 1986). Similarly, in this case, the thermophilic species was later transferred to another genus as *Rhizomucor miehei*. Except for *Mrakia*, few fungal genera contain only psychrophilic species, but genera such as *Alternaria, Chrysosporium, Cladosporium, Cryptococcus, Geomyces, Heteroconidum, Humicola, Keratinomyces, Leptodontidium, Leptomitus, Leucosporidium, Microsphaeropsis, Mortierella, Mucor, Myrioconium, Penicillium, Phialophora, Phoma, Rhizopus*, and *Typhula* contain few psychrotolerant or psychrophilic species (Domsch et al. 1980; Gounot 1986; Gamundi and Spinedi 1988; Carreiro and Koske 1992; Petrini et al. 1992; Bergero et al. 1999).

9.5.2 Yeasts

Yeasts and yeast-like fungi are common in Antarctic and Arctic environments (Di Menna 1960; Goto et al. 1969; Atlas et al. 1978; Vishniac 1987, 1993, 2006). The ascomycetous genus *Candida* is especially common, including *Candida frigida*, *C. gelida*, and *C. nivalis* (Di Menna 1966a,b), but many basidio-yeasts have also been found, including *Cryptococcus albidus, C. antarcticus, C. consortionis, C. friedmannii, C. laurentii, C. lupi, C. socialis*, and *C. vishniacii* (Cameron et al. 1976; Vishniac 1985a;b; 1993; Vishniac and Hempfling 1979; Baharaeen and Vishniac 1982), *Hyphozyma* spp. (de Hoog and Smith 1981), *Leucosporidium scottii* (Rollo et al. 1995), *Mrakia frigida* and *M. psychrophila* (Xin and Zhou 2007), and *Rhodotorula creatinovora* and *Rh. yakutica* (Golubev 1998).

9.5.3 Ascomycetes

Many ascomycetes and anamorphs belonging to ascomycetes from Antarctica have been described. These species are placed in the 12 ascomycete families Arthrodermataceae, Chaetomiaceae, Hypocreaceae, Lasiosphariaceae, Microascaceae, Myxotriaceae, Orbiliaceae, Saccharomycetaceae, Sclerotiniaceae, Thelobolaceae, Trichocomaceae, and Trichospheariaceae and 9 orders: Eurotiales, Hypocreales, Leotiales, Microascales, Onygenales, Pezizales, Saccharomycetales, Sordariales, and Trichosphaeriales. Some of the species are only found as the anamorphic

state, for example *Penicillium jamesonlandense*. The teleomorphic state *Eupenicillium* has not yet been found in very cold climates. The species include *Apiosordaria antarctica*, *Thielavia antarctica* (Stchigel et al. 2003), *Hypocrea psychrophila* (Neill et al. 1972), *Microascus caviariformis* from a cave (Malloch and Hubart 1987), *Myriosclerotinia borealis* (Saito 1998), *Thelobolus globosus, Thelobolus ellipsoideus* and *Thelobolus microsporus* from dung (de Hoog et al. 2005) and *Thielavia antarctica* (Stchigel et al. 2003). None of these species are strictly psychrophilic, but rather psychrotolerant. Among the ascomycete-related fungi with no teleomorphic state, the following fungi were psychrophilic or psychrotolerant: *Acremonium antarcticum* (Möller and Gams 1993), *Acremonium psychrophilum* (Möller and Dreyfuss 1996), *Antarctomyces psychrophilus* (Stchigel et al. 2001), *Arthrobotrys ferox* (Onofri and Tosi 1992), *Arthrobotrys psychrophilum* (Möller and Dreyfuss 1996), *Arthrobotrys tortor* (Caretta et al. 1994), *Cadophora malorum*, *Candida frigida, C. gelida, C. nivalis* and *C. psychrophila* (Di Menna 1966a), *Chaunopycnis ovalispora* (Möller and Gams 1993), *Cylindrocarpon* sp. (Gounot 1986), *Engyodontium album* (Caretta et al. 1994), *Exophiala psychrophila* (Pedersen and Langvad 1989), *Geomyces pannorum* (Möller and Dreyfuss 1996; Marschall 1998), *Humicola marvinii* (Weinstein et al. 1997), *Hypocrea psychrophila* (Neill et al. 1972), *Keratinomyces ceretanicus* (Punsola and Guarro 1984), *Lichenoconium* sp. (Möller and Dreyfuss 1996), *Monachrosporium psychrophila* (Ahren et al. 2004), *Monocillium* sp. (Möller and Dreyfuss 1996), *Monodictys arctica* (Day et al. 2006), *Myceliophthora* sp. (Möller and Dreyfuss 1996), *Penicillium jamesonlandense* (Frisvad et al. 2006) and *Streptotheca psychrophila* (Bergman and Shanor 1957).

9.5.4 Penicillium

Penicillium species isolated from Antarctica, including the new species *P. antarcticum* (McRae et al. 1999), have proved to be species often isolated from foods, including *P. solitum*, *P. corylophilum* and *P. glabrum. P. antarcticum* was isolated from salami in Denmark in 1982, and has since been isolated from seawater in Denmark, Australia, Slovenia, and in the Atlantic, so this species seems to be a halotolerant marine *Penicillium* species of worldwide distribution (Frisvad, unpublished). However, these species are not true psychrophiles as they grow and sporulate very well at 25°C. The same is the case for a new species from the Arctic island Svalbard, *P. svalbardense* (Sonjak et al. 2007b). Other species isolated from "arctic-alpine" areas in Wyoming and Colorado, USA, and Greenland, including *P. ribium*, *P. soppii* and *P. lanosum*, also grew well and sporulated at 25°C (Frisvad et al. 2006). However, isolates of the new species *P. jamesonlandense* grew very poorly at 25°C. Kurek et al. (2007) listed *Penicillium lanosum* as a psychrophile, but these isolates could also have been one of the new species, such as *P. jamesonlandense*, as these species are morphologically and phylogenetically closely related. They also listed *P. expansum* as either psychrophilic or psychrotrophic.

An endoglucanase-producing *P. expansum* has also been found in huts from Antarctica (Duncan et al. 2006). This species has been found on both Svalbard and Antarctica, and may have arrived there because of use of apples in the diet of people staying in the Antarctic or Arctic, as apples often carry spores of *P. expansum* (Frisvad and Samson 2004). Duncan et al. (2006) also reported the presence of *P. roqueforti*, which may be in Antarctica because of blue cheeses being imported or because it is common in timber (Frisvad and Samson 2004). The strains of *P. roqueforti* may also have been close to *P. ribium*, which is morphologically similar, and has been found in Arctic and alpine habitats (Frisvad et al. 2006).

Penicillium crustosum isolates, isolated from all over the world, were genotypically and phenotypically very similar, but Arctic isolates did differ in that some of the isolates of this species produced the secondary metabolite andrastin A and some isolates, from one glacier, did not grow well on creatine-sucrose agar in contrast to all other isolates of *P. crustosum* (Sonjak et al. 2005; 2006; 2007a).

Isolates of *Penicillium* that grow better at 15°C than 25°C and also grow well at 5°C include *P. marinum*, *P. thymicola* and *P. verrucosum* (Frisvad and Samson 2004). However, many other food-borne penicillia also grow nearly as well at 15°C as at 25°C or at least have some isolates in the species growing better at 15°C than at 25°C. The food-borne terverticillate *Penicillium* species mostly grow well at 5°C, but most of them grow poorly at 30°C (Table 9.1). Those that grow well at 30°C and even somehow at 37°C, such as the mesophilic species *P. chrysogenum* and *P. aethiopicum*, have also been isolated from foods. *P. chrysogenum* has also been isolated from glacier ice, but not as a major species there (Gunde-Cimerman et al. 2003).

9.5.5 Cladosporium

Cladosporium cladosporioides and *C. herbarum* have been listed as psychrophiles by Kurek et al. (2007), but even though isolates of these species can grow at temperatures as low as −6°C (Brookes and Hansford 1923; Joffe 1962) or even −10°C (Bidault 1921), they have an optimum growth between 18 and 28°C (Domsch et al. 1980) and maximum temperature at 32°C, and thus can be classified as psychrotolerant to mesophilic. Most *Cladosporium* species seems to be psychrotolerant, and they are able to grow at very low temperatures.

9.5.6 Zygomycetes

Many zygomycetes have been found in cold ecosystems. Psychrotolerant species belong to Mortierellaceae and Mucoracea. Isolates belonging to Mucor strictus have been particularly well studied (Schipper 1967; Dexter and Cooke 1984, 1985), but most major genera have some psychrophilic or psychrotolerant species, including

Table 9.1 Growth diameters of psychrotolerant *Penicillium* subgenus *Penicillium* species at lower range temperatures (after Frisvad and Samson 2004), after 1 week of incubation in darkness

	Growth diameter (mm)				Ratio
Species	5°C	15°C	25°C	30°C	15°C/25°C
P. thymicola		19–24	9–20	2–6	1.0–2.3
P. marinum		17–24	17–27	0	1.0–1.3
P. verrucosum	2–4	17–23	9–24	0	1.0–1.2
P. nordicum	2–4	8–21	8–21	0	0.6–1.2
P. bialowiezense	1–4	17–22	11–25	0	0.8–1.9
P. brevicompactum	1–4	17–21	8–30	0–3	0.7–1.0
P. olsonii	2–5	23–27	26–40	0–2	0.7–0.8
P. expansum	3–5	26–34	26–50	0–3	0.8–1.3
P. echinulatum	3–5	25–31	20–40	0–1	0.8–1.3
P. discolor	3–6	24–30	21–36	0–12	0.8–1.2
P. solitum	3–5	18–30	16–34	0	0.7–1.1
P. camemberti	2–6	14–26	19–27	0–3	0.8–1.3
P. commune	2–5	23–29	15–35	0–4	0.8–1.3
P. concentricum		15–20	12–25	0	0.8–1.3
P. caseifulvum	3–8	18–23	15–24	0	0.8–1.1
P. cavernicola		16–27	23–33	0	0.6–0.9
P. atramentosum	0–2	20–25	23–39	0	0.6–1.0
P. digitatum	0–3	8–22	15–55	0	0.3–1.1
P. allii	0	24–30	26–40	1– 5	0.9–1.1
P. cyclopium	2–5	19–24	18–34	2–6	0.7–1.0
P. freii	2–5	17–23	15–27	0	0.7–1.1
P. palitans	2–4	21–30	15–31	0–7	0.9–1.1
P. glandicola	2–5	17–23	15–32	0	0.6–1.0
P. hirsutum	2–4	32–36	19–43	2–9	0.8–1.9
P. gladioli	2–4	17–23	27–41	0	0.7–0.9
P. radicicola	2–4	27–34	29–41	1–10	0.7–1.2
P. coprobium		14–19	21–29	0–4	0.5–0.9
P. coprophilum		14–19	18–29	0–4	0.6–0.8
P. formosanum	0	19–21	18–42	0	0.8–0.8
P. ulaiense	2–4	4–17	13–26	0	0.5–1.1
P. italicum	2–4	17–34	26–50	0–12	0.6–0.8
P. tricolor	2–4	4–15	20–32	0	0.1–0.5
P. venetum		26–33	18–34	2–7	0.8–1.3
P. tulipae		28–37	39–48	2–9	0.7–0.9
P. albocoremium	2–4	32–35	28–38	8–14	0.9–1.1
P. hordei	2–4	19–31	27–41	10–17	0.7–1.0
P. polonicum	2–5	27–30	24–43	10–15	0.7–1.0
P. viridicatum	2–4	21–24	19–35	6–18	0.6–0.9
P. aurantiogriseum	2–5	16–23	11–32	12–17	0.7–1.3
P. melanoconidium	2–4	18–23	17–27	13–18	0.8–1.0
P. neoechinulatum	2–4	24–28	24–35	13–17	0.8–0.9
P. nalgiovense	1–4	14–25	18–34	1–16	0.6–0.8
P. roqueforti	2–4	28–38	17–77	0–11	0.4–1.7
P. carneum		32–36	36–53	11–26	0.8–0.9
P. paneum	2–4	27–36	38–41	10–39	0.6–0.9
P. crustosum	2–6	28–31	32–46	15–26	0.7–0.7
P. dipodomyicola	1–4	17–22	20–30	17–21	0.6–0.8
P. griseofulvum	2–4	18–21	19–32	17–22	0.6–0.8

Mortierella alpina (Latter and Heal 1971), *Mortierella ramaniana* = *Umbelopsis ramaniana* (Martseniuk and Mazilkin 1972), *Mortierella verticillata* (Kobayashi et al. 1967), and *Mortierella vinacea* (Rall 1965). Even though the latter species have been found in alpine areas of Europe and USA, it must be considered as a mesophile, as its temperature optimum is 30°C (Domsch et al. 1980). Cold-adapted species of *Mucor* include the psychrotolerant *Mucor hiemalis* (Schipper 1973) and the psychrophilic *M. strictus* (Schipper 1967). Other psychrophilic species include *Absidia psychrophila* (Hesseltine and Ellis 1964) and *Zygorhynchus psychrophilus* (Schipper and Hintikka 1969). These fungi are common in soil in cold ecosystems, but *Mucor* and other psychrotolerant fungi are also found on refrigerated meat (Brookes and Hansford 1923).

9.5.7 Basidiomycetes

Basidiomycetous yeasts are common psychrophiles found in Antarctica (see Sect. 9.5.2), but because mycorrhiza are rare in Antarctica, as there are no trees, there are few basidiocarp-forming fungi. Some huts are being degraded by soft rot basidiomycetes, however (Inglis et al. 2000; Blanchette et al. 2004; Held et al. 2006). Most psychrophilic and psychrotolerant species belong to Filobasidiaceae, Typhulaceae and Sporidiobolaceae. Many different basidiomycetes have been found in Arctic and Alpine areas (Nemergut et al. 2005). For example, Laursen and Miller (1977) found *Galerina subannulata*, *Clitocybe polygonarum*, *Naematolomma udum*, *Panaeolus accuminatus* and *Russula emetica* in tundra soil in Barrow, Alaska. Lepiotaceous fungi are rare in Arctic and tropical regions, but are abundant in temperate regions (Vellinga 2004). Also *Boletus* spp. or other basidiomycetes can produce mycorrhizal associations with dwarf *Betula* and *Salix* spp. in Greenland, but high Antarctic plants are essentially non-mycorrhizal (Gardes and Dahlberg 1996). This may be based on asymmetric symbiont adaptation according to Kytöviita (2005). AM and VA mycorrhizal fungi have some resistance to freezing and thawing (Addy et al. 1997, 1998).

9.5.8 Lichens

Lichens, a symbiosis of fungi and algae or photosynthetic bacteria, are widespread in Antarctic, Arctic and alpine areas (Longton 1988; Øvstedal and Lewis Smith 2001; Seymour et al. 2005; Weber et al. 2007; Gadd 2007). Lists of lichens from Greenland (Hansen and Andersen 1995; Hansen 2006), the Alps (Hafellner and Türk 2001; Nashimbene et al. 2006), the Russian Arctic (Andrev et al. 1996), USA and Canada (Thomson 1984, 1997), Antarctica (Øvstedal and Lewis Smith 2001) and other cold areas have been collected, and a large number of genera have been found. Several species in some of these genera have a circum-polar, “arctic-alpine”

distribution, while species such as *Candelariella xanthostigma*, *Catinaria atropurpurea* and *Mycobilimbia hypnorum* are also found in boreal and temperate areas (Hansen, 2006; Nascimbene et al. 2006). Ornitocoprohilic lichens have also been found in Antarctica (Olech 1990). Being of such a diverse composition in cold ecosystems, lichens are very strong indicators of climatic change, pollution and technocoenosis (Nascimbene et al. 2006). However, other parts of the funga including zygomycetes, basidiomycetes and ascomycetes also appear to be good indicators of such changes, as are the bacterial and archaeal biota, protozoal biota, flora and fauna, but lichens are particularly widespread in cold areas of the world. While the lichens and macromycetes have been studied extensively in the last 120 years, the yeast and mould funga of cold ecosystems has been under-explored.

9.6 Conclusions

Many fungi are adapted to cold ecosystems, and psychrophilic fungi are only found in Arctic, Antarctic, Alpine or cold marine areas of the world. There are many more psychrophilic and psychrotolerant fungi and even some mesophilic fungi to be found also in other cold ecosystems, and most of these fungi are all capable of growing at 0°C. Water activity, radiation resistance and other physico-chemical factors also play a role for the establishment of these species in cold ecosystems. Basidiomycetes are mostly present in the more extreme cold ecosystems as basidio-yeasts, and mushroom basidiomycetes are rare as plants there are slow-growing and non-mycorrhizal. Zygomycetes and ascomycetes and their imperfect forms are very common in cold ecosystems. Chytridiomycetes and oomycetes appear to be rare, if found at all, in the Arctic, Antarctic and alpine areas, only the oomycete *Leptomitus lacteus* is found as a psychrophile in cold waste water. It appears that the fungi play a major role in the ecology of cold areas and that we may find new interesting species, enzymes and secondary metabolites (see Chap. 22) and biotechnological uses of some of these fungi.

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Chapter 10
Cold-Active Viruses

Llyd E. Wells

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10.1 Introduction

Virus-like particles (VLP) are abundant in the aquatic biosphere, typically ranging between 10^5 and 10^8 ml^{-1}, including in the cold environments of the deep sea, polar oceans, sea ice and high-latitude lakes. In these places, viruses play important ecological roles, e.g., equaling or surpassing grazers as agents of bacterial mortality in some Arctic waters (Steward et al. 1996; Wells and Deming 2006a). Systematic investigations of viral ecology in snow, permafrost or glaciers are only beginning, but viruses are present, preserved or even active there (e.g., Castello et al. 1999; Säwström et al. 2007b). Yet, despite the prevalence on Earth of environments ≤4°C and the high abundance of viruses in them, little is known about corresponding viral characteristics or ecology. Instead, viral studies have largely been restricted to higher temperatures convenient for experimenters but of little ecological or evolutionary relevance to many viruses.

Here, I review information about viruses from cold environments, focusing on bacteriophages when hosts are known. (For a more inclusive review specific to Antarctic systems, see Pearce and Wilson 2003.) Although these viruses are termed "psychrophilic" (e.g., Olsen 1967; Olsen et al. 1968) or "psychrotrophic" (Greer 1982, 1983; Patel and Jackman 1986), such words imply growth characteristics not directly applicable to obligate parasites like viruses. I therefore follow Wells and Deming (2006b) and call viruses capable of infection and production at temperatures ≤4°C "cold-active." The

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4°C upper limit includes perennially cold ocean regions and accords with the few studies of viral activity at near-0°C temperatures. The term "cold-active" does not necessarily designate an ecologically or evolutionarily coherent group, however.

10.2 Characterization of cold-active viruses and their hosts

10.2.1 Cold-active phages isolated from sewage or food

The filterable "lytic principle" active at 2 and 4°C reported by Elder and Tanner (1927, 1928) was probably the first cold-active virus described. Isolated from sewage, it infected an unidentified bacterium; no further characterization of the phage-host system (PHS) was apparently done. Since then, thousands of phages have been described, but only 42 (18 from Delisle and Levin 1969b) have been studied sufficiently to ascertain cold activity (Table 10.1). This paucity probably reflects isolation and characterization strategies, usually done at or above room temperature, rather than true rarity.

Among the earliest detailed studies of cold-active phages were Olsen (1967) and Olsen et al. (1968), albeit using pseudomonad-infecting viruses initially isolated from sewage at 20–37°C. Working with five "psychrophilic" phages—i.e., phages produced at 3.5°C but not 37°C—Olsen et al. (1968) found that all had longer latent periods (6–12 h vs 0.5–1 h; the latent period is the time from infection to phage release) and smaller burst sizes (6–25 vs 23–120; the burst size is the average number of viruses released per infected cell) at 3.5°C than 25°C. Reduced burst sizes, protracted latent periods and overall slower phage production rates at low temperatures (3.5–4°C) than high temperatures (25–26°C) have also been reported for other cold-active phages (Kulpa and Olsen 1971; Whitman and Marshall 1971b; Sillankorva et al. 2004). In contrast, using one of the PHS described in Olsen et al. (1968), Olsen (1967) reported highest burst size (300–350 viruses) at 3.5°C rather than 25°C, but only if the hosts were grown at that temperature before infection. Later work revealed other PHS characteristics affected by pre-infection growth temperature, including adsorption rate (Delisle and Levin 1972b) and latent period (Sillankorva et al. 2004), possibly due to bacterial phenotypic responses to the *transition* to low temperature. Unfortunately, few cold-active phage studies specified the host's pre-infection growth temperatures (e.g., Olsen et al. 1968; Kulpa and Olsen 1971; Delisle and Levin 1972b), making unambiguous interpretation of PHS characteristics problematic. Thus, Olsen (1967) and Olsen et al. (1968) can be reconciled only by assuming that the 1968 host was grown at a warmer temperature prior to infection at 3.5°C.

The most comprehensive cold-active virus work was that of Delisle and Levin (1969a, 1969b, 1972a, 1972b), who isolated 180 pseudomonad-infecting phages from fish fillets, raw sewage and Boston Harbor water. Although they used two isolation temperatures (2 and 20°C), their report (Delisle and Levin 1969a) is unclear whether all 180 phages were cold-active; their companion study of 40 of these phages only clearly established cold-activity for 18 of them (Delisle and Levin 1969b). Of these, three were examined in detail, one of which (phage 27) formed plaques at 2 but not 20°C. Further study of phage 27 determined that its

Table 10.1 Characteristics of known cold-active phage-host systems (ordered by publication date). Phages or hosts in **bold** were isolated from the marine environment. Plaque temperatures: temperature range of plaque formation; an * indicates that this range is less than the known range for host growth. LP and BS: latent period and burst size, determined by one-step growth experiments at the temperature in **bold**. GS: genome size. G+C: Molar G+C ratio. EM: electron micrograph of phage. AD: adsorption measurements undertaken (not necessarily at the temperature in bold). +: determined; –: not determined. The table is adapted from Wells (2006)

Phage	Host	Plaque temperatures (°C)	LP (h)	BS	GS (kb)	G+C (%)	EM	AD	Reference
Unnamed	Unidentified	2, 4	–	–	–	–	–	–	Elder and Tanner (1927, 1928)
P/14	***Photobacterium phosphoreum***	0, 20, 21*; not 22	–	–	–	–	–	–	Spencer (1963)
P/L/34	***Cytophaga sp. A***	0, 20	–	–	–	–	–	–	Spencer (1963)
P/SW31	***Pseudomonas sp.***	0, 20	–	–	–	–	–	–	Spencer (1963)
P/SW34	***Pseudomonas sp.***	0, 20	–	–	–	–	–	–	Spencer (1963)
P/SW38	***Pseudomonas sp.***	0, 20	–	–	–	–	–	–	Spencer (1963)
P/SW1/a[a]	***Cytophaga sp. B***[b]	0, 20, **r.t.**[c]	2.5	28	–	30	–	–	Spencer (1963), Chen et al. (1966)
P/SW1/b[a]	***Cytophaga sp. B***[b]	0, 20, **r.t.**[c]	3	20	–	30	+[d]	–	Spencer (1963), Chen et al. (1966), Valentine et al. (1966)
PX14	*Pseudomonas geniculata*	**3.5**, 25, 33, 34; not 35	6	325	–	–	–	–	Olsen (1967)
PX14	*Ps. geniculata*	**3.5**, 25; not > 32	6	25	–	53.8	+	–	Olsen et al. (1968)
PX1	*Ps. putida*	**3.5**, 25; not > 32	12	6	–	52.4	+	–	Olsen et al. (1968)
PX10	*Ps. fluorescens* 22	**3.5**, 25; not > 32	8	6	–	53.0	+	–	Olsen et al. (1968)
PX12	*Ps. fluorescens* 35	**3.5**, 25; not > 32	7	10	–	55.8	+	–	Olsen et al. (1968)
PX4[e]	*Ps. fluorescens* 14	**3.5**, 25; not > 32	11	16	–	44.4	+	–	Olsen et al. (1968)
PX4[e]	*Ps. aeruginosa* 2	3.5, **25**; not > 32	0.9	103	–	44.4	+	–	Olsen et al. (1968)

(continued)

Table 10.1 (continued)

Phage	Host	Plaque temperatures (°C)	LP (h)	BS	GS (kb)	G+C (%)	EM	AD	Reference
Unnamed	***Aeromonas sp.***	0, 5, 10, 15, 20, 23*; not 25	–	–	–	–	+	–	Wiebe and Liston (1968)
C1	*Micrococcus cryophilus*	**3.5**, 20, 25	5–6	50	–	41	+	+	Kulpa and Olsen (1971)
ps_1	*Ps. fragi* PS_1	2, **7**, 21*; not 32	2	118	–	–	+	+	Whitman and Marshall (1971b)
wy	*Ps. fragi* WY	2, **7**, 21*; not 32	4	529	–	–	+	+	Whitman and Marshall (1971b)
23	*Ps. putrefaciens*	2, 13, 20, 26	–	–	–	40.0	+	–	Delisle and Levin (1972a)[f]
25F	*Ps. putrefaciens*	2, 13, 20, 26	–	–	–	48.8	+	–	Delisle and Levin (1972a)[f]
27	*Ps. putrefaciens* P10	**2**, 13, 20*; not 26	8.5	10	–	36.4	+	+	Delisle and Levin (1972a,b)
27	*Ps. putrefaciens* P19X	–5, –2, **2**, 4, 6, 8, 10, 13*; not 20	14	10	–	36.4	+	+	Delisle and Levin (1972a,b)
A19	*Brocothrix thermosphacta*	1, 7, 25; not 30	–	–	–	–	+	–	Greer (1983)
Unnamed	***Pseudoalteromonas sp.***	**0**	15	18	26	–	–	–	Middelboe et al. (2002)
1a	possible ***Shewanella***	0, 5, 10, 14*; not 19	–	–	70	–	+	–	Borriss et al. (2003)
11b	possible ***Flavobacterium***	0, 5, 10*; not 14	–	–	30	–	+	–	Borriss et al. (2003)
21c	possible ***Colwellia***	0, 5*; not 10	–	–	45	–	+	–	Borriss et al. (2003)
ϕS1[g]	*Ps. fluorescens*	**4**, 26, 37	~1[h]	–	–	–	–	–	Sillankorva et al. (2004)
9A	***Colwellia psychrerythraea 34H***	–6, –3, **–1**, 2, 4* (8)[i]; not 13	4–5[j]	55[j]	85	–	+	+	Wells and Deming (2006b)
9A	***C. demingiae ACAM 459***	–6, –3, –1, 2, 4, 8*; not 13	–	–	85	–	+	–	Wells and Deming (2006b)

Table 10.1 (continued)

[a] Spencer's designation; Chen et al. (1966) referred to P/SW1/a as NCMB 384 and P/SW1/b as NCMB 385.

[b] Originally classified as *Flavobacterium* sp. by Spencer (1963); re-classified by Chen et al. (1966).

[c] r.t.: room temperature, reported as 22–25°C.

[d] Valentine et al. (1966) used electron microscopy to examine morphological changes in the host over the course of phage infection; their micrographs are not of sufficient magnification to determine phage morphology.

[e] Known to be a generalized transducing phage; Olsen and Metcalf 1968.

[f] In addition to the three bacteriophages that they studied in detail in 1972, Delisle and Levin (1969b) identified 15 others infective at 2°C.

[g] Identified as a Podoviridae (Sillankorva et al. 2004).

[h] Not determined from a one-step growth experiment.

[i] Plaques formed at 8°C only if the host was first starved; microscopically observed phage production was detected from ~−12°C to 8°C, but not at 13°C.

[j] At 8°C, the latent period was 2.5–3 h and the burst size was 5; a low resolution one-step growth curve at −10 to −12°C implied a latent period of 5–10 days and a burst size of 5.

maximal temperature of plaque formation both depended on its host (Table 10.1; Delisle and Levin 1972a) and changed if the virus were passed through different hosts at different temperatures (Delisle and Levin 1972b). Evidence of phage 27's adaptation to low temperature included an adsorption rate at 0°C comparable to or faster than at 26°C (as for other phages at low vs high temperature; e.g., Johnson 1968) and an efficiency of plating on host P19X five orders of magnitude higher at temperatures between −5 and 4°C than at ≥10°C (Delisle and Levin 1972b).

Other viruses active at low temperatures (≤10°C) have been isolated from refrigerated food products (at 10°C; Whitman and Marshall 1971a), especially ground beef (at 10°C; Whitman and Marshall 1971b), cold storage beef (at 7°C; Patel and Jackman 1986) and spoiled rib steaks (at 7°C; Greer 1982; 1983). Of these, only two were extensively characterized, and both were cold-active (Whitman and Marshall 1971b; Table 10.1).

10.2.2 Cold-active phages from marine environments

10.2.2.1 Marine surface waters

To avoid culture bias, Spencer (1955, 1960, 1963) emphasized isolating phages using indigenous bacteria as hosts and temperature and salinity conditions comparable to those in situ. Applying this approach in the North Sea, Spencer succeeded in isolating phages at 0°C (Table 10.1). Surprisingly, given Spencer's emphasis on in situ temperature, only two of the seven PHS that he isolated were extensively characterized, both at room temperature (Spencer 1963; Chen et al. 1966; Valentine et al. 1966; Table 10.1). Sporadic reports of other demonstrably cold-active or possibly cold-active (based on their provenance) phages have likewise been limited because minimal or no characterization work was done (as emphasized by Table 10.1) or characterization was performed at much warmer temperatures than expected in situ (e.g., Johnson 1968).

Polar oceans are obvious sites to search for cold-active viruses, since field studies there show viruses to be abundant and active (e.g., Steward et al. 1996; Guixa-Boixereu et al. 2002; Middelboe et al. 2002). Yet, only five PHS from polar surface waters have been reported, all in the same study (Middelboe et al. 2002; Table 10.1). Moreover, based on denaturing gradient gel electrophoresis and restriction fragment length polymorphism patterns, Middelboe et al. (2002) concluded that the five PHS were identical, perhaps suggesting stable co-occurrence of phage and host over a 50,000-km^2 sampling area and 2-week period. An unpublished temperate PHS from Arctic surface waters will be discussed below. As far as I know, the only other possibly cold-active phages isolated from surface (but not polar) seawater were those described by Moebus (1983) capable of infection at 5°C; temperatures below 5°C were not tested, however.

10.2.2.2 Other marine water and sedimentary environments

Viral adaptations not only to low temperature but to high pressure have largely been overlooked in marine viral ecology, despite high VLP abundance (e.g., Danovaro and Serresi 2000; Ortmann and Suttle 2005) and demonstrated activity at depths from a hundred or a few hundred meters (Steward et al. 1996; Weinbauer et al. 2003; Wells and Deming 2006a) to, possibly, a few thousand (Ortmann and Suttle 2005). Although PHS have been isolated from 1°C sediments as deep as 3,000 m (Johnson 1968), they have rarely been characterized under corresponding pressure and temperature conditions in the laboratory (e.g., the likely cold-active phage and its host described by Johnson (1968) were examined at 1 atm and 6–25°C). To my knowledge, only two laboratory studies, both of cold-active viruses, have considered phage production under deep-sea pressures. The first was the pioneering description of an *Aeromonas*-infecting bacteriophage isolated from 825-m-deep Pacific sediments (Wiebe and Liston 1968). Besides showing that the temperature range of plaque production (0–20°C; not 23°C) was narrower than the host's range of growth (0–30°C; not 32.5°C), a common observation (Table 10.1), they also reported phage production from 1 to 200 atm, unfortunately at unspecified temperature.

The other published laboratory study of virus production under high hydrostatic pressure examined cold-active bacteriophage 9A, isolated from a 128-m-deep Arctic nepheloid layer using a novel isothermal plating method (Wells and Deming 2006b). The isolation host was the psychrophile *Colwellia psychrerythraea* strain 34H (hereafter "strain 34H"), itself originally cultured from 305-m-deep Arctic sediments. Phage 9A has been more extensively characterized than most cold-active phages (Table 10.1). Its morphology and 80–90 kb double-stranded DNA genome identify it as a member of the *Siphoviridae*, most similar to T5-like viruses. On strain 34H, phage 9A forms plaques from −6 to 4 or 8°C (depending on prior host starvation; Table 10.1), with evidence of continued phage production at −10 to −13°C. In terms of pressure, microscopic analysis detected phage production at −1°C to at least 200 atm, but not at 400 or 600 atm. Based on Johnson (1968), Wiebe and Liston (1968) and Wells and Deming (2006b), it seems likely that phages adapted to higher pressures (as well as low temperature) await discovery in the deep ocean. Since 20–40% of cells below ~1,000 m are archaea (e.g., Karner et al. 2001), many of these viruses may infect archaea rather than members of the domain bacteria.

Besides pressure tolerance and cold activity, phage 9A had other curious characteristics. Its temperature and salinity ranges of plaque formation were different on its two identified hosts, strain 34H and *C. demingiae* ACAM 459^{T}, hinting at possible, condition-dependent "niche-partitioning" of viral hosts in the ocean (Wells and Deming 2006b). As already noted, the temperature range could be extended if the host were starved prior to infection. Taken with phage 9A's undetectable adsorption rate, low efficiency of plating and the work of Delisle and Levin (1972a, 1972b), these observations imply the importance of host phenotypic plasticity to overall PHS dynamics, a little-studied subject in marine viral ecology. Such data also emphasize drawbacks of extrapolating general phage characteristics (e.g., host range) from necessarily limited samples of possible environmental con-

ditions. An additional caveat is implied by the detection of, Wells and Deming (2006b) also detected phage production microscopically even when plaques did not form (Wells and Deming 2006b), thus casting doubt upon conclusions about phage infectivity drawn solely from plaque data. Finally, Wells and Deming (2006b) observed that the latent period of phage 9A increased with decreasing temperature. Unlike many cold-active viruses, however, the burst size and overall phage production rate were higher at the low, in situ temperature of −1°C than the optimal growth temperature of strain 34H, 8°C. They concluded that reduced burst size was a reaction to temperature *stress*, not low temperature per se. Thus, the estimated burst size at temperatures between −10 and −12°C was similar to that at 8°C, but approximately ten times lower than that at −1°C (Table 10.1).

10.2.2.3 Sea ice

Among the highest marine VLP concentrations (often $\geq 10^8$ ml^{-1}) are found in sea ice. Visibly infected eukaryotes from summer pack ice (Gowing 2003) and dynamic VLP populations from winter ice incubated in −12°C, 160 psu (practical salinity units) brines (Wells and Deming 2006c) indicate that the low temperatures and possibly high salinities of sea ice do not necessarily preclude viral activity. In fact, the freezing process may promote phage-host interactions: as seawater freezes, it excludes impurities such as salts, most cells (Junge et al. 2001) and, presumably, viruses, concentrating them instead into brine inclusions. Assuming VLP partition into brine, VLP numbers may thus be higher in sea ice than reported, since determined concentrations are normally scaled to the volume of ice sampled (Maranger et al. 1994) or melted (Gowing et al. 2002, 2004). Wells and Deming (2006c) calculated that the concentration effect of freezing could outstrip the temperature-dependent drop in diffusivity, resulting in theoretical contact rates between viruses and cells 3–4 orders of magnitude higher at −30°C than −1°C. Contact rates determined from measured concentrations of bacteria and VLP in winter sea ice were typically lower than theoretical values (presumably because VLP and cells were lost during freezing), but calculated rates at −11 to −29°C were still comparable to or much greater than those in underlying water (Wells and Deming 2006c). The calculated rates rest, however, on the untested assumption that VLP partition into brine.

These observations and arguments suggest sea ice as a site from which to isolate cold-active viruses and their hosts, the accomplishment to date of only one published study consisting of a useful but basic description of three PHS from sea ice or melt ponds (Borriss et al. 2003; Table 10.1). Data on latent period, burst size, or other dynamical aspects of sea-ice PHS are still lacking. Although Wells and Deming (2006b) measured phage production at −10 to −13°C by the strain 34H-phage 9A PHS, they did so in a supercooled medium, not a brine; no information is available about PHS characteristics under the coupled temperature and salinity regimes expected in winter brine inclusions.

10.2.3 Physico-chemical characteristics of cold-active phages

Morphological examination suggests that cold-active viruses include representatives of the double-stranded DNA virus families *Podoviridae*, *Siphoviridae* and *Myoviridae*; none with RNA as their genetic material are yet known. Two viruses described by Spencer (1963) were extremely sensitive to chloroform, perhaps implying inclusion of lipids in their virions; similar information is not available for other cold-active viruses. The limited data do not suggest that cold-active phages as a group are distinguished by particular sensitivity to pH, especially high pH (≤ 11; Olsen et al. 1968); tolerance of acidic values (pH < 4) varies considerably with the phage (Olsen et al. 1968; Whitman and Marshall 1971b) and may be extended at low temperature (Johnson 1968).

The most salient physical feature of cold-active viruses is thermolability. While non-cold-active viruses commonly tolerate ≥ 60 min at 60°C with minimal losses (e.g., Olsen et al. 1968), inactivation of ≥ 99% of cold-active phages (measured by plaque assay) is typical at this or lower temperatures within 10 min (Spencer 1955; 1963; Olsen et al. 1968; Wiebe and Liston 1968; Kulpa and Olsen 1971; Whitman and Marshall 1971b; Delisle and Levin 1972a; Wells and Deming 2006d). Comparisons among cold-active viruses are limited by the qualitative nature of early studies, use of media with different cation and organic concentrations (variables affecting thermal inactivation of viruses, including cold-active ones; Spencer 1955, 1963; Wiebe and Liston 1968), and insufficient data to extrapolate virus-specific Arrhenius curves. A further limitation is culture bias: many phage isolations, including of cold-active ones (e.g., Middelboe et al. 2002; Borriss et al. 2003), used variations of agar overlay methods that briefly expose phages and hosts to temperatures of 35–46°C. The calculated half-life of phage 9A, isolated using an isothermal approach, is 20–50 s at these temperatures (Wells and Deming 2006d), suggesting that such conditions may select against some cold-active phages. Especially in psychrophiles, such exposure may also induce host phenotypic changes (e.g., a shock response) that affect PHS characteristics.

It is unclear why cold-active viruses are more thermolabile than viruses active at warmer temperatures. As judged from melting profiles and G+C content (Table 10.1), their nucleic acid composition is comparable to other viruses. Temperature sensitivity may instead reflect properties of viral proteins. Here, a useful analogy is to cold-active enzymes (Olsen et al. 1968; Wells and Deming 2006d), whose apparent adaptations to environments of low thermal energy include specific compositional and conformational adjustments that confer greater flexibility on the enzyme, maintain high catalytic rates, but result in increased lability (see Chap. 13). To the degree that a virus is also an extracellular enzyme, a similar argument may apply: indeed, many phages contain virion enzymes that hydrolyze peptidoglycan (Moak and Molineaux 2004), enabling cell-surface access. Appropriate binding to cell-surface receptors, changes in the orientation of the head-tail connector, or other features associated with host recognition and nucleic acid injection may also benefit from enhanced protein flexibility and lead to greater thermolability.

As with other viruses, cold-active phage stability is affected by ionic strength and composition (Spencer 1963; Chen et al. 1966; Olsen et al. 1968; Whitman and Marshall 1971b). A phage's ionic sensitivities appear to reflect its selective environment and evolutionary history more than cold activity per se (e.g., marine cold-active viruses are typically more stable in saline than freshwater). Cold activity and salt tolerance may be linked in winter sea-ice brine inclusions, however, with prevailing low temperatures facilitating survival, as suggested by enhanced stability of phage 9A in 161-psu brines at −12 versus −1°C (Wells and Deming 2006d). Besides affecting phage inactivation, specific ions also influence whether a cold-active phage lyses its host (Spencer 1955, 1963; Olsen et al. 1968; Whitman and Marshall 1971b; Delisle and Levin 1972a). Such traits are again better understood as representative of a PHS rather than cold activity per se; indeed, for some PHS, they change as a function of temperature (Wiebe and Liston 1968).

10.3 Molecular and genomic studies of cold-active viruses

Molecular approaches applied to field samples provide information about the majority of viruses which, like their hosts, have eluded isolation. Unfortunately, it is not possible to demonstrate cold-activity solely from sequence data. Here, deductions about cold-active viruses derived from such data are limited to work from perennially cold places, where I assume most viral sequences correspond to cold-active viruses.

Only a handful of studies on molecular or genomic features of uncultured and likely cold-active viruses are available. Short and Suttle (2005) used PCR to amplify a region of a conserved viral (usually cyanophage) structural gene, g20, in samples from Arctic and Antarctic surface marine waters, the deep (≥3,000 m) Chukchi Sea, an Arctic meltwater pond, and several temperate freshwater and marine sites. Surprisingly, they discovered nearly identical nucleotide sequences in dramatically different locales (e.g., amplified products from the Gulf of Mexico, an Arctic meltwater pond, the Southern Ocean and Lake Constance were ≥99% identical), possibly indicating the widespread distribution of closely-related phages in disparate environments. Considerable diversity in the g20 gene was also observed, however, with polar environments contributing sequences to at least seven distinct clusters. Filée et al. (2005) also reported high sequence diversity of the major capsid protein of T4-like phages throughout the world's oceans, including the Arctic, which they interpreted to imply the presence of many uncharacterized subgroups.

Besides specific viral genes, whole viral genomes can be analyzed. For example, Steward et al. (2000) used genome size distributions as a metric of viral diversity, finding that Arctic viral populations showed little seasonal or spatial variability and, along with a lone Antarctic sample, low diversity compared to more temperate marine sites. Their analysis likely missed small (≤26 kb) viral genomes and those composed of RNA or single-stranded DNA (ssDNA). On this count, Angly et al. (2006) did not detect chp1-like microphage ssDNA in the Arctic but found it in

other oceanic regions. Possible filamentous viruses (which have small ssDNA genomes) have been observed by electron microscopy in low abundance (≤3% of VLP) in Arctic waters, however (Wells 2006).

More detailed, sequence- rather than size-based analyses of viral metagenomes from four oceanic regions (the Sargasso Sea, the Gulf of Mexico, the coast of British Columbia and the Canadian Arctic) measured lowest genotype richness (only 532 predicted genotypes vs 5,140–129,000) in the Arctic (Angly et al. 2006), in support of Steward et al (2000). Despite apparently low diversity, the Arctic viral metagenome had the greatest evenness of the four environments: the most abundant genotype was only 2.27% of the population versus 7.28–13.3% at the other sites. Most (86.9%) Arctic viral sequences also had no matches in the SEED nonredundant or environmental databases (the SEED is composed of the GenBank database plus complete and draft genome sequences), as found generally for viral metagenomes (Edwards and Rohwer 2005). Clearly, enormous genetic diversity resides among viruses and awaits detailed study.

Within this diverse, largely unknown collection of phage genes may be some especially responsive to major selective pressures on the *host* (Lindell et al. 2004), since phage reproduction depends on maintaining basic host functions. This hypothesis may explain why genes for phosphate metabolism (e.g., Rohwer et al. 2000) and photosynthesis (e.g., Lindell et al. 2004) are found in viral genomes and sometimes expressed by the host (e.g., Lindell et al. 2005). In this respect, of the few putatively viral genes to which a function could be assigned, those that occurred most frequently in the Arctic included carboxylases and transferases of potential use to maintaining host metabolism during infection (Angly et al. 2006). Additionally, the second most commonly occurring identifiable gene was the alpha subunit of DNA polymerase III, i.e. the subunit with polymerase activity in the DNA polymerase III holoenzyme, congruent with speculation by Wells and Deming (2006b) that cold-active phages (in particular, phage 9A) might encode extremely cold-active DNA polymerases.

Angly et al. (2006) also noted that prophage-like sequences were more prevalent in the Arctic than in the other three oceanic regions. (Prophages are viral genomes integrated into the genomes of their hosts, a circumstance called “lysogeny”; viruses capable of lysogeny are termed “temperate.”) The rare data on lysogeny in perennially cold environments pertain to estimates of its incidence in Antarctic lakes (from undetectable to 73% of cells; Lisle and Priscu 2004; Säwström et al. 2007a; Laybourn-Parry et al. 2007) and in a few depth profiles from the Baltic Sea (from undetectable to 25% of cells; Weinbauer et al. 2003). Similarly, I know of only two psychrophilic lysogens that have been identified as such in culture—a paucity likely due to lysogeny being overlooked rather than absent in other cultured psychrophiles. The first is a marine isolate from Arctic surface waters, 53A3, that spontaneously formed turbid plaques (Wells 2006). By 16S rRNA analysis, 53A3 was most closely affiliated to *C. psychrerythraea* (R.E. Collins and J.W. Deming, unpublished data); further characterization of host or phage has not been done.

The second known psychrophilic lysogen is strain 34H. Although Wells (2006) presented data based on microscopy that strain 34H produced VLP when exposed

to supramaximal temperatures of 43°C, the most compelling evidence was genomic. Wells (2006) found two ~8 kb regions in the published 34H genome (Methé et al. 2005) that were organizationally homologous to several filamentous phage genomes, including the cholera-toxin encoding phage CTXΦ (Fig. 10.1). Each region contains 12 proposed open reading frames (ORFs), 9 of which are identical in the two phages, including recognizable homologs of genes found in other filamentous phages [e.g., *zonula occludens* toxin (zot; ORF 4687/5274) and rolling circle replication (ORF 4681/5268) genes; Fig. 10.1 and Table 10.2]. In the regions where the two possible phages diverge (Fig. 10.1) are found putative repressors, but the repressors are not closely related to each other (Table 10.2). Also found there are ORFs (4680/5267) distinguished between the two viruses by the presence or absence of the first nine amino acids. On the other end of each genome is a large intergenic region with high secondary structure, the initial 401 nucleotides of which are identical in the two phages (Fig. 10.1).

The apparent recombination event recorded in the strain 34H genome is an unusual example of viral evolution caught in flagrante delicto and emphasizes the evolutionary significance of lysogens as genetic depots for viruses or other elements, even at low temperature. The capacity of filamentous phages to transfer large DNA segments (they are often used as cloning vectors) may make the putative strain 34H phages attractive for the development of psychrophilic genetic or phage display systems or for the study of the ecological and evolutionary importance of analogous processes, about which little is known. Note, however, that expression of the putative phages has not been demonstrated. Both are in genomic regions with other evidence of horizontal gene acquisition (i.e., integrases, transposes, and other apparently

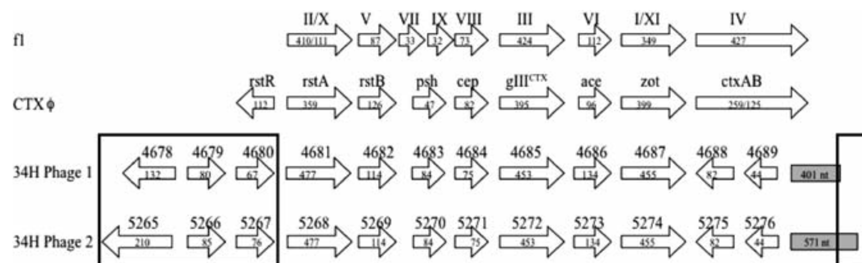

Fig. 10.1 Gene organizations of two representative filamentous phages (f1 and CTXϕ) as well as of two proposed filamentous phages found in the *C. psychrerythraea* strain 34H genome. *Arrows* represent open reading frames (ORFs) and the direction of transcription; the *number* shown inside each arrow is the ORF size in amino acids. Above each arrow is the ORF designation (see Table 10.2 for the predicted products of strain 34H genes). No attempt has been made to indicate intergenic regions, except for the major intergenic region of the two strain 34H genomes (gray boxes with size indicated in nucleotides). The *black boxes* indicate regions where the two putative strain 34H phages diverge. Note the correspondence of predicted rolling circle replication genes in the proposed 34H phages (ORF 4681/5268) with rolling circle replication genes in f1 (gene II/X) and CTXϕ (rstA); likewise for the major capsid gene (ORF 4684/5271, as argued by Wells (2006); gene VIII [f1] and cep [CTXϕ]) and *zot* (ORF 4687/5274). Figure adapted from Davis and Waldor (2003) and Wells (2006)

Table 10.2 Genomic neighborhood and organization of two putative filamentous prophages (in **bold**) of *C. psychrerythraea* strain 34H. ORF: Open reading frame. Size: size of predicted gene product in amino acids (aa). −log P: the negative of the logarithm of the P-value; the P-value describes the probability of finding a match with a higher score. Table adapted from Wells (2006)

Phage 1:				Phage 2:			
ORF	Size (aa)	Best match [% identity/% similarity]	−log P	ORF	Size (aa)	Best match [% identity/% similarity]	− log P
4667	334	Phage integrase (*Salmonella enterica* serovar Typhi CT18) [48.1/62.3]	67.4	5253	151	Putative transmembrane protein (*Ralstonia solanacearum* GMI1000) [48.9/63.0]	25.9
4668	68	gp75 (Mycobacteriophage Bxz1) [48.5/63.6]	1.3	5254	205	None	
4670	226	None		5255	33	None	
4671	150	None		5257	342	ISCps9, putative transposase (*Klebsiella pneumoniae*) [36.8/60.6]	55.0
4672	130	Conserved hypothetical protein (*Pseudomonas syringae*) [30.2/53.4]	1.5	5258	125	None	
4673	120	ISCps3, transposase orfA (*Leptospira interrogans* serovar Lai strain 56601) [36.6/54.5]	7.8	5260	68	Polyprotein (Norwalk-like virus) [34.8/65.3]	1.4
4674	295	ISCps3, transposase orfB (*Vibrio vulnificus* CMCP6) [34.4/54.3]	42.9	5261	138	None	
4675	36	None		5262	130	None	
4676	156	None		5263	176	None	
4677	53	None		5264	68	None	
4678	132	cI-like repressor protein (*Streptococcus pneumoniae* bacteriophage MM1) [32.3/54.5]	3.7	**5265**	210	Putative repressor protein (*Neisseria meningitidis* serogroup A Z2491) [32.4/51.1]	19.6
4679	80	cox (bacteriophage K139) [36.4/58.2]	2.7	**5266**	85	None	
4680	67	None		**5267**	76	None	

(continued)

Table 10.2 **(continued)**

Phage 1:				Phage 2:			
ORF	Size (aa)	Best match [% identity/% similarity]	–log P	ORF	Size (aa)	Best match [% identity/% similarity]	– log P
4681	477	Phage replication protein (*Neisseria meningitidis* Z2491) [32.4/51.1]	19.6	**5268**	477	Phage replication protein (*Neisseria meningitidis* Z2491) [32.4/51.1]	19.6
4682	114	None		**5269**	114	None	
4683	84	None		**5270**	84	None	
4684	75	None[a]		**5271**	75	None[a]	
4685	453	Streptococcal hemagglutinin protein (*Staphylococcus epidermis*) [21.8/35.3]	1.9	**5272**	453	Streptococcal hemagglutinin protein (*Staphylococcus epidermis*) [21.8/35.3]	1.9
4686	134	Accessory cholera enterotoxin (*Vibrio cholerae* phage KSF-1ϕ) [24.8/48.6]	1.7	**5273**	134	Accessory cholera enterotoxin (*Vibrio cholerae* phage KSF-1ϕ) [24.8/48.6]	1.7
4687	455	Zonula occludens toxin-like protein (*Vibrio parahaemolyticus*) [40.4/57.1]	60.8	**5274**	455	Zonula occludens toxin-like protein (*Vibrio parahaemolyticus*) [40.4/57.1]	60.8
4688	82	None		**5275**	82	None	
4689	44	None		**5276**	44	None	
4690	68	None		5277	35	None	
4691	343	None		5278	33	None	
4692	31	None		5279	219	None	
4693	701	Elongation factor G (*Vibrio cholerae* El Tor N16861) [80.4/89.4]	281	5280	55	None	
				5281	355	Hypothetical protein (*Pseudomonas syringae* pv. pisi) [34.9/58.2][b]	30.0

[a] Wells (2006) proposed that this gene encodes the major capsid protein, based on its size, genomic context and a characteristic distribution of charge in the translated product.

[b] In addition to the hypothetical protein, several integrases of phages and shufflons are identified with high similarity (~ 50%) and – log P values of 14–19.

viral genes; Table 10.2), and perhaps are defective or parts of larger genetic elements.

10.4 Conclusions

Viruses are important ecological and evolutionary agents in cold environments as elsewhere, contributing substantially to overall microbial mortality and affecting both the genotypic (e.g., Weinbauer and Rassoulzadegan 2004) and phenotypic (Wells and Deming 2006b) diversity of the microbial assemblage. Whether by transduction (Olsen and Metcalf 1968) or phage conversion, they may also impact evolution by mobilizing selectively important genes. The vast genetic diversity represented by viruses, most presently of unknown function, likely hosts proteins and enzymes of biogeochemical and biotechnological interest, including particularly cold-active ones. Further understanding of the ecological and evolutionary roles of cold-active viruses, as well as of their physical characteristics, will require the application of temperature-sensitive methods, integration of field and laboratory studies, and investigation of currently under-explored realms like the deep sea, glacial ice and genomes of psychrophilic or psychrotolerant cells in culture.

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Part III
Molecular Adaptations to Cold Habitats

Chapter 11
Membrane Components and Cold Sensing

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11.1 Introduction

Like all microorganisms, psychrophiles are too small to insulate themselves from the cold or to use avoidance strategies by moving away from thermal extremes. Therefore, the only recourse is to alter their cellular composition. This chapter focuses on the cold adaptation of membranes, particularly how the lipid composition of membranes is changed so that the fluidity and phase properties are retained within functional limits of passive permeability and the activity of integral membrane proteins, including how cold is sensed. Most information concerning cold adaptation of membranes derives from work on bacteria. When possible, comparative information on bacteria, archaea, yeasts, filamentous fungi and algae will be given.

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11.2 Phylogeny of membrane lipid cold-dependent changes

The main changes in lipid composition that occur in all microorganisms as a result of the lowering of growth temperature are in the fatty acyl (mainly C_{14}-C_{18}) composition of complex glycero-acyl lipids, which are the predominant membrane lipids in all microorganisms except archaea. In comparison, the cold-dependent changes in head-group composition of lipids are much smaller, and do not follow a set pattern (Russell 1989, 2003). The outer membrane of Gram-negative bacteria has lipopolysaccharide (LPS) instead of phospholipid in the outer leaflet of the lipid bilayer. The LPS contains diagnostic hydroxyl (C_{12}-C_{16}) and hydroxyacyl fatty acids as well as the more usual saturated and monounsaturated fatty acids and, although the outer membrane does not act as a semi-permeable membrane, its fatty acid composition is thermally regulated, probably to modulate the function of porins and other proteins such as receptors (Kumar et al. 2002).

Several types of anaerobic bacteria contain ether lipids with (C_{14}-C_{18}) alkyl or alkenyl chains, e.g. the plasmalogens in clostridia (Harwood and Russell 1984). The small amount of available data indicates that low temperature has similar effects on the alkyl/alkenyl and acyl compositions, which vary considerably between different phylogenetic groups of anaerobes (Goldfine 1984). However, a number of the analytical methodologies used frequently for lipid analysis fail to give information about the alkyl/alkenyl chains in gas chromatographic analyses. Thus, in what follows the terminology “fatty acid” or “fatty acyl chain” will be adopted, since most information is derived from microorganisms containing acyl glycerolipids.

In contrast to bacteria, the archaea contain ether-linked glycero-alkyl lipids that are based on C_{20} (or C_{25}) phytanyl units instead of fatty acids. The focus of attention has been on (hyper)thermophilic archaea, in which the temperature-dependent changes in lipid composition are either much smaller or non-existent compared with those in bacteria. Little is known about cold adaptation of membranes in psychrophilic archaea, even though they are abundant, particularly in cold oceanic waters and wet soils (Cavicchioli 2006).

Eukaryotic microorganisms (yeasts, filamentous fungi and algae) plus a few exceptional species of bacteria contain sterols that are phylogenetically specific, with ergosterol being the most common (Harwood and Russell 1984). The sterol composition does not usually change significantly with alterations of temperature: instead, the relative proportions of total sterol and phospholipid is a key feature of membrane fluidity regulation in the cold in eukaryotic microorganisms.

11.3 Thermal (cold) control of membrane lipid changes

The various cold-dependent lipid changes found in different microbial phylogenetic groups are summarized in Table 11.1. Clearly, it is bacteria that utilize the broadest range of structural changes to maintain lipid fluidity at low temperatures. An

Table 11.1 The cold-dependent lipid changes in different groups of microorganisms

Type of lipid change	Group of microorganism
Increased FA unsaturation	Bacteria, archaea, yeasts, filamentous fungi, algae
Increased FA methyl branching	Bacteria
Increased *anteiso/iso*-branched ratio	Bacteria
Decreased FA average chain length	Bacteria, yeasts, filamentous fungi, algae
Decreased sterol/phospholipid ratio	Bacteria, yeasts, filamentous fungi, algae

FA, fatty acid (acyl)

increase in fatty acid unsaturation is the most common change in the cold, and a number of mechanisms are found in different microbial groups.

11.3.1 Desaturases

Desaturases generally form part of a membrane-bound complex, associated with other proteins and cofactors that function as a short respiratory chain to transfer the hydrogen atoms removed stereospecifically from the fatty acid substrate to a terminal acceptor that is usually oxygen. Hence, the process is sometimes termed "aerobic desaturation" (to distinguish it from the "anaerobic pathway", described below). However, it is feasible that alternative electron acceptors such as FeIII or nitrate could be used for desaturation during anaerobic growth. Thus, by analogy with bacterial respiration, the desaturase mechanism may be aerobic or anaerobic. The most common positional specificity for hydrogen removal is Δ9 in all microorganisms, giving 16:1Δ9 (palmitoleic acid) and 18:1Δ9 (oleic acid) as frequent products. In bacteria, Δ5 and Δ10 desaturases are also relatively common. In yeasts, filamentous fungi, algae and some cyanobacteria, Δ6, Δ12 and Δ15 desaturases (together with the Δ9 enzyme) are responsible for the synthesis of polyunsaturated fatty acids (PUFA) by repetitive desaturation of the "fatty acid" substrate to give characteristic products such as linoleic acid (18:2Δ9,12) or γ-linolenic acid (18:2Δ6,9,12).

Certain marine psychrophiles, including species of *Shewanella* and *Colwellia*, also contain PUFA, but they are made by a distinctive polyketide mechanism, and will not be considered further as their thermal regulation is not understood (Russell and Nichols 1999).

The substrate for desaturase is generally not the free (unesterified) acid, but is usually a fatty acyl chain of an intact phospholipid, although it is not known if it is lipid acyl chains in the inner leaflet of the bilayer only or both leaflets that are modified. Less commonly, acyl-CoA or acyl-ACP are used as substrates. This means that, for most desaturases, existing membrane lipid can be modified in the face of (sudden) temperature downshift. In bacteria, unsaturated lipids are usually *sn*-1 saturated, *sn*-2 monousaturated, but after a decrease in temperature diunsaturated (i.e., *sn*-1 monounsaturated, *sn*-2 monounsaturated) lipids are formed. Because the desaturase is an integral membrane protein, its activity will be influenced by membrane fluidity changes via lipid-protein interactions. If reduced fluidity activates the enzyme, the

desaturation system could be autoregulatory, since increased unsaturation would restore lipid fluidity and desaturase activity would decline.

Some desaturases are cold-inducible, but this does not imply that the adaptive change would be slower, because bacterial gene induction and expression and enzyme synthesis take only a few minutes for completion under normal growing conditions. In *Bacillus licheniformis*, immediately following a decrease in growth temperature, there is (hyper)induction of a Δ5 desaturase enzyme (the usual desaturase has Δ10 specificity and is made constitutively), which uses membrane lipid fatty acyl chains as its substrate; the products are mostly monounsaturated (Δ5), plus a smaller amount of diunsaturated (Δ5, 10) lipids, since under isothermal growth conditions the bacterium contains only a few percent of unsaturated (Δ10) lipids. However, this is not a permanent effect and desaturase activity declines as growth continues at the low temperature (Fulco and Fujii 1980). More than two decades later, the mechanism was elucidated by Mansilla and de Mendoza (2005) who showed that the Δ5 desaturase in *Bacillus subtilis* is a transmembrane protein that interacts with a two-component regulatory system. Their elegant study has revealed that the protein kinase component (DesK) responds to lipid fluidity (e.g., caused by a temperature down-shift) by phosphorylating the cytoplasmic DesR response regulator that is a transcriptional activator of the Δ5 desaturase gene. Subsequent desaturation of membrane phospholipids restores correct membrane fluidity and DesK resumes its dominant phosphatase state, whilst desaturase activity declines. As growth continues at the new (lower) temperature, the unsaturated lipid would be diluted by new membrane synthesis, but a second mechanism based on branched-chain fatty acids takes over to maintain fluidity.

Desaturase action is essentially unidirectional and therefore can only function in the adaptation to thermal downshifts. The reverse reaction requires a different enzyme, a fatty acid hydrogenase. This is found in some microorganisms, particularly in anaerobic habitats such as the rumen, but such enzymes appear to be involved in accessing lipid substrates for oxidation rather than in thermal adaptation of membranes (Harwood and Russell 1984).

11.3.2 Anaerobic mechanisms of unsaturated fatty acid synthesis

The so-named Anaerobic Pathway was the first anaerobic mechanism to be elucidated. It uses a modified Type II fatty acid synthase (FAS), in which the pathway is branched, with one "arm" leading to monounsaturated fatty acids and the other to saturated products (Harwood and Russell 1984). The double bond positions are diagnostic and reflect the elongation reaction of the FAS system, e.g., 16:1Δ9 and 18:1Δ11 (*cis*-vaccenic acid), which is particularly common in Gram-negative bacteria. The definitive work of Cronan and colleagues showed that after thermal downshift in *Escherichia coli* the proportion of *cis*-vaccenic acid increases, because the enzyme β-ketoacyl-ACP synthase II, which catalyses the condensation (elongation)

reaction whereby 2C units are added, is more efficient at elongating 16:1Δ9 (compared with 16:0) and is more thermolabile than synthase I, which carries out the other elongation reactions (Cronan and Rock 1996). Therefore, in the cold, synthase II activity rises compared to synthase I, which diverts substrate down the unsaturated branch of the anaerobic pathway to give the increase in18:1Δ11. Thus, a decrease in temperature has two effects, increasing unsaturated fatty acid biosynthesis but increasing the average fatty acid chain length (because less 16:0 is made). Mutants lacking or with a defective ß-ketoacyl-ACP synthase II are unable to modify their membrane lipid fatty acid composition at low temperature, whilst revertants can, proving that this is the thermally-regulated enzyme.

Recently, another anaerobic mechanism of introducing a double bond has been discovered in *Streptococcus pneumoniae* and a number of anaerobes, using combined biochemical and genome mining approaches (Marrakchi et al. 2002).

Unsaturated phytanyl chains have been found in the psychrophilic archaea *Methanococcoides burtonii* and *Halorubrum lacusprofundi* (Cavicchioli 2006). However, on the basis of a combined proteomics and genomics study, the mechanism of their synthesis in *M. burtonii* appears to be different to that in other microbial groups in that it results from the incomplete saturation of unsaturated precursors rather than by the introduction of a double bond into a saturated intermediate. More such organisms need to be investigated and the biochemistry elucidated to determine if this is indeed a unique archaeal cold-adaptive mechanism.

11.3.3 Branched-chain fatty acids

Another important mechanism for regulating membrane fluidity is found particularly, but not exclusively, in Gram-positive bacteria. This involves changes in the amount and type of methyl-branched fatty acids. Compared with *cis*-unsaturated lipids, those with methyl-branched acyl chains have a relatively more ordered liquid-crystalline phase but a more disordered gel phase—i.e., they have an "intermediate" fluidity (Russell 1989). *Anteiso*-methyl-branched acids are more fluidizing than are *iso*-branched acids, so an increase in the ratio of *anteiso/iso*-branched acids as well as the total amount of branched fatty acid (relative to straight-chain acids) will increase fluidity.

The molecular basis of thermal regulation of branched-chain fatty acid synthesis is not well characterized. Bacteria that contain branched fatty acids have a modified type II FAS that prefers branched-chain acyl-CoA primers for fatty acid biosynthesis (Kaneda 1991). The β-ketoacyl acyl carrier protein synthase III enzyme found in these bacteria has a strong preference for branched-chain acyl-CoA primers relative to acetyl-CoA, which is the usual primer for straight-chain (C_{even}) fatty acids (Choi et al. 2000). It seems likely that the relative amounts of *iso*-C_{odd}, *anteiso*-C_{odd} and *iso*-C_{even} fatty acids are controlled by the temperature characteristics (thermostability vs cold activity) of the branched-chain α-keto acid dehydrogenase (involved in the formation of the branched primers from leucine, isoleucine and

valine) and the β-ketoacyl acyl carrier protein synthase III enzymes. Differential cold activity of the initiation enzymes that recognize the branched-chain acyl-CoAs and acetyl-CoA would similarly regulate the flow of precursors into branched-chain and straight-chain fatty acids.

11.3.4 Fatty acid chain length

The shortening of fatty acyl chains decreases van der Waals interactions between them, lowering lipid viscosity and increasing fluidity (Russell 1984b). In the psychrotolerant bacterium *Psychrobacter uratovorans* (formerly misclassified as *Micrococcus cryophilus*), in which >97% of the total fatty acids are made up of just two components (16:Δ9 and 18:1Δ9) at all growth temperatures, thermal regulation of membrane fluidity across the growth temperature range is achieved exclusively through changes in fatty acyl chain length by adjusting the relative proportions of the C_{18} and C_{16} fatty acids (Russell 1984b). In *Listeria monocytogenes*, which is also psychrotolerant with a particularly wide thermal range of growth, the content of *anteiso* 15:0 appears to be important for growth in the cold, with the proportion of this acid rising at the expense of *anteiso* 17:0 at low temperatures, i.e. there is also cold-triggered fatty acid shortening in this bacterium (Zhu et al. 2005).

The chain length effect on lipid fluidity is exaggerated if one chain of a lipid molecule but not the other is shortened, because the longer acyl chain is free of van der Waals interactions at its distal (methyl) end and has more space in which to flex and vibrate, so further lowering the bilayer viscosity. The effect also depends on the positional-isomeric distribution of the fatty acyl chains, because the glycerol backbone to which they are attached in glycero-acyl complex lipids lies at an angle in the plane of the membrane with the *sn*-1 chain "dipping" deeper into the membrane than the *sn*-2 chain. Consequently, lipids with a *sn*-1 long, *sn*-2 short structure are significantly more fluidizing than those with a *sn*-1 short, *sn*-2 long configuration (Russell 1989). However, the positional isomeric distribution of fatty acids has seldom been reported in studies of microbial lipid thermal adaptation, and the significance or not of so-called "lipid tailoring" in psychrophiles by switching acyl chains to regulate membrane fluidity in the cold is not known. The psychrotolerant *P. uratovorans* contains unsaturated membrane phospholipids in which the preference is for the more fluidizing *sn*-1 long, *sn*-2 short isomer, but this preference is maintained throughout the growth temperature range and there is no evidence of lipid tailoring. Instead, it seems that this isomeric distribution is a cold adaptation to provide a fluid membrane at all growth temperatures.

11.3.5 Some fatty acid thermal "red herrings"

Some microorganisms, particularly bacteria, contain *trans*-unsaturated fatty acids, but they are not involved in the thermal regulation of membrane fluidity. They seem

to play a role in stabilizing the membrane against toxic agents, particularly hydrophobic compounds such as solvents (Heipieper et al. 2003). They are anyway most unlikely to play a role in cold adaptation, because *trans*-unsaturated acids are formed by isomerization of the *cis*-unsaturated homologue, a reaction that decreases membrane fluidity. The reverse reaction, which would be required for a thermal downshift, does not occur. However, they might contribute to the wide growth temperature range of psychrotolerant bacteria, enabling them to stabilize their membranes against hyperfluidity near the upper limit (Härtig et al. 2005).

The same appears to be true of cyclopropane fatty acids, which are synthesized from their unsaturated homologue and also function to reduce fluidity and strengthen the membrane (Harwood and Russell 1984). There has been much speculation about their role in membranes, but they too are very unlikely to play a part in cold adaptation, because they cannot be converted back to the corresponding unsaturated fatty acid.

11.3.6 Sterols and hopanoids

In eukaryotic microorganisms that contain sterols, membrane fluidity depends not only on the composition of the fatty acyl lipids, but also critically on the sterol/phospholipid ratio, which can decrease by up to 10-fold from the maximum to minimum growth temperature (e.g., see Miller and Barran 1984; Watson 1984). Sterols have complex interactions with phospholipids, but in physiological systems they generally stabilize and strengthen the lipid bilayer. Thus, at low temperatures, psychrophiles require less sterol in their membranes, but how the sterol/phospholipid ratio is regulated thermally is not known.

The hopanoids found in some bacteria are saturated analogues of sterols and they may perform a similar function in thermal regulation of membrane fluidity, but that has not been demonstrated unequivocally (Kannenberg and Poralla 1999). They do reinforce lipid bilayers, but several growth parameters besides temperature influence their proportions in membranes. Carotenoids and related terpenoids in pigmented microorganisms may perform similar roles (Morgan-Kiss et al. 2006).

11.4 Do psychrophiles have specific "cold" fatty acid compositions?

Given the considerations of thermal dependence of fatty acid composition discussed above, one might expect that psychrophiles would contain larger amounts of the more highly fluidizing fatty acids in their membrane lipids, and that this would be particularly evident if phylogenetic differences were minimized by comparing psychrophilic, mesophilic and thermophilic species of the same genus. Using this approach, Chan et al. (1971) showed that a psychrophilic species of *Clostridium* contained 52% unsaturated fatty acids, whereas a mesophilic and two thermophilic

species had 37% and 10%, respectively. However, it is usually difficult to pick out a logical trend in fatty acid composition, and even the example above is not without its problems because the psychrophilic species was grown in a different medium to the others, which probably influenced fatty acid composition and may explain the absence of branched-chain fatty acids in the psychrophile but not the mesophile and thermophiles that were grown in complex media.

Generally, the trends can be difficult to discern and may even be in the "wrong" direction. For example, a psychrotrophic strain of *Clostridium botulinum* has 11% and 23% unsaturated fatty acids when grown at 37°C and 8°C, respectively (Evans et al. 1998), which is comparable to the thermophilic/mesophilic clostridia in the study by Chan et al. (1971). Such comparisons may be further complicated by qualitative differences in fatty acids in what seem to be closely-related species, e.g. the clostridia studied by Chan et al. (1971) contain significant proportions of an unsaturated cyclopropane fatty acid that is not found in *C. botulinum*. Comparisons between genera and other broader groupings may become impossible due to large qualitative differences in composition and the complexities of changes in individual components.

Psychrophiles alter their fatty acid compositions in a manner analogous to mesophiles or thermophiles and, although there will be a heightened requirement for the presence of fluidizing fatty acids for growth at low temperatures, there does not appear to be any cold-regulatory mechanism of lipid composition that is unique to cold-adapted microorganisms. Thus, all the changes given in Table 11.1 are found in psychrophiles as well as other thermal groupings. The types of fatty acid changes may be different over the upper and lower portions of the growth temperature range, indicating that regulation of fluidity is complex.

11.5 Capacity for cold adaptation: psychrophiles versus psychrotolerants

The ability to adapt to the cold by changing lipid composition will contribute to whether a microorganism is psychrophilic or psychrotolerant. Although there are examples of psychrotolerant species that can grow at sub-zero (°C) temperatures, psychrophiles are generally better at adapting to very cold temperatures. Nonetheless, the major difference between the two groups of cold-adapted microorganisms is the ability of psychrotolerants to grow at higher temperatures than psychrophiles, often as high as 40°C, which one might consider as being a mesophilic temperature. Thus, psychrophiles have narrower growth temperature ranges compared with psychrotolerants, and therefore it has been proposed a number of times that the terms stenopsychrophile and eurypsychrophile be used in preference to psychrophile and psychrotolerant (psychrotroph) (discussed recently by Cavicchioli 2006). Whatever we call them, psychrotolerants are capable of changing their lipid compositions over a wider thermal growth temperature range compared with psychrophiles. This will be reflected in the metabolic capability of the species and the type of fatty acyl

change mechanisms that are employed. Individual adaptive events will also be influenced by the rate at which temperature falls, with slow cooling usually being less disruptive than a sudden cold shock.

11.6 Rate of lipid cold adaptation

The different mechanisms of lipid change can be categorized as those which modify existing membrane lipid fatty acyl chains and those which require de novo fatty acid, phospholipid and sterol biosynthesis (Table 11.2). The former has been termed “modification synthesis”, since it is the existing membrane that is altered, and the latter “addition synthesis”, because the lipid compositional change requires new membrane to be made and the existing molecules diluted by the new, thermally-adapted lipids (Russell 1984a). Even if lipids with a completely different fatty acid composition are synthesized after a decrease in temperature, the greatest change that can be effected in membrane composition by addition synthesis after one generation is, of course, a 50% dilution of existing membrane lipid. Consequently, modification synthesis is a more rapid process than addition synthesis. This is especially so when the temperature drop is sudden, when microbial populations stop growing so there is little or no new membrane synthesis. In contrast, it is possible for modification synthesis to occur in non-growing cells, as long as enough energy is available.

Bacilli illustrate these different modes of thermal adaptation of their membrane lipids in response to temperature shifts. These bacteria have complex membrane lipid compositions consisting of phospholipids and glyco(phospho)lipids with different combinations of straight-chain, *anteiso*-branched and *iso*-branched, saturated and unsaturated fatty acyl chains. Initially, after thermal downshift, lipid desaturation is used to restore membrane fluidity, but then a mechanism based on fatty acid branching and chain length takes over.

Besides inter-species differences in the thermal response of branched-chain fatty acid composition, there may also be a distinction between the response to shift-up and shift-down in the same bacterium. For instance, in *P. uratovorans* the change in C_{18}/C_{16} chain-length ratio that controls membrane fluidity in this bacterium is two-fold slower after thermal downshift from 20 to 0°C compared with the reciprocal

Table 11.2 A categorization of cold-adaptive fatty acid changes

Type of lipid change	Metabolic basis of change	Category of change
FA unsaturation by desaturase	Metabolism of existing lipid	Modification synthesis
FA unsaturation by anaerobic mechanisms	De novo biosynthesis	Addition synthesis
FA methyl branching	De novo biosynthesis	Addition synthesis
Altered FA chain length	De novo biosynthesis	Addition synthesis
Sterol/phospholipid ratio	De novo biosynthesis	Addition synthesis

FA, fatty acid (acyl)

upshift. The changes after downshift rely on dilution of existing membrane lipid (i.e., there is no cold stimulation or induction of elongation activity), whereas the changes after upshift involve stimulation of fatty acid elongation (Russell 1984b). The highly unsaturated fatty acid composition of this psychrotolerant bacterium appears to relieve the need for rapid alteration of fatty acid composition after a sudden shift to low temperature.

The fatty acid changes in *Bacillus megaterium* are not only slower after shift-down compared with shift-up, but the fatty acid compositional changes seen under the two conditions are not the same and do not have the same time course. This may not simply reflect differences in the thermal properties of the fatty acid synthesizing enzymes, but also those for phospholipid synthesis and incorporation into membranes (Russell 2003). This will be true for any adaptive mechanism that uses an addition synthesis mechanism. There will also be an interplay with intracytoplasmic events, particularly protein synthesis in relation to cold adaptation, because the initiation of translation is markedly sensitive to low temperature. If cells stop growing because of inhibition of protein synthesis after cold shock, so too will membrane growth and phospholipid synthesis, because fatty acid synthesis is tightly coupled to phospholipid and macromolecular synthesis (Cronan and Rock 1996). This is observed in *E. coli* after cold shock: although the increase in *cis*-vaccenate biosynthesis is observed within seconds of the thermal shift, the change in membrane lipid fatty acyl composition takes several hours depending on the extent of the cold shock and growth inhibition.

11.7 Lipid phase behavior and cold adaptation

The Fluid Mosaic Model of membrane structure is now well established, but it is less well appreciated that lipid phase may be just as important for membrane stability and efficient function, particularly in relation to cold adaptation. The identity of both the head-group and the fatty acyl chains influences what phase is adopted, as expounded by the Lipid Shape Model of Israelachvili et al. (1980) and the Homeophasic Adaptation Model described by McElhaney (1984). Cylindrical-shaped lipids tend to form bilayers (lamellar phase), whereas (truncated) cone-shaped ones adopt non-bilayer phases (e.g. hexagonal II).

This is pertinent to growth at low temperatures, because cold-triggered changes such as increased unsaturation and methyl branching, as well as acyl chain shortening, generally favor the formation of non-bilayer phases. Therefore, the cellular response to a decrease in growth temperature has to counteract not only a lowering of membrane fluidity but also must prevent the formation of non-bilayer phases. Indeed, sometimes a simple analysis of the temperature dependence of fatty acid composition in terms of fluidity might indicate that the change is in the “wrong” direction until lipid phase behavior is taken into consideration. For example, the low-temperature adaptation of membranes in photosynthetic microorganisms involves changes in the relative proportions of glycolipids that are the major lipids, as well as alterations in their fatty acyl compositions, which

can be interpreted in terms of the avoidance of formation of non-bilayer lipid phases (Morgan-Kiss et al. 2006).

11.8 How do microorganisms sense the cold?

A change in temperature will have an immediate effect on all of the cellular processes of a microorganism, since it is too small to insulate itself. The many intracellular enzymes of an organism vary in their thermal sensitivities, so their rates of reaction will change by different amounts and lead to shifts in metabolic patterns. The conformation of key regulatory proteins may alter sufficiently to make the change in their binding affinity for a ligand into a "switch"–i.e., the metabolic consequence is one in which the activity increases from a "tickover" value to one that is essentially full on, or vice versa for a decrease in activity. Protein conformational changes are frequently highly cooperative and there will be a threshold temperature at which they are triggered. In addition, large changes in enzyme activity can occur by thermally-triggered enzyme induction or repression, for instance via alterations in the conformation of transcription factors. This is the general basis for the Cold Shock Response (see Chap. 12).

Of interest in the present context, are the mechanisms that control the activities of membrane lipid-metabolizing enzymes. For each of the cold adaptations in fatty acid composition, described above, there is one particular enzyme whose activity is the key thermoregulated one. For example, the anaerobic pathway of unsaturated fatty acid synthesis is controlled by the thermal sensitivity of ß-ketoacyl synthase II (Sect. 11.3.2). This enzyme is a "molecular thermometer" (Russell 1984a) and is effectively the way in which temperature is sensed as far as this metabolic pathway is concerned.

However, this begs the question as to how membrane fluidity is regulated globally within the microbial cell. There must be thermal coordination of incorporation of new products made during cold adaptation into the membrane, as well as feedback mechanisms to prevent hyperfluidization of the lipid bilayer. For membrane desaturases, this could occur by autoregulation via lipid fluidity changes, but other thermoregulated fatty acid changes are mediated by cytoplasmic enzymes, so there must be "thermal communication" between the membrane and cytoplasm. There is no direct evidence for general thermosensing membrane proteins in microorganisms, but in mammals several ion channels have been implicated in cold sensing at nerve endings; these channels open below a threshold temperature to trigger ion movements and the cold response (de la Peña et al. 2005). A precedent for specific thermosensors in microorganisms does exist in the mechanosensitive ion channels that respond to osmotic stress. Some, such as MscL, are responsive to increasing tension in the membrane and their channel opens when the lateral pressure in the lipid bilayer falls below a threshold value (Poolman et al. 2004). The channels respond to both local changes in bilayer curvature and thickness, as well as to long-range pressure differences. Specific lipid-protein interactions can also

influence channel opening. For instance, the transporters for several amino compounds involved in osmosensing are associated with anionic lipids (e.g., phosphatidylglycerol) at the membrane surface, which will also influence local deformation events. If the decrease in temperature is enough to cause the formation of gel-phase lipid domains, this too will modify membrane curvature and local lateral pressure (Parthasarathy and Groves 2007).

Therefore, it is theoretically and practically feasible for cold-induced changes in membrane lipid physical properties and chemical structure to control the activation of membrane protein thermosensors. If these thermosensors are linked to effector proteins then you have a system for responding to the cold. Such systems are well known as two-component regulatory systems. This is how desaturase activity is regulated in bacilli (see Sect. 11.3.1), plus the fact that the desaturase gene is also a cold shock gene (see Chap. 12). The cyanobacterium *Synechocystis* regulates membrane fluidity largely by changes in lipid unsaturation that are linked to a signal transduction pathway involving histidine kinases. The two-component regulatory system includes a cold sensor (Hik33) in the cytoplasmic membrane, which is linked to several target proteins that regulate transcription of cold-sensitive desaturase genes (Susuki et al. 2000). The aspartate chemoreceptor (Tar) of *E. coli* also serves as a thermosensor, a function that is controlled by reversible methylation, which alters its thermal sensitivity (Nishihara et al. 1999). The last example shows, moreover, the potential for membrane proteins to evolve into cold sensors by relatively simple structural changes that could be achieved by point mutations.

11.9 Conclusions

Commensurate with their diversity, microorganisms use a wide variety of mechanisms to regulate the fluidity and phase behavior of their membrane lipids. Whilst the biochemical mechanism of some changes is understood in detail (Cronan 2003), overall knowledge is patchy. Surprisingly, molecular biology has not had the same impact that it has in elucidating many other areas of metabolism. Understanding is poor of how membrane homeostasis is managed in relation to cytoplasmic fatty acid synthesis, and we are only just beginning to unravel how temperature is sensed. More organisms need to be investigated, which perhaps might be achieved in the near future through a combined genomics/proteomics approach (e.g., see Goodchild et al. 2004; Methe et al. 2005).

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Chapter 12
Cold-Shock Proteins

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12.1 Introduction

Change in temperature is one of the most common stresses faced by all living organisms. Microorganisms, which encounter significant shifts to either high or low temperatures in their natural habitats, are equipped with cellular mechanisms to respond and adapt to these changes. The focus of research in previous years had been heat shock response and adaptation, as heat shock causes well-defined changes in cell such as protein unfolding. On the other hand, effect of cold shock was thought to be more of a general nature, for example slowing down of metabolic activities. Recent observations have changed this outlook on cold-shock response and shown it to

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be a specific response of cell at various levels such as cytoplasmic membrane, ribosomes, nucleic acids, and proteins. Cold-shock proteins are induced upon temperature downshift and play a significant role in acclimation of cells to cold (for reviews, see Yamanaka et al. 1998; Phadtare et al. 1999, 2000; Ermolenko and Makhatadze 2002; Weber and Marahiel 2003; Phadtare 2004). In this chapter, we describe salient features of bacterial cold-shock response and cold-shock proteins.

12.2 Cellular response to cold shock

Several changes take place inside the cell when it is exposed to low temperature. These include (1) loss of membrane flexibility, (2) stabilization of secondary structures in nucleic acids, (3) increase in negative supercoiling of DNA, and (4) unfolding or improper folding and methylation of some proteins. Loss of membrane flexibility affects the membrane-associated functions such as transport. In order to adapt to low temperature, bacteria have to restore the flexibility of membranes by various mechanisms, such as increasing proportion of unsaturated fatty acids (UFAs) with low melting points and high flexibility (homeoviscous adaptation) (Sinensky 1974), shortening the fatty acid chain length or by altering fatty acid branching from iso to anteiso (Kaneda 1967, 1991). Stabilization of secondary structures in nucleic acids leads to impaired transcription and translation due to hindered movement of RNA polymerase or of ribosomes, respectively. Increase in negative supercoiling of DNA is another change caused by temperature downshift. This affects DNA related functions, such as replication, transcription and recombination (Wang and Syvanen 1992; Krispin and Allmansberger 1995; Mizushima et al. 1997). The effect on transcription is mainly due to the fact that changes in supercoiling influence relative orientation of the −35 and −10 regions, which in turn influences recognition of some σ^{70} promoters by RNA polymerase (Wang and Syvanen 1992). Certain proteins also undergo changes such as reversible methylation of cytoplasmic signaling/adaptation domain as seen in case of aspartate chemoreceptor (Tar) of *E. coli* (Nishiyama et al. 1999). Folding of some proteins is also affected at low temperature, albeit to a lesser degree than that by heat shock. As will be discussed in detail in this chapter, cells produce cold-shock proteins to combat the damaging changes caused by temperature downshift.

All types of bacteria, such as psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria, exhibit cold-shock response. Typically, in mesophilic bacteria such as *Escherichia coli*, following the temperature downshift there is a lag period of growth in which cold-shock proteins are transiently induced. The growth resumes after the acclimation phase is over and synthesis of cold-shock proteins is reduced to a new basal level. Some bacteria, such as *Lactobacillus lactis* (Chapot-Chartier et al. 1997) and *Bacillus subtilis* (Graumann et al. 1997), do not exhibit acclimation phase upon temperature downshift. Cold-shock proteins that are

significantly induced upon temperature downshift in a mesophilic bacterium such as *E. coli* include:

- the major cold-shock protein-CspA (Goldstein et al. 1990) and its homologues CspB (Lee et al. 1994),
- CspG (Nakashima et al. 1996),
- CspI (Wang et al. 1999),
- ribosome-associated RNA helicase-CsdA (Toone et al. 1991),
- a transcription factor-NusA (Friedman et al. 1984),
- ribosome binding factor-RbfA (Dammel and Noller 1995),
- ribonuclease-polynucleotide phosphorylase(PNPase) (Donovan and Kushner 1986),
- and moderately induced proteins such as:
- dihydrolipoamide transferase (Jones and Inouye 1994),
- subunit of topoisomerase DNA gyrase-GyrA (Sugino et al. 1977),
- presumed cold-shock molecular chaperones-Hsc66 and HscB (Lelivelt and Kawula 1995),
- nucleoid-associated, DNA-binding protein-H-NS (Dersch et al. 1994),
- initiation factors IF1 and IF2 (Gualerzi and Pon 1990),
- a ribosome-associated protein, pY (Agafonov et al. 2001),
- pyruvate dehydrogenase (Jones and Inouye 1994),
- recombination factor-RecA (Walker 1984),
- trigger factor-TF (Kandror and Goldberg 1997),
- trehalose-6-phosphate synthase (OtsA), and
- trehalose-6-phosphate phosphatase (OtsB) (Kandror et al. 2002).

In addition to these, several other proteins involved in sugar uptake, general metabolism or chemotaxis are induced to a certain extent upon cold shock (Graumann et al. 1996; Graumann and Marahiel 1999). DNA microarray analyses of cold-shock response of *E. coli* (Phadtare and Inouye 2004) and *B. subtilis* (Beckering et al. 2002; Kaan et al. 2002) have also demonstrated cold-shock induction of several genes involved in various cell processes. The cold-shock response of cold-loving (psychrophilic and psychrotrophic) bacteria and thermophilic bacteria shares common basic principles of cold-shock response of mesophilic bacteria. The cold-shock response of the cold-loving bacteria, however, exhibits certain differences from that of mesophilic bacteria (see review by Hebraud and Potier 1999). For example, in cold-loving bacteria, synthesis of housekeeping genes is not repressed upon temperature downshift, large numbers of cold-shock proteins are present, cold-shock induction of these proteins is moderate and mostly due to post-translational modification, while continued growth at low temperature is characterized by persistence of cold-shock proteins and induction of cold-acclimation proteins (Caps) (Berger et al. 1997; Hebraud and Potier 1999). In this chapter, we mainly focus on mesophilic cold-shock proteins, which have recently received attention of researchers for their tremendous biotechnological potential.

12.3 CspA family of cold-shock proteins

12.3.1 Functional redundancy

CspA is the major cold-shock protein of *E. coli*, and its homologues have been reported from a number of Gram-positive and Gram-negative bacteria including psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria. Interestingly, archaea and cyanobacteria do not contain CspA homologues. *E. coli* contains nine homologous proteins, CspA to CspI, among these CspA, CspB, CspG and CspI are cold-shock inducible. *E. coli* cells harboring double or triple deletions of the *csp* genes (Δ*cspA*Δ*cspB*, Δ*cspA*Δ*cspG*, Δ*cspB*Δ*cspG*, Δ*cspA*Δ*cspI* or Δ*cspA*Δ*cspB*Δ*cspG*) are not cold sensitive, and in the triple deletion strain, CspE is overproduced at low temperatures (Xia et al. 2001). On the other hand, a quadruple deletion strain (Δ*cspA*Δ*cspB*Δ*cspG*Δ*cspE*) is cold sensitive, and this defect can be complemented by overproduction of any one of CspA homologues except CspD. This suggests functional overlap among these proteins. In the case of *B. subtilis*, a *cspB/cspC/cspD* triple deletion mutation is lethal, indicating that a minimum of one CspA homologue is essential for the survival of the organism, CspB being the most important of the three proteins (Graumann et al. 1997). Expression of cold-shock induction of CspA homologues is mainly regulated at levels of mRNA stability and translation.

12.3.2 Regulation of cold-shock induction

Previously, transcription was thought to play a significant role in cold induction of CspA. However, subsequent studies proved it not to be the case. The 4- to 5-fold increase seen in the level of *cspA* transcript is mainly attributed to the stabilization of its mRNA upon temperature downshift. The *cspA* mRNA is dramatically stabilized immediately following cold shock. Cold-shock-inducible CspA homologues contain an unusually long 5' untranslated region (5'-UTR). This region is presumably responsible for the extreme instability of *cspA* mRNA at 37°C (half life 12 s), and influences its stabilization upon cold shock (half life >20 min) (Mitta et al. 1997). Thus, CspA production is low at 37°C due to extreme instability of its mRNA. The cold-shock induction of *cspA* is also regulated at the level of translation. The mRNAs for *cspA*, *cspB*, *cspG*, *cspI*, *csdA* and *rbfA* contain an AT-rich sequence (Translation-Enhancing Element) located 14-bases downstream of their initiation codons. This element is presumed to enhance translation initiation in cold-shock mRNAs. However, the exact mechanism is not known (Mitta et al. 1997; Moll et al. 2001).

12.3.3 Structure

The three-dimensional structure of CspA from *E. coli* and CspB from *B. subtilis* has been resolved by X-ray crystallography and NMR-analysis (Schindelin et al. 1993,

1994; Schnuchel et al. 1993; Newkirk et al. 1994; Feng et al. 1998). The protein consists of five antiparallel β-strands (β1 to β5) that form a β-barrel structure with two β-sheets. The two putative RNA-binding motifs, RNP1 and RNP2, are located on the β2 and β3 strands, respectively, and include a surface patch of aromatic residues (RNP1 W11, F18 and F20, and RNP2 F31, H33 and F34). These residues probably contribute to nucleic acid binding by intercalating between DNA or RNA bases. There is functional differentiation among these residues; three residues F18, F31 and H33 being essential for the nucleic acid melting function of Csps (Phadtare et al. 2002a, 2002b).

12.3.4 Function

CspA homologues have been termed as RNA chaperones (Jiang et al. 1997; Bae et al. 2000; Phadtare et al. 2002a). As explained above, one of the detrimental effects of low temperatures is stabilization of the secondary structures of RNA. This affects both transcription elongation and ribosomal movement on RNA and thus translation. CspA homologues can bind and melt secondary structures in nucleic acids, are induced significantly immediately upon temperature downshift, and are important for adaptation of cells to low temperature. Thus, these proteins destabilize the secondary structures in nucleic acids facilitating transcription and translation at low temperature. Their structure is ideal for their role as RNA chaperones. These proteins have overall negative surface charge with a positively charged aromatic patch on the surface. After binding to RNA by virtue of stacking of the aromatic side chains with RNA bases, the approach of other RNA for intramolecular or intermolecular base pairing will be prevented by charge repulsion (Graumann and Marahiel 1998). CspA from *E. coli* binds RNA and single stranded (ss) DNA without sequence specificity and with low binding affinity (Jiang et al. 1997). CspB, CspC and CspE from *E. coli* selectively bind RNA/ss DNA (the preferred sequences being UUUUU, AGGGAGGGA and AU-rich regions, respectively), while CspB from *B. subtilis* binds to T-rich regions preferentially, although the magnitude of this selectivity is small (Phadtare and Inouye 1999; Lopez et al. 2001). Certain CspA homologues were shown to act as transcription antiterminators at *rho*-independent terminators and thus participate in cold acclimation of cells (Bae et al. 2000; Phadtare et al. 2002a). As mentioned above, three surface aromatic patch residues are essential for this function, and their mutations lead to loss of melting, transcription antitermination and cold acclimation activity (Phadtare et al. 2002a, 2002b). Interestingly, CspA homologues from higher organisms exhibit similar RNA chaperone activities as seen in case of wheat Csp protein WCSP1 (Nakaminami et al. 2006).

Although the primary role of CspA homologues is cold acclimation of cells, recent advances in the study of cold-shock proteins have attributed several roles to CspA homologues. These observations suggest that these proteins may play a more complex role in the stress response network of cells (Phadtare and Inouye 2001). This is exemplified by following observations. In addition to the role in cold acclimation

of cells by virtue of its RNA chaperone activity, *E. coli* CspE has been proposed to possess number of other activities, such as camphor resistance and chromosome condensation(Hu et al. 1996; Sand et al. 2003), downregulation of poly(A)-mediated 3' to 5' exonucleolytic decay by PNPase (Feng et al. 2001), downregulation of λ Q-mediated transcription antitermination (Hanna and Liu 1998), and effect on UV sensitivity (Mangoli et al. 2001). The mechanism(s) by which CspE performs these diverse functions are not well defined. *E. coli* CspC and CspE are also shown to regulate the expression of a number of RpoS-regulated stress proteins such as OsmY (osmotic stress, stationary phase), Dps (osmotic, oxidative stress and stationary phase), ProP (osmotic stress) and KatG (oxidative stress), possibly thorough regulation of *RpoS* itself. CspE and CspC also regulate expression of Universal protein A, UspA, a protein responding to numerous stresses (Phadtare and Inouye 2001). *Staphylococcus aureus* CspA is involved in susceptibility of the organism to an antimicrobial peptide derived from human neutrophil cathepsin G. This regulation probably occurs through the genes regulated by CspA which encode proteins that influence susceptibility of this organism to the antimicrobial peptide (Katzif et al. 2003). CspD in *E. coli* is induced by starvation and upon stationary phase (Yamanaka and Inouye 1997). It inhibits the initiation and elongation steps of mini-chromosome replication in vitro and presumably acts as a novel inhibitor of DNA replication and plays a regulatory role in chromosomal replication in nutrient-depleted cells (Yamanaka et al. 2001). Overproduction of *Lactobacillus plantarum* CspL, CspP, and CspC leads to improved adaptation of the organism to cold-shock, stationary phase, and freezing stresses (Derzelle et al. 2003). Involvement of CspA homologues at a broad range of cellular processes emphasizes complexity of regulation and functions of these proteins.

12.3.5 Biotechnological applications

Two features of the cold-shock proteins, (1) ability to efficiently express at low temperature at which synthesis of most of the cellular proteins is diminished and (2) RNA chaperone activity are attractive from biotechnological point of view.

12.3.5.1 Cold-shock vectors

Expression of proteins at large scale in soluble form and their purification has been a limiting factor for studies, especially structural studies of several proteins. Overexpression of proteins in *E. coli* at low temperature improves their solubility and stability. Studies on CspA, the major cold-shock protein of *E. coli*, revealed several unique features present in its gene that enable its cold-shock induction. It was shown that some of these elements can be used to produce proteins at low temperature (Vasina and Baneyx 1996). This is especially helpful if the target proteins are insoluble or are not stable at higher temperatures. We further exploited these

elements to create vectors termed cold-shock vectors or pCold vectors (Qing et al. 2004). With these vectors, one can achieve high expression of cloned genes upon induction by cold shock. Using these vectors, several proteins were produced with very high yields, such as *E. coli* EnvZ ATP-binding domain (EnvZ-B) and *Xenopus* *laevis* calmodulin (CaM). An example of the use of pCold vectors for producing proteins at low temperature is shown in Fig. 12.1. The gene encoding initiator factor IF3 was cloned in pColdI. This vector contains 5' and 3' UTR regions and promoter of *cspA*, translation enhancement element (TEE) described above, His-tag and Factor Xa sites. The BL21 cells transformed with this construct were grown at 37°C till the A_{600} of 0.5 and then cold-shocked at 15°C. The protein was induced by addition of IPTG (isopropyl ß-D thiogalactopyranoside) 1 h after cells are acclimatized to low temperature. Figure 12.1 shows production of IF3 at different time intervals after induction. Lane 1 shows control sample before induction and lanes 2-5 show samples removed 1.5, 2, 4 and 5 h after induction, respectively. As can be seen, IF3 is induced at a significant level using this vector. Thus, pCold vectors are complementary to the conventional pET vectors used for protein production. Another exciting aspect of the pCold vector system is that it can be used to selectively enrich target proteins with isotopes to study their properties in cell lysates using nuclear magnetic resonance (NMR) spectroscopy. A modification of this method that is immensely useful in terms of cost cutting for NMR studies is that cell culture can be concentrated 4-fold. This reduces the amount of expensive ^{15}N and ^{13}C isotopes required for NMR analysis. For this, the cells are first grown in medium without isotopes and, after the desired density is achieved, the cells are washed and resuspended in one-fourth volume of medium containing isotopes and protein is induced. Using these vectors, NMR spectra of *E. coli* EnvZ and *X. laevis* CaM were obtained with cell lysates without purification (Qing et al. 2004). NMR structural studies

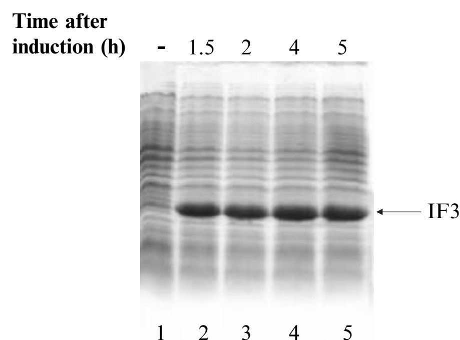

Fig. 12.1 Production of IF3 using cold shock vector. The *E. coli* BL21 cells transformed with pColdI containing gene encoding IF3 were grown in M9-casamino acids medium at 37°C until the A_{600} of 0.5 and then transferred to 15°C. One hour after cold shock, the cells were treated with 1 mM IPTG to induce IF3. Aliquots were removed at designated time intervals and subjected to 17.5% SDS-PAGE. The gel was stained with Coomassie Blue stain. Lane 1 shows control sample before addition of IPTG and lanes 2-5 show samples removed 1.5, 2, 4 and 5 h after induction with IPTG, respectively. Position of IF3 is indicated by an arrow

without protein purification will be especially attractive for proteins that are hard to purify such as membrane proteins.

12.3.5.2 Single protein production (SPP) system

Recently, we established a system for single protein production termed SPP system (Suzuki et al. 2005). This system utilizes the unique properties of MazF, a bacterial toxin that is ssRNA and ACA-specific endoribonuclease (mRNA interferase) (Zhang et al. 2003). Almost all the *E. coli* genes contain ACA sequences and are thus susceptible to MazF. Interestingly, however, the cells overproducing MazF assume a quasi-dormant state in which the growth is completely stopped, but the cells retain all the biochemical machinery in an active form. Thus, a protein encoded by a gene in which the ACA sequence is changed to render resistance to cleavage by MazF can be specifically produced in the absence of background protein synthesis. The ACA sequence in the gene can be altered without changing the amino acid sequence of the protein. This system contains two vectors; one is pCold vector with the target gene to be expressed, and the second vector produces MazF. The SPP system was successfully used for production of several proteins; one such example is illustrated in Fig. 12.2. *E. coli* cells were transformed with pColdI vector with gene encoding EnvZb and pACYC containing *mazF* gene. The ACA sequences were removed from the *cspA* 5' UTR region and the coding region of *envZb* in this construct. The cells were grown at 37°C to A_{600} of 0.5 and then cold-shocked

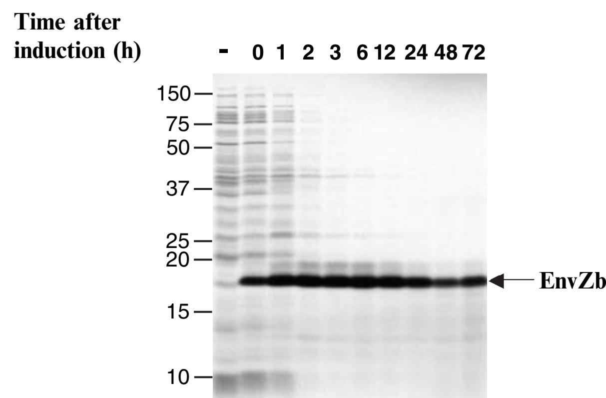

Fig. 12.2 Production of EnvZb using single protein producing system. *E. coli* BL21DE3 cells harboring pColdI vector with gene encoding EnvZb and pACYC containing *mazF* gene were grown at 37°C to A_{600} of 0.5 and then cold shocked at 15°C. One hour after the cold shock, cells were treated with 1 mM IPTG to induce both proteins, aliquots were removed at different time intervals, labeled with ^{35}S methionine for 15 min and subjected to SDS-PAGE and autoradiography. Lane 1 represents sample before addition of IPTG. Position of EnvZb is shown with an arrow. Position of molecular weight marker proteins is shown on the left

at 15°C. Samples were labeled with ^{35}S methionine at different time intervals after induction with IPTG. Figure 12.2 shows autoradiography pattern of SDS-PAGE analysis of the samples producing EnvZb. The dramatic lack of background protein synthesis in this system opens up several new, exciting avenues, such as structural studies of membrane proteins, production of expensive, medically important human proteins, etc. Recently, a condensed SPP system (cSPP) was created, in which the culture can be concentrated 40-fold before labeling by isotopes. This is made possible by the fact that the cells do not grow in the SPP system. This will significantly reduce the amount of isotopes needed for NMR studies (Suzuki et al. 2006).

12.3.5.3 Use of RNA chaperone activity of CspA homologues

As described above, CspA homologues can melt secondary structures in nucleic acids and are thus termed as RNA chaperones, an activity critical for their function in cold acclimation of cells. This activity has tremendous biotechnological potential especially in instances wherein the secondary structures in nucleic acids prove to be a limiting factor for a reaction. In our studies with mRNA interferases from *Mycobacterium tuberculosis* (Zhu et al. 2006), we encountered difficulties in determination of individual specificity of ssRNA cleavage due to secondary structures present in the substrate RNAs. We decided to include CspA

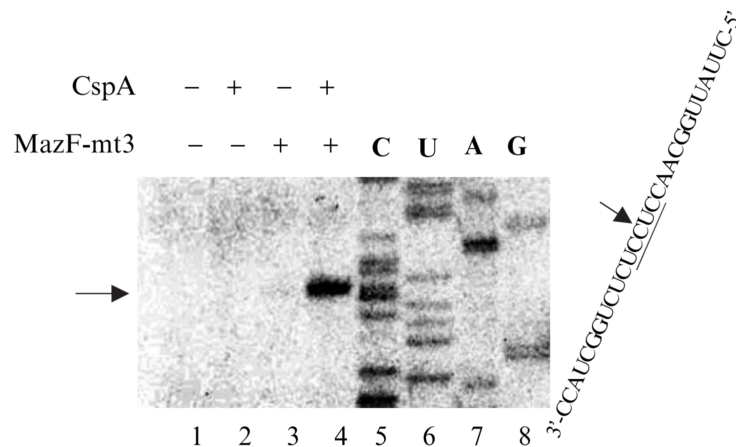

Fig. 12.3 RNA chaperone activity of CspA helps RNA cleavage by mRNA interferases. The substrate MS2 RNA was incubated with MazF-mt3 protein with or without CspA in 37°C for 15 min. Then primer extension was carried out to detect the cutting sites. Lane 1 represents control reaction carried out in the absence of added protein. In the reaction represented by lane 2, CspA was incubated with MS2 RNA in the absence of MazF-mt3. Lanes 3 and 4 represent cleavage reactions carried out with MazF protein without and with CspA, respectively. Cleavage site is indicated by an arrow. Lanes 5 to 8 represent sequencing ladder

in the reactions to melt these structures, which will then facilitate cleavage of the substrate RNAs by mRNA interferases. *M. tuberculosis* has seven MazF homologues-designated as MazF-mt1-MazF-mt7. The substrate used for the reaction, MS2 RNA containing extensive secondary structures, was incubated with MazF-mt3 protein with or without CspA at 37°C for 15 min. Then primer extension was carried out to detect the cutting sites. As seen in Fig. 12.3, CspA alone (lane 2) does not cleave the RNA. MazF-mt3 did not show cleavage activity in the absence of CspA (lane 3). However, addition of CspA in the reaction facilitated melting of the secondary structure in MS2, which was subsequently cleaved by MazF-mt3 (lane 4). With the help of sequencing ladder, the cleavage site of MazF-mt3 is determined to be CUCCU. Using this method, all the CUCCU cutting sites in the MS2 RNA were cleaved by MazF-mt3. In addition, the assay also revealed that MazF-mt3 can also cleave at UUCCU sequence. This suggests that RNA chaperone activity of CspA and its homologues can be used to rescue reactions that are hindered due to secondary structures in the substrate nucleic acids. The following characteristics of CspA homologues make them ideal candidates for exploitation in a variety of biotechnological applications: (1) they can act on both RNA and DNA, (2) have low substrate specificity, thus can act on a wide range of substrates, and (3) CspA homologues from thermophilic bacteria are significantly stable at high temperatures.

12.4 Molecular chaperones

Protein misfolding was previously not considered a major problem upon cold shock. But recent reports suggest that proper folding of proteins as well as refolding of cold-damaged proteins is important after cold shock (Kandror and Goldberg 1997). Global transcript profiling of *E. coli* cells undergoing cold shock showed that certain molecular chaperones such as Caseinolytic proteases (Clps), trigger factor and GroEL and GroES (Phadtare and Inouye 2004) were induced upon cold shock.

12.4.1 Caseinolytic proteases

Caseinolytic proteases (Clps) are expressed constitutively in some cases and induced by stress in others. These are induced in cyanobacteria after temperature downshift. ClpP1 is significantly induced within 24 h of cold shock (Porankiewicz et al. 1998). It is also induced upon exposure to UV. Its deletion severely affects growth at low temperatures. ClpB in *Synechococcus* sp. PCC 7942 is induced by both heat and cold shock (Porankiewicz and Clarke 1997).

12.4.2 *Trigger factor*

12.4.2.1 Cold-shock induction and function

Another molecular chaperone that is induced albeit at a modest level 2–3 h after cold shock is trigger factor TF (Kandror et al. 2002). This is a peptidyl prolyl isomerase that catalyzes the *cis/trans* isomerization of peptide bonds N-terminal to the proline residue (Kandror and Goldberg 1997). Similar to other cold-shock proteins, its synthesis is induced after temperature downshift from 37°C to °C or exposure to chloramphenicol. Reduction in cellular levels of TF leads to reduced cell viability during storage at 4°C; on the other hand, its overexpression leads to increased viability. It was suggested that TF helps protein synthesis and folding to continue at low temperature and accelerates proline-limited steps in protein folding with a very high efficiency (Kandror and Goldberg 1997). It associates with ribosomes and influences the folding of newly-formed protein chains (Maier et al. 2003). It has been proposed to be the first chaperone to interact with the nascent polypeptide chain as it emerges from the tunnel of the 70S ribosome and thus probably plays an important role in co-translational protein folding (Blaha et al. 2003). It also interacts with other chaperones such as GroELS (Kandror and Goldberg 1997).

12.4.2.2 Biotechnological potential

Coexpression of molecular chaperones may be helpful in situations where target proteins cannot be expressed well in a soluble form. Expression and solubility of a target protein can be substantially enhanced if it is produced at low temperature along with a known cold-shock protein chaperone. TF was used as a possible candidate for this approach and a vector termed pColdTF vector was constructed by modification of the pColdI vector described above (Takara Mirus Bio). This vector has additional elements as compared to pColdI such as sites for HRV 3C protease and thrombin and coding sequence of trigger factor. The target protein used for this study was reverse transcriptase domain consisting of 86 amino acids, designated as RT-86. This protein cannot be efficiently produced in a soluble form using conventional vectors. The gene encoding this protein was cloned into pColdTF vector. *E. coli* cells harboring this plasmid were grown at 37°C until the A_{600} of 0.8 and then cold-shocked at 15°C. As seen from Fig. 12.4, the fusion protein of trigger factor and RT-86 is well expressed in soluble form (lane 2). The His-tagged fusion protein was partially purified by passing the cell extract through nickel column (Ni-NTA) (lane 3). RT-86 protein was then separated from trigger factor by thrombin digestion (lane 4) and purified by ion exchange chromatography (lane 5). This method has proven to be extremely useful for production and labeling of RT-86 for its structural studies by NMR analysis and will prove to be immensely useful in rescuing proteins, which cannot be produced in soluble form using conventional vectors.

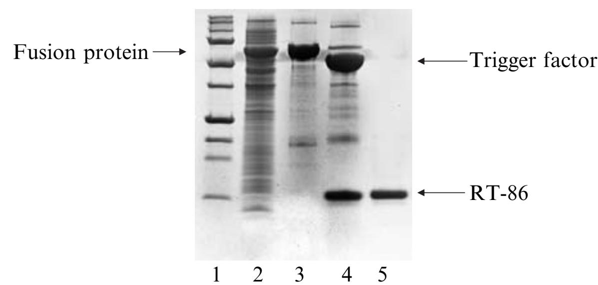

Fig. 12.4 Production of soluble RT-86protein by coexpression with trigger factor. The *E. coli* BL21 cells harboring pCold-TF plasmid containing gene encoding RT-86 protein were grown at 37°C till the A_{600} of 0.8 and then cold shocked at 15°C. After 45 min, cells were treated with IPTG (1 mM) and incubated overnight. The sample was analyzed by SDS-PAGE. The gel was stained with Coomassie Blue stain. Positions of respective proteins are indicated. Lane 1 represents standard molecular weight markers. Lane 2 represents soluble fraction. Lane 3 represents fraction after passing the cell extract through nickel (Ni-NTA) column (lane 3). Lane 4 represents sample after thrombin digestion. Lane 5 is RT-86 fraction purified by ion exchange chromatography

12.5 Desaturases

As mentioned above, increase in the proportion of unsaturated fatty acids (UFAs) in membrane lipids is one of the mechanisms by which bacteria adapt to temperature downshift. Desaturases are exclusively present in photosynthetic bacteria with the exception of *Bacillus*.

12.5.1 Desaturases from cyanobacteria

Desaturases in cyanobacteria are acyl-lipid desaturases that introduce double bonds into fatty acids that have been esterified to glycerolipids and are bound to the thylakoid membrane (Murata and Wada 1995). The cyanobacterium *Synechococcus* has cold-inducible desaturase genes, *desA* and *desB*. These are induced after temperature downshift from 38°C to 22°C. Their cold-shock expression is tightly controlled by a combination of mRNA synthesis and stabilization (Sakamoto and Bryant 1997). Presence of these genes is critical at low temperature as desaturation of lipids is associated with acclimatization of photosynthetic activity at low temperature. It has also been shown that transcription of *desA* is supported by the energy produced by photosynthesis. Thus, the low temperature-induced desaturation of membrane lipids occurs only in the light, and polyunsaturated fatty acids are important for growth and the ability to tolerate photoinhibition of photosynthesis at low temperature (Gombos et al. 1992, 1994).

12.5.2 Desaturases from **Bacillus**

As mentioned above, *Bacillus* is the only non-photosynthetic bacterium in which the presence and cold induction of desaturase has been reported (Aguilar et al. 1998, 2001). The *des* transcript shows 10- to 15-fold increase 4 h after cold shock. However, unlike cyanobacteria, deletion of the *de*s gene does not result in cold-sensitivity in *Bacillus* growing in rich medium (Aguilar et al. 1998). On the other hand, deletion of *des* together with absence of isoleucine in the growth medium causes a severe cold-sensitive phenotype. The effect of *des* deletion is manifested at two levels, (1) absence of four UFA species of different lengths, branching patterns and positions of the double bond present in the wild-type strain, and (2) significantly altered saturated fatty acid profile at the onset of the stationary phase in the presence of exogenous isoleucine sources. It was reported that during cold-shock adaptation, *des* expression can completely replace the isoleucine-dependent fatty acid branching adaptation mechanism (Weber et al. 2001).

The cold-shock induction of *des* is regulated by a two-component signal transduction system consisting of a sensor kinase DesK and a response regulator DesR. DesR binds to a DNA segment from −28 to −77 positions relative to the start site of *des* gene. DesK regulates the ratio of kinase to phosphatase to ensure dominance of phosphorylated-state at 37°C. On the other hand, lowering temperature results in the ordered membrane lipids and kinase-dominant state. DesK-mediated phosphorylation of DesR results in transcriptional activation of *des*, resulting in synthesis of Des. The newly synthesized UFAs act as negative signal for the *des* transcription (Aguilar et al. 2001). Recently published DNA microarray analysis of cold-shock response in *B. subtilis* showed *des* to be the strongest cold-inducible gene, however the global relevance of DesKR system as a cold-shock induced regulatory system was not emphasized (Beckering et al. 2002; Kaan et al. 2002).

12.5.3 Biotechnological potential

The *desC* gene for the acyl-lipid Delta9-desaturase from the thermophilic cyanobacterium *Synechococcus vulcanus* was introduced into tobacco plant *Nicotiana tabacum*. This resulted in significant increase in lipid content and the extent of fatty acid unsaturation in leaves of transgenic plants and concomitant increase in chilling tolerance of these plants. Seeds of plants that expressed the *desC* gene also demonstrated higher chilling tolerance than those of the control plants (Ishizaki-Nishizawa et al. 1996; Orlova et al. 2003). This observation has tremendous commercial significance in agricultural industry, as cold stress is one of the common causes of economic loss in this industry.

12.6 Trehalose synthesis machinery

Recent studies have shown beneficiary effects of sugars in cold acclimation of organisms. Global transcript profiling of *E. coli* cells undergoing cold shock showed that several genes encoding proteins involved in sugar transport and metabolism are induced by cold shock (Phadtare and Inouye 2004). These sugars include maltose, mannose, ribose, xylose, and trehalose. Physiological relevance of this observation is suggested by the fact that cold-shock induction of mannose and maltose transport systems was prominently repressed in a cold-sensitive *csp* quadruple deletion mutant that has significantly prolonged (4 h) lag period as opposed to 1 h lag period of the wild-type strain (Xia et al. 2001) at 15°C. Maltose was also shown to accumulate and confer protection during cold stress in plants (Kaplan and Guy 2004).

Studies to date have been focused on protective effects of trehalose. Trehalose biosynthesis pathways are widely distributed in nature in eubacteria, archaea, plants, fungi and animals. In bacteria, there are five different biosynthetic routes, whereas in fungi, plants and animals there is only one (Avonce et al. 2006). In addition to cold shock, trehalose is also known to protect the cells against heat and osmotic stress (Kandror et al. 2002). Cellular levels of trehalose increase significantly (8-fold) upon temperature downshift from 37°C to 16°C. Deficiency of trehalose leads to reduced viability at 4°C. Complementation by *otsA* and *otsB*, the genes involved in trehalose synthesis correct this deficiency. Although the mechanism of protection is not known at present, it is suggested that trehalose acts at several levels such as denaturation and aggregation of proteins, protection against oxidative damage, and also stabilizes cellular membrane (Kandror et al. 2002). It has also been shown that trehalose stabilizes a cold-adapted protease by preventing its autolysis (Pan et al. 2005). The protective effect of trehalose is not restricted to bacteria and is also manifested in higher organisms such as *Drosophila*, *Caenorhabditi*s *elegans*, yeasts and plants.

12.6.1 Biotechnological potential

Trehalose-producing, transgenic rice plants were generated by the introduction of a gene encoding a bifunctional fusion (TPSP) of the trehalose-6-phosphate (T-6-P) synthase (TPS/OtsA) and T-6-P phosphatase (TPP/OtsB) of *E. coli*. The trehalose levels in leaf and seed extracts from rice plants were increased up to 1.076 mg g^{-1} fresh mass. This trehalose accumulation resulted in increased tolerance to drought, salt, and cold, as shown by chlorophyll fluorescence and growth inhibition analyses. It was thus concluded that trehalose acts as a global protectant against abiotic stress (Jang et al. 2003). Similar to desaturase system, the trehalose synthesizing machinery too has tremendous potential for agricultural application to reduce losses caused by cold temperatures.

12.7 Conclusions

Study of cold-shock response and adaptation of organisms is a classic example of link between research in basic science to uncover underlying principles of cellular physiology and exploitation of these principles for commercial purposes. For example, using *E. coli* and *Bacillus* as model systems it was shown that cold shock improves freezing tolerance. Based on this observation, the shelf life of refrigerated food is increased by directly freezing the food instead of stepwise decrease in temperature. This decreases the efficiency of cold-shock response of food-borne pathogens. The nitrogen fixing bacterium *Rhizobium* is used as biofertilizer for legume plants. By using cold-adapted *Rhizobium* cultures, losses due to reduced efficiency caused by low temperatures can be avoided, which in turn results in significant favorable impact on economy of the agricultural process. Several cold-adapted enzymes have been used in processes, which are carried out at low temperature in the presence of biocatalysts. Use of genetic elements that are critical for cold-shock response and adaptation such as translation enhancement element, 5' UTRs of cold-inducible genes, cold-shock proteins such as RNA chaperones, desaturases or enzymes involved in synthesis of cold-protectant sugars in various applications has proven to be immensely useful. The new systems now available for synthesis of single proteins not only facilitate structural studies of problematic proteins such as membrane proteins, but also pave a way to produce medically important, expensive proteins and peptides in a large quantity with little or no purification required. It is obvious that these applications will have tremendous impact on overall strategies for protein production and purification and will revolutionize biotechnology. Thus, it can be concluded that recent novel discoveries in the field of cold-shock response and adaptation have opened up new frontiers and have proven to be an important tool in basic research, medical field, and different aspects of biotechnology including applications in agricultural industry.

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Chapter 13
Fundamentals of Cold-Adapted Enzymes

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13.1 Introduction

As already discussed in the preceding chapters, life at low temperatures is abundant, diverse and widespread, with organisms from all three domains of life being represented. Bacteria and archaea at thermal equilibrium with their environment are found to be preponderant, and these cold-adapted, or psychrophilic, microorganisms have been found to not only endure, but to flourish under the harsh conditions of permanently low-temperatures. In fact, for some, this environment is not only optimal, but mandatory for sustained cell proliferation, with moderate to high temperatures (e.g., >12°C) being deleterious (Xu et al. 2003c).

Clearly, adaptations at all levels of organisation, both structural and physiological, have occurred which allow these psychrophilic microorganisms to overcome key obstacles inherent to life at low temperatures (D'Amico et al. 2006a). The current knowledge of the adaptive features characteristic to psychrophilic organisms are addressed in this book (see Chaps. 11–19) with the present chapter being focused on the enzymes produced by these and on their adaptation to the cold. Here, the challenges imposed by low temperatures on enzyme activity and stability will be briefly introduced, whereafter the general characteristics of the cold-adapted enzymes hitherto investigated will be presented and discussed.

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13.2 The low temperature challenge

Low temperatures have a strong negative effect on most biological reactions; reducing reaction rates, provoking alterations in the strength of enzyme-substrate interactions, inducing an increased viscosity of the solvent and an altered solubility of proteins, salts and gases and ultimately leading to protein cold-denaturation (Makhatadze and Privalov 1994; Georlette et al. 2004).

All chemical reactions, including enzymatic reactions, are strongly temperature dependent as described by the Arrhenius equation (Arrhenius 1889):

$$k_{cat} = Ae^{-Ea/RT} \quad (1)$$

From this equation, it can be clearly seen that any decrease in the temperature (T) leads to an exponential decrease in the enzymatic reaction rate (k_{cat}) with the magnitude of this decrease being dependent on the value of the activation energy for the reaction (*Ea*). Here, *R* is the universal gas constant (8.314 J mol^{-1} K^{-1}) and *A* is a frequency factor related to the frequency of collision of the reactants and to the probability of the reactants being in the appropriate orientation to react. In fact, the rate constant can also be expressed according to the transition state theory to give:

$$k_{cat} = \frac{\kappa k_B T}{h} e^{-\Delta G^*/RT} \quad (2)$$

From this and equation (1), one can also derive that (Garcia-Viloca et al. 2004; Collins et al. 2007):

$$A = \frac{\kappa k_B T}{h} e^{-\Delta s^*/R} e^1 \quad (3)$$

Thus, it can be seen that *A* is related to the activation entropy (ΔS^*). In the above equations, κ is the transmission coefficient expressing the probability that the activated state will not always give rise to the product, but will return to the ground state, k_B is the Boltzmann constant and *h* the Planck constant (Garcia-Viloca et al. 2004; Collins et al. 2007). The transmission coefficient κ can be negatively influenced by the viscosity of the medium through possible re-crossing and non-equilibrium effects so that the frequency factor *A* will also be dependent on the viscosity of the reaction medium, and the increase in this latter, concomitant to a temperature decrease, can indeed be quite significant. For example, the average cytoplasmic viscosity is augmented from a mean of 2.5 cP at 20°C (Mastro and Keith 1984) to approximately 5 cP at 0°C. This will obviously have a negative effect on enzyme activity, and such a viscosity increase has been shown to lead to an approximately 5-fold loss of activity for a lactate dehydrogenase at 25°C (Demchenko et al. 1989). Thus, it is important to keep this inverse relationship of viscosity with temperature and reaction rate in mind when monitoring temperature effects on enzyme activity. A model which takes this viscosity effect into account has even been recently proposed (Siddiqui et al. 2004).

The effect of low temperatures on enzyme–substrate binding is much less clear-cut than that on reaction rates, with both positive and negative outcomes being possible. In effect, electrostatic interactions are formed exothermically and are stabilized by low temperatures at least down to those temperatures where the dehydration of the individual ions can still be achieved. On the other hand, hydrophobic interactions are formed endothermically in the positive temperature range up to approximately 40°C and hence are destabilised at low temperatures. Thus, the effect of temperature (either detrimental or beneficial) is determined by the types and contributions of the bonds involved in substrate binding and hence by the particular enzyme and substrate concerned.

Finally, cold denaturation, while being a scantly studied phenomenon, is believed to be due to the hydration of charged, polar and non-polar amino acid residues that leads, in particular, to a weakening of critical hydrophobic forces. While this should typically occur at temperatures below the freezing point of water, it obviously poses a further predicament to enzymes produced by organisms inhabiting permanently low temperature environments (Makhatadze and Privalov 1994).

As a result of these diverse adverse effects, most biological reactions display an approximately 16- to 80-fold drop in activity on reducing the temperature from 37°C to 0°C. In contrast, psychrophilic microorganisms have been found to maintain relatively high metabolic fluxes at low temperatures, indicating that adaptation of the enzymatic repertoire has taken place to allow for appropriate reaction rates. Reported mechanisms of cold-adaptation include an increased enzyme production (Crawford and Powers 1992; De Vos et al. 1998), yet this is energetically expensive, and while an expression of specific isotypes adapted to different temperatures has also been reported (Lin and Somero 1995), this suffers the disadvantage of requiring the presence of multiple gene copies and is most apt for organisms requiring seasonal adaptation (e.g., fish and nematodes). Finally, the synthesis of enzymes specifically adapted to operate at permanently low temperatures is another putative cold-adaptation mechanism and this in fact appears to be the main physiological adaptation used by psychrophiles at the enzyme level.

We have attempted to tabulate all the currently known enzymes isolated from psychrophilic organisms, both prokaryotic and eukaryotic, and the listing of these can be found at http://www.ulg.ac.be/biochlab where both the identity of the host organism as well as the level of characterisation of the enzymes are also indicated. Only those enzymes purified from psychrophilic organisms inhabiting permanently low temperature environments have been listed and it currently appears that 108 prokaryotic and 51 eukaryotic enzymes have been isolated and studied. These originate from various sources and have been characterized to varying levels in terms of their physical, chemical, kinetic and structural properties. The majority of these have been found to have evolved strategies for overcoming the temperature challenge. In the subsequent sections, the principle results of the various studies of these enzymes and in particular the comparative studies of the activity, stability and flexibility will be presented and discussed.

13.3 Activity

In relation to activity, the dominant features of most cold-adapted enzymes are an increased specific activity (k_{cat}) or catalytic efficiency (k_{cat}/K_m) at low and moderate temperatures and a shift in the apparent optimal temperature towards low temperatures as compared to their mesophilic and thermophilic homologs (Fig. 13.1).

The apparent optimum temperature of an enzyme is obviously dependent on the experimental conditions used and in particular on the assay time. Nevertheless, analysis of the available data shows that the thermodependence of activity of psychrophilic enzymes is highly variable, with diverse apparent optimum temperatures for activity and both limited and broad temperature ranges being reported. For example, while apparent temperature optima around 30°C are preponderant, optima at approximately 15°C (Huston et al. 2000; Arnorsdottir et al. 2005) and as high as 64°C (Birolo et al. 2000) have also been reported. In fact, the defining feature of these cold-adapted enzymes is a shift in the apparent optimum temperature towards lower values and the retention of a high activity at low temperatures, in particular when compared to that of their mesophilic and thermophilic homologs (Bae and Philliops 2004). Examples include a psychrophilic xylanase (Collins et al. 2002b) and chitinase (Bendt et al. 2001) which retain, respectively, 60% and 40% of their

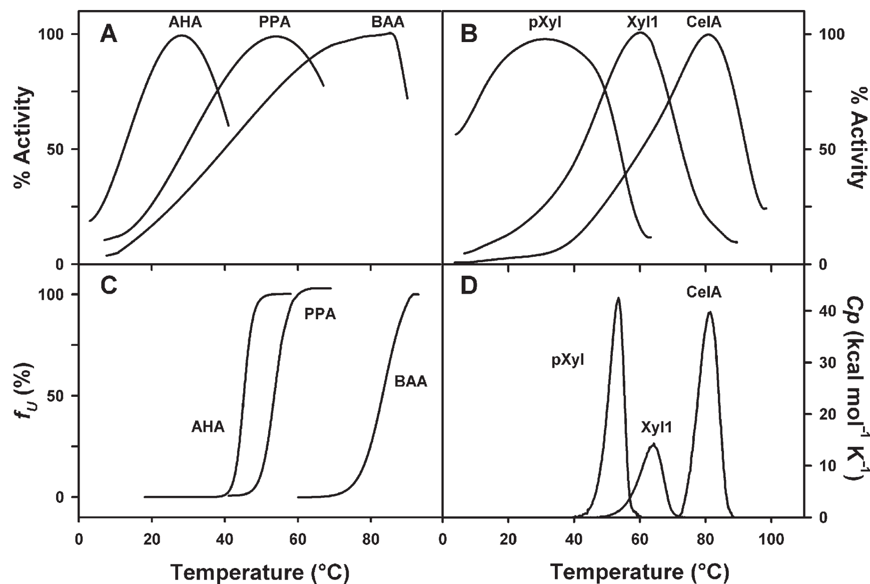

Fig. 13.1 Thermodependence of activity (**A** and **B**) and thermal stability as determined by fluorescence monitoring (**C**) and differential scanning calorimetry (**D**) of homologous α-amylases (**A** and **C**) and glycoside hydrolases (**B** and **D**). AHA, PPA and BAA are, respectively, psychrophilic, mesophilic and thermophilic α-amylases. pXyl, Xyl1 and CelA are, respectively, psychrophilic, mesophilic and thermophilic glycoside hydrolases. Taken from Georlette et al. (2004)

maximum activity at 5°C, and display apparent optimum temperature shifts towards lower temperatures of approximately 25°C. Indeed, this shift in the apparent optimum temperature for activity is related to the thermal lability of these enzymes (see Sect. 13.4), while the reduced temperature dependence and high activity at low temperatures is brought about by a reduction of the activation energy barrier for the reaction ΔG* (i.e. the energy difference between the activated enzyme–substrate transition state and the ground state enzyme–substrate complex).

Extensive comparative thermodynamic analysis of the activity of psychrophilic, mesophilic and thermophilic enzymes (Lonhienne et al. 2000; Siddiqui and Cavicchioli 2006) indicates that, as compared to their higher temperature adapted homologs, the low ΔG^* of cold-adapted enzymes is characterized by a reduced activation enthalpy partially compensated for by a less favorable activation entropy. The decrease of the former reflects the lower dependence of these enzymes on temperature and indicates a reduction in the number or strength of weak interactions that are broken during activation. Accordingly, this favorable enthalpy change is the driving force for low temperature activity, but the magnitude of its effect is reduced by the unfavorable entropy change observed (i.e. $\Delta G^*=\Delta H^*-T\Delta S^*$). It is important to note that, while the entropy change on activation is often negative due to a more organised transition state, positive values are also encountered. Therefore, the unfavorable entropy change observed during the activation process in psychrophilic enzymes indicates, in the former case, the necessity for a greater change in order to reach the activated state (i.e. a higher negative entropy change as compared to mesophilic and thermophilic homologs) and, in the latter case, a lower degree of disordering (i.e. a lower positive entropy change). Both of these observations can be explained by a more disordered enzyme–substrate ground state complex for the cold-adapted enzyme as compared to its high temperature homologs. This is postulated to be the result of a more flexible enzyme structure leading to a broader distribution of conformational states for the ground state complex (Lonhienne et al. 2000).

In agreement with the apparent increased disorder of the enzyme–substrate ground state complex in psychrophilic enzymes, these have been found to be frequently characterized by an increased K_m in addition to a high k_{cat} (Collins et al. 2002a). Furthermore, this high K_m value frequently observed for psychrophilic enzymes may be conjectured as further proof of a highly flexible structure (Fields 2001), in particular at or around the active site, which perturbs the geometry and enzyme–substrate complementarity and leads to the observed decreased substrate affinity (i.e. increased K_m). Similarly, the frequently observed decreased affinity for various ions (Davail et al. 1994; D'Amico et al. 2006b), cofactors (Okubo et al. 1999) and inhibitors (Alvarez et al. 1998), and the reduced substrate specificity of some cold-adapted enzymes (Smalas et al. 2000), also points to an increased flexibility of the molecular edifice of these. Finally, and in a similar vein of thought, recent comparative studies of a psychrophilic α-amylase with its mesophilic counterpart has indicated that the former, in contrast to the latter, is capable of forming an enzyme–substrate–inhibitor ternary complex and has a higher activity on large macromolecular substrates but a decreased activity on small substrates (D'Amico et al. 2006b). These observations, in conjunction with the structural identity of the

catalytic cleft of these enzymes are also compatible with a more flexible active site in the psychrophilic enzyme.

For enzymes operating at high substrate concentrations, for example extracellular and in particular digestive enzymes (Narinx et al. 1997; Collins et al. 2002b), the high K_m value discussed above is of little importance as optimization of k_{cat} is the only relevant parameter. In contrast, for enzymes typically operating at low substrate concentrations, for example certain intracellular enzymes, both K_m and k_{cat} are important and indeed both of these parameters have been found to be optimized in a number of cases (Bentahir et al. 2000; Hoyoux et al. 2001; Lonhienne et al. 2001). Obviously, a different strategy must be employed by these proteins whereby the adaptive drift of the K_m is counteracted. For example, comparison of the kinetic parameters of a cold-adapted and mesophilic chitobiase (Fig. 13.2) reveals that these enzymes have been fine tuned so as to enable optimization of their kinetic parameters in accordance with their physiological temperature (Lonhienne et al. 2001). In particular, the crossed K_m plots for these enzymes illustrate the improved substrate binding for the psychrophilic enzyme on reduction of temperature, and this is believed to be achieved by the subtle replacement of two tryptophan residues (endothermic interactions) involved in substrate binding in the mesophile by polar residues (exothermic interactions) in the psychrophile, which, as discussed above, improves the enzyme-substrate interactions at low temperatures.

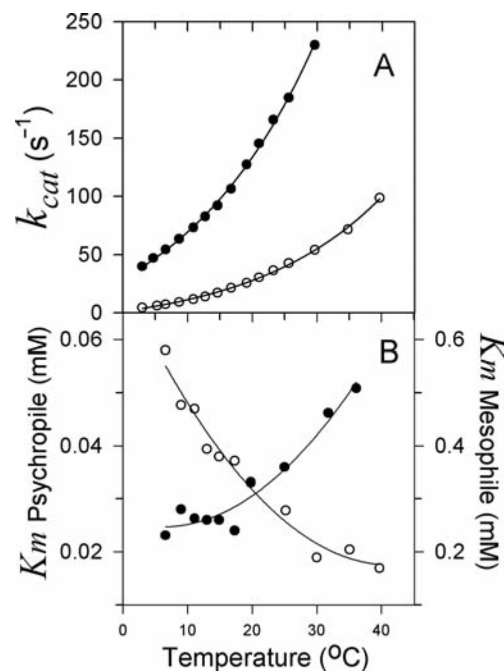

Fig. 13.2 Thermodependence of the k_{cat} (**A**) and K_m (**B**) for a psychrophilic (•) and mesophilic (o) chitobiase. Note the different scales used. Taken from Lonhienne et al. (2001)

A further interesting observation of a number of comparative studies is that both the K_m and k_{cat} of psychrophilic and mesophilic enzymes are often highly similar at their respective physiological temperatures. This gives rise to the corresponding state hypothesis suggested by Somero (1995) whereby enzymes have evolved so as that both of these parameters are optimized at the environmental temperature of the enzyme. However, this kinetic optimization has not always been possible and the kinetic parameters of some psychrophilic enzymes at their environmental temperature are sometimes found to be inferior to that of their high temperature homologs (Feller et al. 1992; Xu et al. 2003a; Xu et al. 2003b), hence pointing to an 'incomplete' adaptation of these enzymes to their environment.

13.4 Stability

In light of the essential role of protein stability in thermal adaptation, extensive studies have been performed in order to compare the conformational stability of psychrophilic enzymes with their mesophilic and thermophilic counterparts, and have shown that cold-adapted enzymes are generally the most unstable. As demonstrated by techniques such as fluorescence spectroscopy and differential scanning calorimetry (Fig. 13.3) their unfolding typically occurs at lower temperatures and the calorimetric enthalpy, $\Delta H_{cal,}$ i.e., the enthalpy of disruption of bonds involved in maintaining the protein structure, is generally reduced. Furthermore, thermal unfolding curves for cold-adapted enzymes are typically sharp and symmetrical and thereby reflect a highly cooperative process without any stable intermediate, while in contrast more stable enzymes exhibit distinct thermodynamic domains of varying stability (Fig. 13.3).

13.4.1 Reversible and irreversible unfolding

While the majority of proteins unfold irreversibly, some have been found to show completely reversible thermal denaturation and thus allow calculation of the stabilization energy (ΔG) curves as presented in Fig. 13.4. These curves represent the energy difference between the free energy of the unfolded state and that of the native state, in other words, they represent the energy required to unfold the molecular structure at each temperature. It can be clearly seen that the increase of the melting points of the more thermostable proteins is obtained by an uplifting of the stability curves and that this is mainly achieved through an increase of the stabilization enthalpy, hence demonstrating the major involvement of enthalpic factors in the stabilization of protein structures (Makhatadze and Privalov 1995; Kumar et al. 2001). Furthermore, the environmental temperatures of mesophiles and thermophiles are found to be above those corresponding to the ΔG_{max} and, consequently, it can be suggested that these enzymes use the thermal dissipative forces

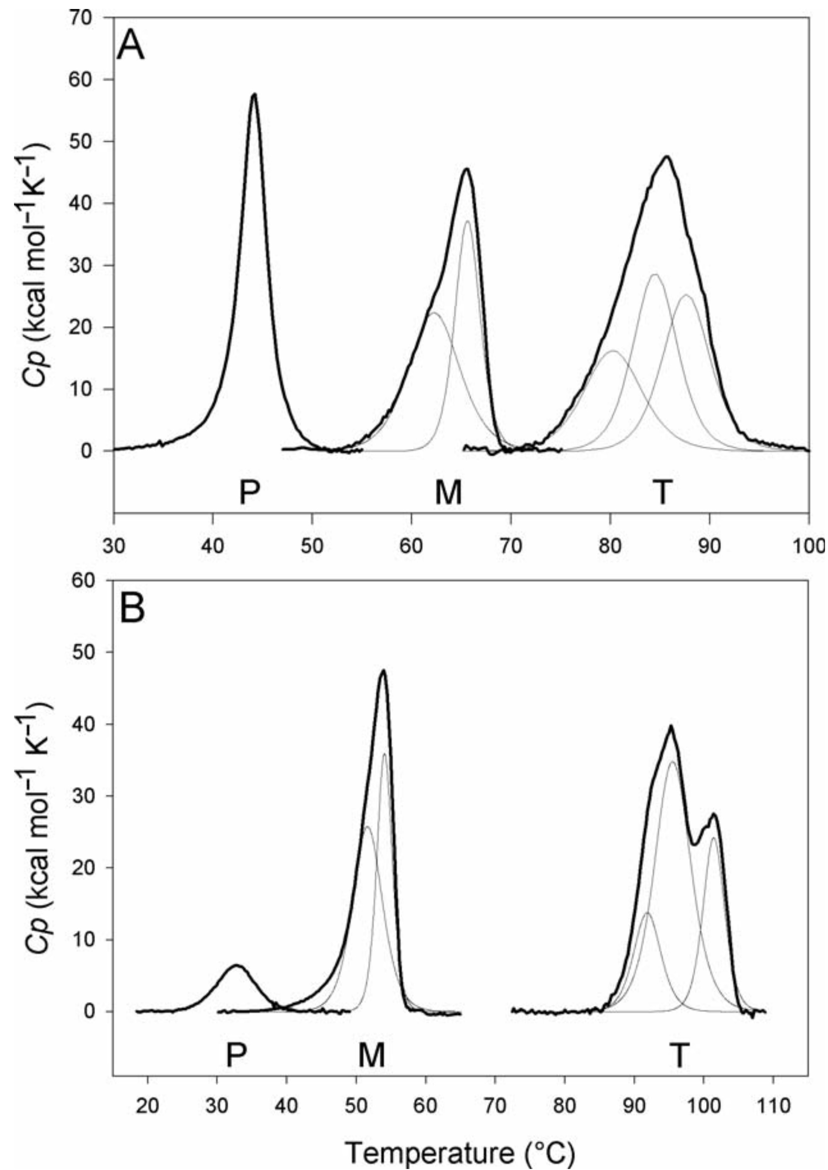

Fig. 13.3 Thermal unfolding of psychrophilic (*P*), mesophilic (*M*) and thermophilic (*T*) enzymes recorded by differential scanning calorimetry. **A** From left to right: α-amylases from *Pseudoalteromonas haloplanktis*, pig pancreas and *Bacillus amyloliquefaciens*. **B** From left to right: DNA ligases from *Pseudoalteromonas haloplanktis*, *Escherichia coli* and *Thermus scotoductus*. Deconvolution of the peaks into two or three domains for the mesophilic and thermophilic enzymes respectively are shown in thin lines. Taken with modification from D'Amico et al. (2001) and Georlette et al. (2003)

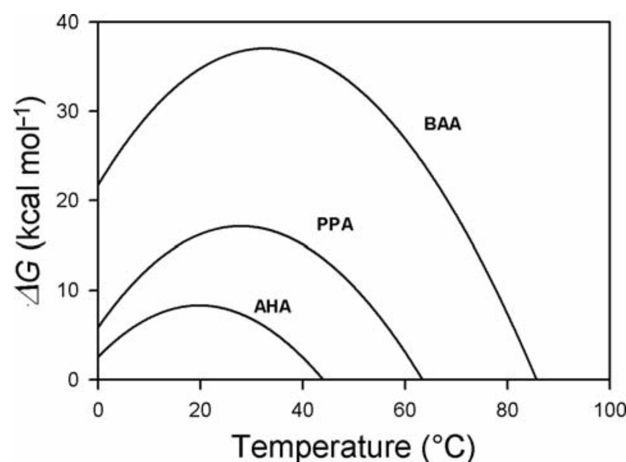

Fig. 13.4 Stabilization energy curves of three α-amylases: AHA, psychrophilic, PPA, mesophilic and BAA, thermophilic, as calculated from microcalorimetric data. Taken from D'Amico et al. (2003)

of high temperatures to promote the molecular motions needed for enzyme function. In contrast, for psychrophiles, the environmental temperatures are below that of the ΔG_{max}, indicating that molecular motions at low temperatures are gained from the hydration of ionic-, polar- and non-polar interactions, i.e. the factors ultimately leading to cold unfolding (Feller 2007). In fact, the stability curves shown in Fig. 13.4 also allow the prediction (by extrapolation) of a temperature of cold-unfolding and it can be seen that, contrary to what would be intuitively expected, cold-adapted enzymes are also the least resistant to cold denaturation. This poorly investigated observation must be kept in mind when considering the lower temperature limit of life and indeed further studies in this field are called for.

As has already been mentioned, the majority of proteins unfold irreversibly, thereby preventing extraction of thermodynamic equilibrium data but nevertheless allowing for determination of the activation parameters for these irreversible processes. Irreversible denaturation and/or inactivation have been found to be characterized by a low activation free energy value ($\Delta G^{\#}$) in cold-adapted enzymes (Collins et al. 2003; D'Amico et al. 2003; Georlette et al. 2003) and simply reflect the rapid unfolding of these proteins. The small differences observed in the $\Delta G^{\#}$ arise from large differences in the enthalpic and entropic contributions and are the consequence of classical enthalpy–entropy compensations. The high activation enthalpy ($\Delta H^{\#}$) for psychrophilic enzymes, as a result of the high slope of the Arrhenius plots used to calculate these parameters, simply reflects a higher cooperativity of the denaturation and inactivation processes in these enzymes. Furthermore, the lower value of the activation entropy ($\Delta S^{\#}$) for the thermostable enzymes suggests that the activated state is reached with less disordering and thus reflects the higher resistance of the thermophilic enzyme to irreversible denaturation.

13.4.2 Global versus local stability

From an evolutionary point of view, several studies have discussed the possibility that the instability of psychrophilic enzymes is the result of a random genetic drift due to the lack of a selective pressure on stability (Miyazaki et al. 2000; Wintrode et al. 2001). The fact is, however, that enzymes displaying a high stability as well as a high specific activity at low temperatures are not apparently found in nature, even though this appears to be possible to obtain in vitro, with small size synthetic substrates and after several rounds of random mutagenesis where a selective pressure on stability is imposed (Wintrode et al. 2001). Furthermore, it is important to note that while psychrophilic enzymes found in nature are generally characterized by a reduced stability, this destabilization is not always found to involve the whole protein structure and, indeed, a number of proteins with localized zones of reduced stability have been reported. Obviously this observation questions the possibility of random genetic drift playing a part in the poor stability of psychrophilic enzymes as it is improbable that this could affect only a specific part of the protein structure.

In particular, this destabilization in cold-adapted proteins appears to be concentrated at or near the active site. In multi-domain enzymes, such as the psychrophilic chitobiase (Lonhienne et al. 2001), phosphoglycerate kinase (Bentahir et al. 2000) and FKBP 22 (Suzuki et al. 2005), as well as in the precursor of the cold adapted α-amylase (Claverie et al. 2003), the catalytic domain is always found to be the least stable domain whereas other non catalytic domains can be as stable as that of the mesophilic homologs. Furthermore, although the thermal unfolding of the mature α-amylase is highly cooperative, it has been shown using transverse urea gel electrophoresis that the active site is the least stable structural entity in the presence of urea (Siddiqui et al. 2005).

Further strong support for this concept of a reduced stability at or near the active site can be found on comparing enzyme activity and stability (Collins et al. 2003; D'Amico et al. 2003; Georlette et al. 2003). As can be seen from Fig. 13.1, the initial loss of activity for the mesophilic and thermophilic enzymes corresponds quite well with the thermal protein unfolding, whereas in the case of the cold-adapted enzymes the activity decreases well before any unfolding process as recorded by fluorescence spectroscopy or microcalorimetry. This simply means that here the active site or the enzyme–substrate complex is more heat-labile than the whole protein structure and as a consequence the enzyme is inactivated several degrees before the melting temperature. This again points to a localized adaptation in psychrophilic enzymes whereby the active site and/or its surroundings are destabilized and may again be proposed to be due to the requirement for an increased flexibility of these specific regions for cold-adaptation.

13.5 Flexibility

From the previous discussions of the activity and stability of psychrophilic enzymes it is obvious that a number of studies indirectly point to the high activity and low stability of these to being a consequence of an increased flexibility of the whole or

of a particular part of the molecular edifice. Indeed, it is believed that this high flexibility, which derives from the observed low stability of psychrophilic enzymes, would allow for the molecular motions necessary for activity at a low energy cost, i.e. at low temperatures.

In this concept of an activity–stability–flexibility relationship, stability and activity can be experimentally demonstrated quite easily. In contrast, the determination of protein flexibility, mobility or plasticity, as it is also known, is much more difficult and, in particular, if it is limited to a small part of the whole structure. Moreover, we must consider the molecular motions in terms of amplitude as well as time scale. One approach which has been used to evaluate this peculiar characteristic of proteins has been to carry out a comparative study of fluorescence quenching in extremophilic enzymes so as to assess the propensity of the whole molecular structure to being penetrated by a small quencher molecule (acrylamide being the most frequently used). This technique gives an idea of all conformational opening processes occurring in the whole protein and averaged over a large time scale, and it has been successfully used to compare the flexibility of homologous Ca^{2+}-Zn^{2+} proteases (Chessa et al. 2000), xylanases (Collins et al. 2003), DNA ligases (Georlette et al. 2003), α-amylases (D'Amico et al. 2003) and cellulases (G. Sonan, personal communication). These experiments unequivocally demonstrated the decrease in protein permeability on going from psychrophilic to thermophilic enzymes.

Another approach is neutron scattering and this has been used to compare the mean macromolecular dynamics of whole crude extracts obtained from bacteria adapted to different environmental temperatures (Tehei et al. 2004). Here, the authors showed that at a given temperature, the resilience (which is approximately equivalent to rigidity) increased from psychrophile to thermophile. However, at their respective environmental temperatures, the measured resiliencies were similar and therefore in perfect agreement with the "corresponding state" theory (Somero 1995). Other methods which have been used more or less successfully to evaluate protein flexibility include hydrogen/deuterium exchange (Zavodszky et al. 1998; Svingor et al. 2001) and tryptophan phosphorescence (Fischer et al. 2000; Gershenson et al. 2000).

Finally, molecular dynamics studies are becoming increasingly common (Brandsdal et al. 1999; Olufsen et al. 2005; Papaleo et al. 2006; Spiwok et al. 2007) and a recent study of an elastase family (Papaleo et al. 2006) showed that (1) loop regions clustered around the active site are characterized by an enhanced flexibility in the cold-adapted enzyme, and (2) some scattered regions distant from the functional sites are more rigid in the cold-adapted enzyme, thus in perfect agreement with the theory we developed previously in this review.

Thus, it can be seen that while still suffering from the lack of a widely accepted technique to unequivocally characterise protein dynamics, it appears that many psychrophilic enzymes are characterised by an increased flexibility of at least a part of their structure and it is this increased flexibility, originating from the low stability of crucial parts of the molecular structures, which allows for the high activity at low temperatures.

13.6 Structural adaptations

The crystal structures of 22 psychrophilic enzymes have been reported up to now (Table 13.1) and many of these have been subjected to comparative structural analyses with their mesophilic and/or thermophilic homologs. The first observation from these studies is that the overall fold of proteins adapted to different thermal environments are very similar, and many residues, in particular those involved in catalysis, are found to be conserved (Aghajari et al. 1998b). A closer comparative examination does, however, reveal subtle structural modifications, with cold-adapted proteins being most frequently characterized by a reduced number and/or strength of stabilizing interactions. Indeed, any of a large number or combination of a variety of structural adjustments, such as a reduction of salt-bridges, hydrogen

Table 13.1 Cold-adapted enzymes for which the crystal structure has been determined

Enzyme	Host organism	Reference
Adenylate kinase	*Bacillus globisporus*	Bae and Phillips (2004)
Alkaline phosphatase	Bacterial strain TAB5	Wang et al. (2007)
Alpha-amylase	*Pseudoalteromonas haloplanktis*	Aghajari et al. (1998a)
Aspartate carbamoyltransferase	*Moritella profunda*	De Vos et al. (2007)
Beta-galactosidase	*Arthrobacter* sp. C2-2	Skalova et al. (2005)
Catalase	*Vibrio salmonicida*	Riise et al. (2007)
Cellulase	*Pseudoalteromonas haloplanktis*	Violot et al. (2005)
Citrate synthase	*Arthrobacter* sp. strain DS2-3R	Russell et al. (1998)
Lipase B	*Candida antarctica*	Uppenberg et al. (1994)
Malate dehydrogenase	*Aquaspirillium articum*	Kim et al. (1999)
Phosphatase (alkaline phosphatase)	*Pandalus borealis*	de Backer et al. (2002)
Phosphatase (protein-tyrosine phosphatase)	*Shewanella* sp.	Tsuruta et al. (2005)
Protease (alkaline metalloprotease)	*Pseudomonas* sp. strain TAC II 18	Aghajari et al. (2003)
Protease (anionic trypsin)	*Salmon salar*	Helland et al. (1998)
Protease (elastase)	*Salmon salar*	Berglund et al. (1995)
Protease (pepsin Iib)	*Gadus morhua*	Karlsen et al. (1998)
Protease (proteinase K-like)	*Serratia* sp.	Helland et al. (2006)
Protease (subtilisin-like serine proteinase)	*Vibrio* sp. PA-44	Arnorsdottir et al. (2005)
Protease (trypsin)	*Oncorhynchus ketav*	Toyota et al. (2002)
Triose phosphate isomerase	*Vibrio marinus*	Alvarez et al. (1998)
Uracil-DNA glycosylase	*Gadus morhua*	Leiros et al. (2003)
Xylanase	*Pseudoalteromonas* sp. strain TAH3a	Van Petegem et al. (2003)

bonds, core hydrophobicity, aromatic interactions, arginine and/or proline content, density of charged surface residues, an increased surface hydrophobicity and an increased clustering of glycine residues etc., have been observed, with each enzyme adopting a specific strategy (Feller et al. 1997; Smalas et al. 2000). Indeed, these are the main structural modifications involved in cold-adaptation and it is obvious how such alterations can give rise to a decreased stability but also an increased flexibility of the molecular structure and hence allow for the molecular motions required for activity at low temperatures.

In addition to the above common observations a number of other more enzyme specific structural adaptations have also been reported. These include a larger and more accessible active site (Russell et al. 1998; Aghajari et al. 2003) an improved electrostatics of and in the vicinity of the reactive centre (Brandsdal 2001; Leiros et al. 2003), a long and highly flexible linker region (Violot et al. 2005) and a reorganization of the quaternary structure (Skalova et al. 2005)

13.7 Conclusions

It is important to remember that while only cold-adapted enzymes have been discussed in this chapter, life at low temperatures requires a multitude of adaptations at all levels within the cell, from single molecules up to supra-molecular structures.

Enzymes achieve cold adaptation by means of fine tuning the activity, flexibility, stability balance. They efficiently catalyze reactions at low temperatures as a result of an inherent increased flexibility, in particular at or near the catalytic site and this enables the molecular motions necessary for activity in this low energy environment. On the other hand, this increased low temperature activity and flexibility is achieved at the expense of stability and frequently also an increased K_m. Of course there is a limit to this adaptation process since the stability of a protein cannot decrease below a certain limit, i.e. it must maintain a properly folded form and this is probably one of the reasons why enzyme adaptation appears incomplete for some proteins. Finally, it appears that cold-adaptation is mainly achieved by means of quite discrete structural modifications and primarily by removal of stabilizing interactions, with each enzyme using a specific strategy to achieve its goal.

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Chapter 14
Cryoprotectants and Ice-Binding Proteins

Hidehisa Kawahara

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14.1 Introduction

Some bacteria have developed a variety of strategies to survive and colonize extremely cold environments such as the Antarctic and the Arctic (Russell 1997; Chattopadhyay 2006). In these frozen environments, bacteria are exposed to conditions that necessitate the partial removal of water from the intracellular space in order to maintain the structure and function of the cell. As water is essential for the function of various macromolecular structures, i.e. proteins, polysaccharides, lipids, and nucleic acids, any significant deviation in the accessibility of water due to dehydration, desiccation or the alteration of its physical state, from the aqueous phase to an ice crystal, will pose a severe threat to the normal function and survival of organisms (Beall 1983).

It has been suggested that substances such as trehalose, glycerol, and sorbitol are major cryoprotectants (CRP) for prokaryotic cells exposed to freezing conditions. They allow the maintenance of enzyme activities in vivo (Storey and Storey 1986) and also prevent the cold denaturation of proteins (Phadtare 2004).

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As shown in Fig. 14.1, the degree of injury in various intact cells is affected by the freezing rate and the recrystallization of ice in the intra- and extracellular space that can occur when the freezing rate is lower than 10°C min^{-1} (Fujikawa 1987).

The intracellular freezing of yeast and bacteria requires a freezing rate of 1–10°C min^{-1} (Mazur 1970). Freezing rates between 10 and 100°C min^{-1} can damage the cytoplasmic membrane, whereas a rate of over 100°C min^{-1} is less damaging since ice appears as microcrystals.

For many organisms, and especially some bacteria, specific proteins or/and saccharides can be produced to counteract or minimize the deleterious effects of ice crystal formation (Kawahara 2002). As shown in Fig. 14.2, ice nuclei can be formed by the so-called homogeneous (no particle present) or heterogeneous nucleation (particle-induced nucleation) in a first step. In a second step, the ice crystals grow from their ice nuclei due to the binding of free water molecules. The formation of ice nuclei through heterogeneous ice nucleation is promoted by foreign particles called ice nucleation activators. Various types of ice nucleation activators of biogenic origin (Hew and Yang 1992) are known to exist in plant bacteria, insects, intertidal invertebrates, plants and lichens. The highest level of ice-nucleation activation is provided by ice-nucleating proteins (INP) produced by some ice-nucleating bacteria.

Paradoxically, the inhibitors of heterogeneous ice nucleation which can favour supercooling have been poorly studied. These inhibitors can contribute to minimize the threats of intra- or extracellular ice formation. These anti-nucleating materials (ANM) include proteins and polysaccharides, and are also classified as ice crystal-controlling materials.

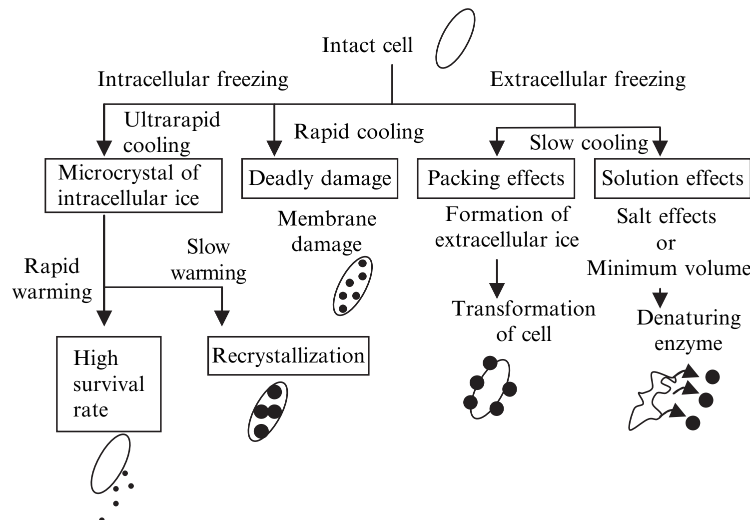

Fig. 14.1 Mechanism of freezing injury against an intact cell

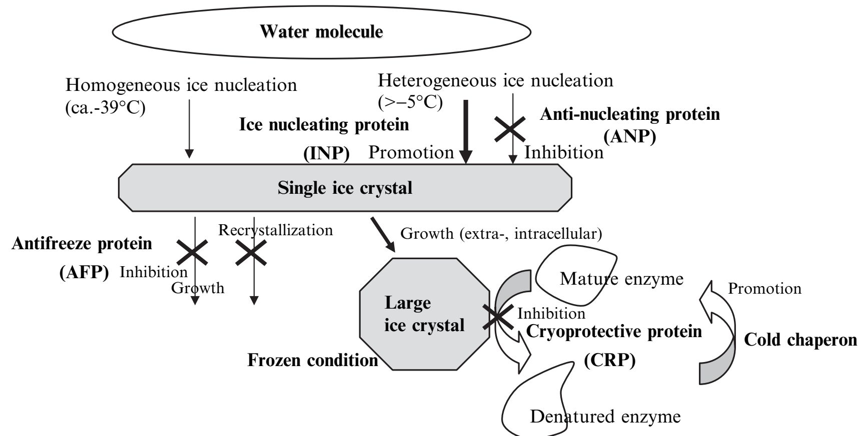

Fig. 14.2 The scheme of action modes of various ice crystal-controlling proteins

Other ice crystal-controlling proteins, which can play a crucial role in the second step of ice formation, are antifreeze proteins (AFP). The function of the AFP is to inhibit ice formation by suppressing the growth of ice nuclei. In the past two decades, all these proteins were found in bacteria from various environments. INPs have been discovered in bacteria such as *Pseudomonas*, *Pantoea,* and *Xanthomonas*; in fungi such as *Fusarium*; in insects and in lichens such as *Umbilicaria esculenta*, and *Anaptychia obscurata*. Anti-Nucleating Materials (ANMs) have been found in bacteria such as *Acinetobacter* and *Bacillus* and in coniferous trees. AFPs have been first discovered in fishes, plants, insects, fungi such as *Penicillium* and *Typhpula*, in lichens such as *Thamnolia*, in mushrooms such as *Coprinus* and *Flammulina,* as well as in bacteria such as *Pseudomonas*, *Moraxella,* and *Marinomonas*. Cryoprotectants (CRPs) have been found in bacteria such as *Pseudomonas*, *Pantoea* and *Lactobacillus*, in plants such as spinach, soybean and *Chlorella* and also in vertebrates such as frogs.

14.2 Cryoprotection in cold-adapted microorganisms

14.2.1 Cryoprotectants of low molecular mass

Cryoprotectants are known to accumulate in the body fluids of some wintering frogs and insects (Storey 1983). Examples of these substances include sugars (glucose, fructose, sucrose, trehalose, etc.), alcohols derived from sugars (sorbitol, glycerol) and amino acids (alanine, proline) (Morita et al. 2003). As an example, glycine betaine which is a well-known osmolyte can enhance the growth of *Listeria*

monocytogenes at low temperatures (Ko et al. 1994). The cryoprotective roles of glycine betaine in bacteria are believed to prevent cold-induced aggregation of proteins and to maintain optimum membrane fluidity at low temperatures (Chattopadhyay 2002). Other examples can be found in ice-nucleating bacteria, such as the genera *Pantoea* (Lindow et al. 1983), *Pseudomonas* (Maki et al. 1974; Obata et al. 1987), and *Xanthomonas* (Kim et al. 1987). They can induce the nucleation of ice in supercooled water at −2 to −3°C and incite frost damage in many crops (Lindow et al. 1983). The ability of ice-nucleating bacteria to survive on the surface of a leaf after freezing and thawing has been shown to be due to ice-nucleating activity (Hirano et al. 1982). This phenomenon is responsible for the high degree of cryotolerance of ice-nucleating bacteria as shown in Fig. 14.3. The temperature changes imposed during cold-acclimation are very close to those causing frost damages in plants on spring mornings. *Pantoea agglomerance* NBRC12686 has a high rate of survival (65%) after cold acclimation at 10°C as well as *Pantoea ananatis* KUIN-1 (79%) (Koda et al. 2002). *Pseudomonas fluorescens* KUIN-1 has the highest rate of survival (94%) after cold acclimation at 4°C. These high survival rates in ice-nucleating bacteria are partly due to the accumulation of cold-acclimation proteins that possess cryoprotective or chaperone activity, but also to the accumulation of glucose in *Pantoea ananatis* KUIN-3 (1.9 mg g^{-1} cell wet mass) (Table 14.1). The glucose-6-phosphatase, related to the glucose accumulation, has a 1.44-fold higher activity (117 mU mg^{-1}) after cold acclimation for 24 h at 10°C following growth for 12 h at 30°C (81 mU mg^{-1}). Furthermore, *Pa. agglomerans* NBRC12686 accumulates ribose-1-phosphate and glucose in cells following a shift in temperature (12°C) from the optimum growth temperature (30°C) (Table 14.1). The uridine phosphorylase, related to the ribose-1-phosphate and glucose accumulation, has a 1.48-fold higher

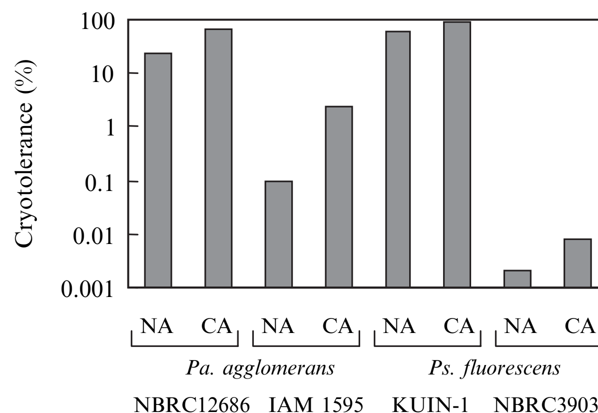

Fig. 14.3 Effects of cold acclimation (*CA*) on the cryotolerance of ice-nucleating bacteria, *Pa. agglomerans* NBRC12686 and *Ps. fluorecens* KUIN-1, and non ice-nucleating bacteria, *Pa. agglomerans* IAM 1595 and *Ps. fluorescens* NBRC3903. *NA* Non-acclimated strains (Obata et al. 1998; Koda et al. 2000)

Table 14.1 Accumulations of various cryoprotectants in some ice-nucleating bacteria

Strains	Cryoprotectants	Functions	Reference
Pseudomonas fluorescens KUIN-1	COR26	Cryoprotection of enzymes	Obata et al. (1998)
Pantoea agglomerans NBRC12686	CRP	Cryoprotection of enzymes	Koda et al. (2001)
	Uridine phosphorylase	Cryoprotection of enzymes	Obata et al. (2004)
	Ribose-1-phosphate	Cryoprotection of enzymes Depression of freezing points	Obata et al. (2004)
Pantoea ananatis KUIN-3	HSC25	Refolding of denatured enzymes	Kawahara et al. (2000b)
	Glucose	Depression of freezing points	Koda et al. (2002)

activity after cold acclimation for 48 h at 12°C following growth for 12 h at 30°C. Both enzymes play an important role as some of the various regulators for glucose accumulation during cold acclimation to achieve high survival rates in ice-nucleating bacteria.

14.2.2 Cryoprotective proteins and cold chaperones from ice-nucleating bacteria

Many enzymes in low concentrations are easily inactivated during storage, especially frozen storage (Chilson et al. 1965; Anderson et al. 1978). The factors involved in this inactivation, and the relationship between freeze denaturation and conformational changes, have been investigated in details, particularly for lactate dehydrogenase (LDH) (Seguro et al. 1989). The cryoprotective effects of albumin on common metabolic enzymes including LDH and mutarotase, etc., have been reported (Tamiya et al. 1985). Indeed, albumin (0.1%) prevents the freeze inactivation of LDH in response to cold-acclimation at 12°C by surrounding the enzyme molecules thereby protecting them at a molar ratio of 10 BSA for 1 LDH from conformational changes and aggregation.

The COR15 (15 kDa polypeptide) from *Arabidopsis thaliana* is also known as a cryoprotectant (Lin and Thomashow 1992). The gene *cor15* is expressed during cold acclimation processes; in vitro, COR15 has a cryoprotection activity about 10^2 to 10^3 times higher than albumin (Table 14.2). The psychrotrophic ice-nucleating bacterium *Pseudomonas fluorescens* KUIN-1 accumulates COR26, a 26 kDa polypeptide, induced in response to a decrease in temperature (Obata et al. 1998). Also, *Pantoea agglomerans* NBRC12686 produces a 29 kDa cryoprotective protein (CRP) (Koda et al. 2001). This CRP protects freeze-labile enzymes such as LDH,

Table 14.2 Comparison of various cryoprotective proteins

Protein	MW	CP_{50} (μg ml^{-1})	CP_{50} (nM)	Reference
BSA	66,000	2.8×10^{1}	4.0×10^{2}	Lin and Tomashow (1992)
Sucrose	340	2.7×10^{5}	8.0×10^{8}	Lin and Tomashow (1992)
COR 15	15,000	8.3×10^{-2}	5.6×10^{0}	Lin and Tomashow (1992)
COR 85	350,000	1.5×10^{1}	4.3×10^{1}	Tomashow (1990)
COR 26	159,000	1.6×10^{4}	1.0×10^{5}	Obata et al. (1998)
HIC 6	14,700	1.1×10^{-1}	7.4×10^{0}	Honjoh et al. (2000)
CRP 29	29,000	8.0×10^{-4}	2.7×10^{-2}	Koda et al. (2001)
CRP 62	62,000	2.0×10^{-1}	3.2×10^{0}	Neven et al. (1993)
AS 26k	26,000	2.0×10^{1}	7.7×10^{2}	Momma et al. (1997)

CP_{50}, the concentration required to protect for 50% of LDH activity

alcohol dehydrogenase (ADH) and isocitrate dehydrogenase against freezing and thawing denaturation. The activity of CRP as cryoprotectant was about 3.5×10^{4} times higher than that of COR26 (Table 14.2) (Tamiya et al. 1985). In contrast to BSA, the molar ratio of protected LDH and CRP was 6.49×10^{-4}, but it is impossible for 1 mole of CRP to surround 1,540 moles of LDH. Although this mechanism of cryoprotection remains unexplained, it seems that the association between the CRP molecules and water molecules may play an important role. Recently, Obata et al. (2004) have reported that uridine phosphorylase (M_W=29,700 Da) from *Pantoea agglomerans* NBRC12686 has also a weak cryoprotective activity at a concentration of 5.0×10^{-3} mg ml^{-1} (Table 14.2).

Another ice-nucleating bacterium, *Pa. ananatis* KUIN-3 produces Hsc25 as one of the cold-acclimation proteins (Kawahara et al. 2000b). This Hsc25 has a refolding activity similar to GroEL; it is composed of 8 subunits of 25 kDa each. It has a refolding activity towards denatured enzymes induced by heat-treatment at 100°C and guanidinium chloride, and also a cryoprotection effect at −20°C for 24 h. The affinity of Hsc25 for cold-denatured enzymes is higher than that of GroEL for heat-denatured enzymes. The high rate of survival after freezing–thawing of ice-nucleating bacteria is therefore also achieved by some components of the cytoplasmic space different from the ice nucleating activity present at the surfaces of their cell membranes.

14.3 Ice crystal-controlling proteins

14.3.1 Structure and function of ice-nucleation proteins

When pure liquid water is cooled at atmospheric pressure, it does not freeze spontaneously at 0°C. Due to density fluctuations in liquid water, the water molecules form clusters with the same molecular arrangement as ice crystals but remain in a

liquid state due to the fluctuation of energy. This is called supercooling. A drop of pure water that is perfectly cleared from all foreign particles can display a supercooling temperature or freezing temperature of about −40°C. This process has been called 'homogeneous ice-nucleation'. In practice, however, impurities or foreign particles that are usually present in water attach water molecules on their surface. As water molecules may be oriented in a way such as to resemble an ice nucleus, these become compatible with the critical dimension of ice-nucleation. This process is called 'heterogeneous ice-nucleation; it always occurs at a temperature between −2°C and −15°C. Some bacteria produce proteins which can induce ice-nucleation (INP) at temperatures higher that −3°C; they have been called ice-nucleating bacteria (Fig. 14.2). At least six species of ice-nucleating bacteria have been found; some strains of *Fusarium* and related genera of fungi are also active in ice nucleation (Pouleur et al. 1992). Recently, the ice-nucleating components from some lichens have been partially purified (Kawahara et al. 2005; Obata et al. 2006). These substances have different properties when compared to those of bacterial and fungal INPs. Although almost all microorganisms producing INPs were found at the surface of plants, a strain of *Pseudomonas* sp. KUIN-5 was isolated from the marine algae, *Monostroma latissum*. It produces a bacterial cellulose in the culture broth which can act as an ice-nucleating component (Kawahara et al. 1996a).

Genes conferring ice-nucleating activity have been sequenced from six bacterial strains and all encode INPs (120–150 kDa) with similar primary structures (Green and Warren 1985; Warren et al. 1986; Abe et al. 1989; Warren and Corotto 1989; Zhao and Orser 1990; Michigami et al. 1994). As shown in Table 14.3, all INPs are composed of three domains, the N-, R-, and C-domains. The N-domain (15% of the

Table 14.3 Comparison of the amino acid sequences of various Ina proteins

Bacteria	Gene	Total amino acids	Predicted MW	Amino acid residue in each domain			Reference
				N-domain	R-domain	C-domain	
Ps. fluorescens KUIN-1	*ina*F	1,352	132,472	183	1,120	49	Kawahara (unpubl.)
Ps. fluorescens	*ina*W	1,210	111,881	164	992	54	Warren et al. (1986)
Ps. syringae	*ina*Z	1,200	118,587	175	976	49	Green and Warren (1985)
Pa. ananatis	*ina*A	1,322	131,094	161	1,120	41	Abe et al. (1989)
Pa. ananatis	*ina*U	1,034	103,387	161	832	41	Michigami et al. (1994)
Pa. agglomerans	*ice*E	1,258	125,076	161	1,056	41	Warren and Corrotto (1989)
X. xampestris	*ina*X	1,567	152,548	219	1,280	68	Zhao and Orser (1990)

total sequence) is at least responsible for the binding of lipids, polysaccharides and INP (Kozloff et al. 1991); it is relatively hydrophobic and contains a membrane anchor with a mannan-phosphatydylinositol. The R-domains play an important role as a template for ice formation; their length (832—1,280 amino acids) is correlated with the amplitude of ice nucleation activity. (Table 14.3). They are made (ca. 80%) of contiguous repeats of a consensus octapeptide, Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr, mainly consisting of hydrophilic amino acids. The C-terminal domain (less than 40–50 residues) is rich in basic amino acid residues and is very hydrophilic. It is necessary for the ice-nucleating activity since, for example, in the INP from *Pantoea ananatis,* the deletion of the last 12 C-terminal residues does not modify the activity while another mutant with Met29 as new C-terminal amino acid displays a nearly complete loss of activity (Michigami et al. 1995a). It was also shown that one of the residues of this domain, Tyr27, is important for the activity, although not exclusively required, since the activity was lost to a great extent when this residue was replaced by Gly or Ala, but to a much lesser extent when it was replaced by Leu. These results point to the importance of the secondary and/or tertiary structure of the C-terminal region for the ice-nucleation activity.

The characterization of INPs has involved sequencing, truncation, activity measurements, peptide studies, and theoretical modeling (Waren and Wolber 1991; Hew and Yang 1992; Guriansherman and Lindow 1993). Several research groups have previously presented theoretical structures of the highly repetitive region (R-domain), (Mizuno 1989; Waren and Wolber 1991) and one group has described models consisting of 8, 16, and 24 residue peptides based on NMR data (Tsuda et al. 1997). Recently, it was shown that the recombinant 97 residues polypeptide corresponding to the sequence Tyr176 to Gly273 of INP from *Pseudomonas syringae* has the ability to shape an ice crystal, whose morphology was highly similar to that of the hexagonal bi-pyramid form generally identified for AFPs (Kobashigawa et al. 2005). Also, Wilson et al. (2006) have suggested that the ice affinity of INPs directs the role of bacteria either towards inhibition of ice-recrystallization, ice-nucleation or ice-shaping. The ice-controlling protein from *P. syringae* exhibits only ice-nucleation activity and has no AFP activity.

As shown in Fig. 14.4, the conserved glycine residues, often involved in chain bending, are located at every turn of the proposed R-domain structure while the highly conserved Ser and Tyr residues are only present in the middle of the beta-strands allowing them to act as an ice-like template. They are involved in the aggregation of individual INPs, a process that renders the ice-nucleating properties more efficient (Kajava and Lindow 1993). Based on the ice-binding ability (Kobashigawa et al. 2005), it was suggested that INPs may have a similar beta-helical fold and may interact with water through the repetitive TXT motif (Fig. 14.4) (Graether and Jia 2001).

Proteins and other components located on the outer membranes of these bacteria are responsible for ice nucleation. Two strains of *P. fluorescens* (Obata et al. 1993) and *Pa. ananatis* (syn. *Erwinia uredovora*) (Kawahara et al. 1993) have been reported to release the extracellular ice-nucleating materials into the culture fluid as well as two other strains, *Erwinia herbicola* (Phelps et al. 1986) and *Erwinia carrotovora* (Fukuoka et al. 1992). However, purified INP, contrarily to raw mate-

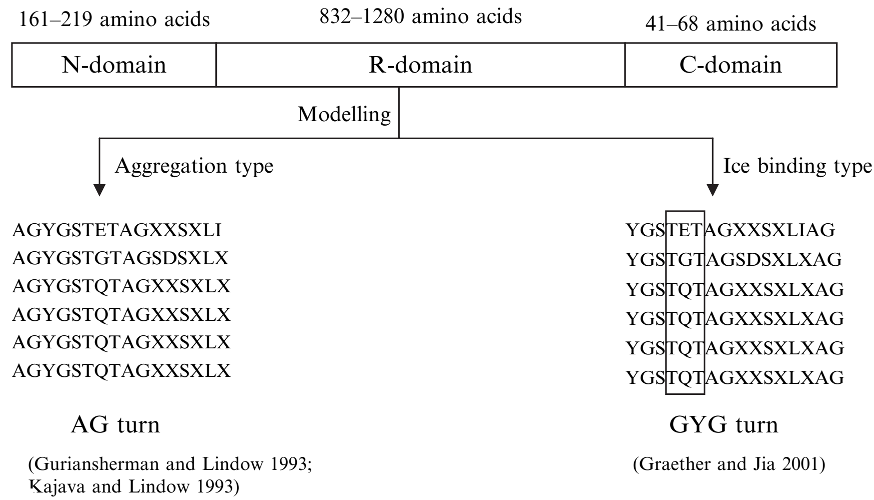

Fig. 14.4 Different models of the central repeat region of ice-nucleating proteins

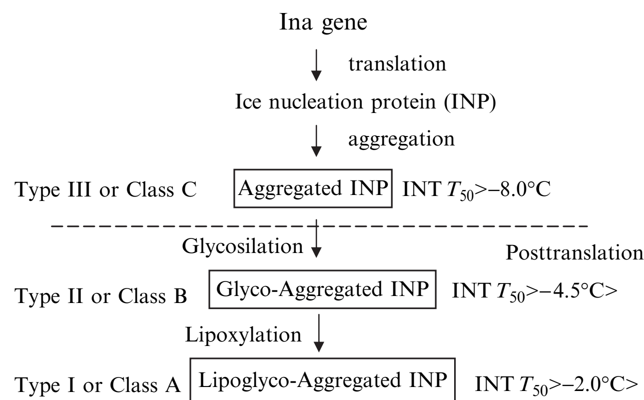

Fig. 14.5 Tentative pathway of the biosynthesis of ice nucleation materials in some ice-nucleating bacteria. *INT* Ice nucleating temperature

rial (−3°C), exhibits an ice-nucleation activity at temperatures below −6°C (Fall and Wolber 1995). This difference between raw and purified material is probably due to a requirement for phospholipids (Govindarajan and Lindow 1988). Bacterial ice activators can in fact be complexes of lipids, saccharides and proteins; they are lipoglycoproteins (Kozloff et al. 1991), and classified into three chemically distinct classes according to their chemical composition: A, lipoglycoprotein; B, glycoprotein, and C, only protein (Turner et al. 1991) (Fig. 14.5). Also, the activation effect of the class C structures is due to the aggregation of INPs giving rise to molecular masses over 1,000 kDa (Govindarajan and Lindow 1988). Franks (1985) reported

that the factors involved in the formation of ice nuclei have to obey to three conditions: similarity to the ice crystal lattice, paucity of the surface charge, and high hydrophobicity. Class A structures apparently display the full set of conditions required for a maximal activity.

The extracellular ice-nucleating material produced by *E. uredovora* KUIN-3 was purified and characterized as a spherical structure (0.2–0.4 μm), which is composed of lipid, protein, saccharide and polyamine (Kawahara et al. 1993). The polyamine plays a role in the surface charge, in the control of hydrophobicity, and in the stability of the protein conformation of class A and B structures. It is a critical component of these structures (Kawahara et al. 1994). Also, the INP purified from the extracellular ice-nucleating matter of *E. uredovora* is a glycoprotein (Michigami et al. 1995b). Not all INPs secreted in the culture broth have the same signal sequences of 15–70 amino acid residues at the N-terminus. Michigami et al. (1995b) have reported that after INP first assembles near the inner membrane, the assembly then enters a vesicle just formed on the surface of the outer membrane and leaves the surface. It has been shown that ATP is essential for the secretion of extracellular ice-nucleating matter into the culture broth (Kawahara et al. 1999). Considering these results, it appears that the secretion of INP or extracellular ice-nucleating matter might be a unique mechanism.

Almost all ice-nucleating bacteria belong to Gram-negative epiphytic genera and are known as pathogenic bacteria, either mesophilic or psychrotrophic. The isolation and characterization of ice-nucleating bacteria from Antarctica are still in an early state of investigation; that is the reason why we have started a research programme to isolate ice-nucleating bacteria from the bacterial collection stock at Ross Island in the McMurdo Dry Valley region of Antarctica (Obata et al. 1999). Eleven ice-nucleating bacteria were discovered out of 135 strains (ca. 8%). Strain IN-74, with the highest level of ice-nucleating activity, was identified as *Pseudomonas antarctica*. This strain also displays a high level of ice-nucleating activity following induction at 0°C, and this temperature was significantly higher than those of other ice-nucleating bacteria. The molecular mass of its INP is over 120,000 (Michigami et al. 1995b).

14.3.2 Structure and function of anti-nucleating proteins

Ice-nucleating inhibitors have the ability to lower the supercooling point of water (Fig. 14.6), and this point, expressed in °C, is defined as the anti-nucleation activity. These components have been up to now poorly studied. An enzyme-modified gelatin (EMG-12) has been reported as an ice-nucleating inhibitor of silver iodide, AgI, a well-known ice-nucleating agent (Arai and Watanabe 1985). Also, hinokitiol, a compound found in the leaves of coniferous trees, has an anti-nucleating activity against *P. fluorescens* (Kawahara et al. 2000a). Furthermore, poly-glycerol, with molecular mass of 750 Da, has been found to apparently bind and inhibit the ice-nucleating activity of *P. syringae* (Wowk and Fahy 2002).

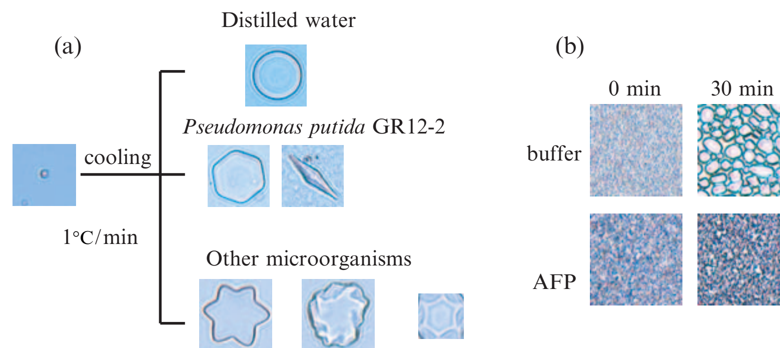

Fig. 14.6 Ice crystal regulation by antifreeze proteins from some microorganisms. **a** Ice crystal morphology; **b** ice crystal recrystallization inhibition

We have also discovered a bacterial strain with anti-nucleating activity (Kawahara et al. 1996b); this strain, KINI-1, was isolated from camphor and identified as *Acinetobacter calcoaceticus*. The anti-nucleating protein (ANP) produced by this strain has a molecular mass of 550 kDa. It exhibits a broad specificity with the capability to lower the nucleating activity of a wide range of ice-nucleators including some bacterial components, and AgI. This ANP is for example able to shift the supercooling point of water to −5°C in a cell suspension of *E. uredovora;* it inhibits the formation of ice nuclei in a way similar to EMG-8 and EMG-12 (Arai and Watanabe 1985). Furthermore, one strain of *Bacillus thuringiensis*, isolated from camphor, produces an anti-ice-nucleating polysaccharide having a molecular mass of 1300 kDa (Yamashita et al. 2002b). The analysis of this compound by LCMS demonstrated that this polysaccharide consists of a polyacetyl-D-glucosamine structure similar to that of chitin. This polysaccharide has an activity ranging from 0.1 to 4.2°C against various ice-nucleating materials including AgI, some organic compounds such as metaldehyde, fluoren-9-one, as well as phenazine, and some ice-nucleating bacteria. It does not have, however, any activity against homogeneous ice-nucleation, that is, pure water.

Organ cryopreservation is hindered by ice-inflicted damages. Non-freezing preservation of livers at subzero temperatures might offer advantages over the current method of preservation. In order to improve the preservation procedure for liver grafts, Matsukawa et al. (2000) have attempted to apply subzero non-freezing (SZNF) storage methods making use of bacterial ANP and ascorbic acid 2-glucoside (AA-2G) as an antioxidant. When the liver graft was kept for 24 h at SZNF storage (−3.0°C) in the above mentioned solution made of ANP (20 μg ml^{-1}) and AA-2G (100 μg ml^{-1}), both the number of apoptotic cells and the serum level of alanine-aminotransferase after 4 h of reperfusion could be greatly diminished. Furthermore, ATP concentrations in grafted liver tissues were significantly greater than those compared with the normal storage at 4°C. From these results, SZNF liver storage using ANPs has the potential to surpass conventional hypothermic liver storage

procedures for long periods. Further studies are carried out to isolate novel ANP-producing bacteria and characterize their ANPs or polysaccharides.

14.3.3 Structure and function of bacterial antifreeze protein

All AFPs and antifreeze glycoproteins (AFGP) lower the freezing point of water without altering the melting point (Fig. 14.6). This activity is defined as the difference between both temperatures; and is called thermal hysteresis (TH) It is caused by a non-colligative phenomenon, the activity of which being as high as 500-fold that of colligatively acting substances such as sucrose, glycerol and trehalose. As shown in Fig. 14.7, the forms of ice crystals in the presence of AFP at the initiation time of growth during cooling at 1°C min^{-1} are different from that of pure water. Primarily, needle-like growth along the c-axis occurs with fish AFP and bacterial AFP from *Pseudomonas putida* GR12-2 (Sun et al. 1995), rather than the usual predominantly highly dendritic growth along the a-axis within the basal plane. Furthermore, AFPs from other microorganisms like fungi, but also mushrooms, etc., can form different ice crystals (Fig. 14.7). These changes in ice crystal forms can also be induced by other materials including saccharides or inorganic compounds. Recrystallization of frozen solutions can be inhibited at very low concentrations (0.1 μg ml^{-1}) (Fig. 14.7).

So far, AFP and AFGP have been identified in polar fish, insects, plants (Hew and Yang 1992) and fungi (Duman and Olsen 1993). Ice-shaping activity, TH activity, and recrystallisation inhibition (RI) are common activities of most AFPs isolated from fishes. A model of the binding sites of AFPs from fishes and insect larvae to ice crystal surfaces is shown in Fig. 14.8. As AFPs isolated from insect larvae have a superactive TH activity (ca. 5°C), these proteins were named thermal hysteresis

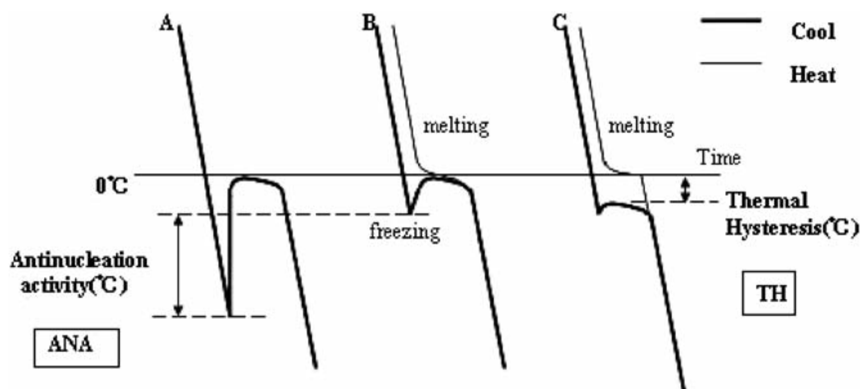

Fig. 14.7 Freezing curves of solutions with anti-nucleation material (**A**), distilled water (**B**), and antifreeze protein (**C**)

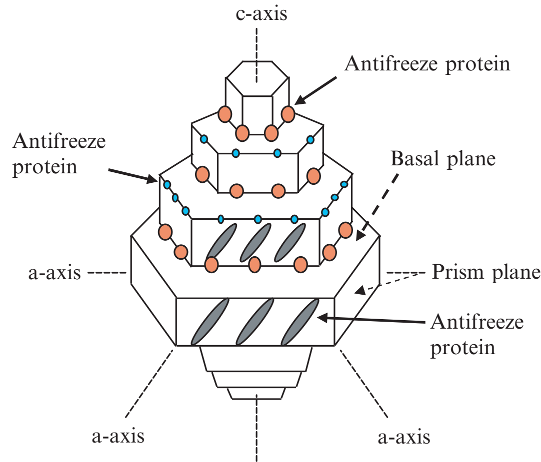

Fig. 14.8 Binding sites of some antifreeze proteins to ice crystal surfaces

Table 14.4 Comparison of some bacterial antifreeze proteins

Bacteria	MW	TH	RI	Compound	Localization	Reference
Pseudomonas putida GR12-2	164 kD	0.1°C	++	Glycolipoprotein	Extracellular	Xu et al. (1998)
Moraxella sp.	52 kD	0.1°C	+	Lipoprotein	Extracellular	Yamashita et al. (2002b)
Marimonas primoryensis	60 kD	3.5°C	+++	Protein	Extracellular	Gilbert et al. (2005)

proteins (THP) (Barrett 2001). Recently, AFPs possessing only RI activity from the Antarctic nematode *Panagrolaimus davidi* and winter wheat grass have been discovered, and named Ice-Active or Ice-Structuring Proteins (ISP) (Wharton et al. 2005; Regand and Goff 2006). Bacteria in which the presence of antifreeze activity was demonstrated are *Rhodococcus erythropolis* and *Micrococcus cryophilus* (Duman and Olsen 1993), *Sphingomonas* sp., *Halomonas* sp., *Pseudoalteromonas* sp., *Stenotrophomonas maltophilia*, *Bacillus aquamarinus, Psychrobacter* sp., *Enterobacter agglomerans* (Gilbert et al. 2004), *Pseudomonas fluorescens* (Gilbert et al. 2004; Kawahara et al.2004) and *Marinomonas protea* (Mills 1999; Gilbert et al. 2004).

The properties of a few antifreeze components produced by some bacteria are shown in Table 14.4; it is worth noting that when *P. putida* GR12-2 is grown at 5°C, it secretes an AFP having also an ice-nucleating activity; It is a lipoglycoprotein with a molecular mass of 164 kDa (Sun et al. 1995; Xu et al. 1998). To the best

of our knowledge, this is the first report of AFP having activities directed towards two opposite actions. So far, all other bacterial strains only exhibit antifreeze activity; they can all grow at low temperatures (Duman and Olsen 1993; Mills 1999; Gilbert et al. 2004; Kawahara et al. 2004). An exception is *Marinomonas primoryensis* (Table 14.4) (Gilbert et al. 2005); almost all the other bacteria secrete AFP into the culture broth. Although the TH activities of most bacterial AFPs are lower than those of THP from some insect larvae, a lysate supernatant of *M. primoryensis* containing 11 mg ml^{-1} of protein had a TH activity of 0.8°C. The activity of this lysate is lost following extensive dialysis using a 3,500 Da MW cut-off membrane. The addition to the dialysate of 10 mM $CaCl_2$ restores full activity. When concentrated, the crude lysate had a TH activity > 2°C, higher than the maximal activity of most fish AFPs. This AFP is a Ca^{2+}-dependent, superactive AFP-like THP. Furthermore, ice crystals formed in the presence of this lysate do not have distinct facets. They are typically rounded in shape and their morphology does not change during the course of the TH measurement. The ice crystal "burst" occurring at the end-point of TH is dendritic with hexagonal symmetry, suggesting growth from primary or secondary prism faces or edges. A DNA fragment from *P. putida* GR12-2 with an open reading frame encoding 473 amino acids was cloned by PCR and inverse PCR using primers elaborated from the partial amino acid sequence of the isolated AFP (Muryoi et al. 2004). The predicted gene product, AfpA, has a molecular mass of 47.3 kDa, a pI of 3.51, and no previously known function. Although AfpA is a secreted protein, it lacks an N-terminal signal peptide and was shown by sequence analysis to have two possible secretion systems: a hemolysin-like, calcium-binding secretion domain and a type V auto-transporter domain typical of Gram-negative bacteria. Also, the expression of *afpA* in *E. coli* yields an intracellular 72 kDa protein modified with both sugars and lipids that exhibits lower levels of antifreeze and ice-nucleation activities. The deduced amino acid sequence from *afpA* shows seven potential sites for N-glycosylation, two sites for O-glycosylation, and 20 sites for myristoylation.

Following transposon Tn5 mutagenesis of strain GR12-2, five mutants that have different freeze-resistance were selected (Kawahara et al. 2001). These mutants secreted low amounts of AFP (0.4–2.2 μg ml^{-1}) into the culture broth compared with the wild type (4.8 μg ml^{-1}). Furthermore, the decreased freeze-resistance of three mutants could be partially restored by adding purified AFP to the mutant cell suspensions. We concluded that accumulation of AFP is one component of the mechanism for freeze-resistance in bacteria.

We were also able to select 6 bacteria out of 130 strains isolated from Ross Island in the McMurdo Dry Valley region in the Antarctic (ca. 5%) capable of producing AFPs (Yamashita et al. 2002a). Strain 82 has the highest antifreeze activity without ice-nucleating activity and was identified as *Moraxella* sp. This AFP is a lipoprotein with a molecular mass of 52 kDa. Its N-terminal amino acid sequence is very similar to that of the outer membrane proteins from *Moraxella (Brahanmella) catarrhalis*. Both the isolation of ice-nucleating bacteria and AFP producing bacteria may provide new insights into the elucidation of the freeze-resistance or cryotolerance mechanisms in bacteria.

14.4 Conclusions

Thus far, we have examined some potential strategies for surviving under extremely cold environments. Among related compounds, various ice crystal-controlling proteins and other materials from some bacteria have been isolated and characterized. These proteins have a high potential in biotechnology. For example, ice-nucleation activators can be used to regulate both the rate of freezing and the texture of frozen foods (Li and Lee 1998) and AFPs can offer a new way of improving the quality of foods. However, the structure and function of these proteins from various organisms, especially from bacteria, are poorly characterized. The use of both proteins in food most likely will depend on the cost of their production, and also on the safety of the bacteria involved. Currently, our work is oriented towards the search of ice-sublimation-controlling proteins from bacteria as a new type of ice-controlling proteins.

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Chapter 15
The Role of Exopolymers in Microbial Adaptation to Sea Ice

Christopher Krembs(✉) and Jody W. Deming

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15.1 Introduction

The cellular exterior that a microbe presents to its surroundings marks its first line of defense against environmental pressures that range from energy deprivation and other extreme conditions, including ionic and thermal stress, to viral and higher-order attack. The production of exopolymers, whether to provide an immediate individual coating of multiple functions or to be freely released and shared by other organisms in consortial arrangements or biofilm formations, is a hallmark of microbial life in soil, water, and host (plant and animal)-associated environments. The basic features of exopolymers and their functions pertain to all manner of environments and microbial adaptation, largely independently of ambient temperature. At extreme temperatures, however, where phase changes come into play, special considerations arise.

In this chapter, we pay particular attention to the small-scale physics and chemistry of the behavior of exopolymers at low temperatures, and specifically within the sea-ice matrix where multiple phases are present in the space of a microbe, influencing its ability to survive. The study of exopolymers and cold adaptation in ice still represents a largely unexplored frontier, so other literature is tapped, including that for biofilms, polymers, the food industry, and medicine.

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By condensing seemingly disparate information into one source focused on exopolymeric substances, we seek to illuminate new directions for understanding mechanisms of cold adaptation in the microbial world, lining the pathway with an appreciation of the physical-chemical complexities of multi-phase systems so well-represented by sea ice.

15.1.1 What are exopolymeric substances?

Exopolymeric substances (EPS) are complex organic materials composed primarily of polysaccharides with carbon backbones of high molecular weight ($1–3 \times 10^5$ Daltons). With major components of hexose and pentose, they are also known as exopolysaccharides. Although variable in composition and shape (Santchi et al. 1998), they typically carry carboxylic acid groups in the form of uronic acids (Fazio et al. 1982; McConville et al. 1999), which along with other components give EPS a predominantly acidic nature. The chemistry of EPS produced by aquatic microorganisms, particularly the marine algae and bacteria on which we focus, show variable sugar compositions, consisting of highly branched heteropolysaccharides that can contain fructose, rhamnose, mannose, D-glucose, xylose, D-glucuronic acid, galactose and half-ester sulphate, among others (Percival et al. 1980), and sometimes significant amounts of protein, up to 50% (Mancuso-Nichols et al. 2005). Of essential importance in this chapter is that exopolymers can form gels hydrated to varying degree depending on environmental conditions (Verdugo et al. 2004). In the hydrated gels of natural biofilms, microbially produced mucopolysaccharides establish networks of largely insoluble polymers, often strengthened by cross-linking between ionizable functional groups on the polymers and ambient calcium or magnesium ions (Koerstgens et al. 2001). Depending on the history of their formation and transformation, EPS can contribute substantially to a wide range of categories of organic material in the ocean, from the dissolved fraction (DOM) to colloidal and particulate classes (Wells 1998; Passow 2000).

15.1.2 Exopolymers in the environment

15.1.2.1 General occurrence

EPS co-occur inseparably with microbial assemblages in both terrestrial and aquatic environments, underscoring their wide-ranging importance and diverse functions in microbial ecology (Passow 2000; Lasa 2006). Their recalcitrant character and tendency to stick to one another and various surfaces can lead to accumulations that significantly alter the physical properties of the environment, including sediments (Yallop et al. 2000) and aquifers (Battin and Sengschmitt 1999). EPS secreted as mucous slime or gels by aquatic microorganisms are known to affect the biogeochemical cycles of temperate lakes and

the ocean (Decho 1990; Heissenberger and Herndl 1994). Their structuring properties in biofilms (Costerton et al. 1995) and aquatic aggregates (Alldredge et al. 1993), their effects on the physical properties of fluids (Marcotte et al. 2001), porewater flow (Stoner et al. 2003) water and ion diffusivity (Hart et al. 1999; 2001), and their applied use in the food industry (Marcotte et al. 2001) all contribute to placing this class of compounds at an active junction of interdisciplinary research.

Although the effects of EPS accumulations cascade through an ecosystem, their roles at the organism level are critical in life histories and the survival of stressful conditions. Among their many roles, EPS gels can aid in locomotion (Wetherbee et al. 1998), adhesion to surfaces (Cooksey and Wigglesworth-Cooksey 1995; Chiovitti et al. 2006), and sequestering of nutrients and small molecular weight organic compounds from solution (Costerton et al. 1995; Decho 1990). Of particular relevance to this chapter, they can provide a protective buffer zone around a cell against unfavorable shifts in the environment, including changes in ionic, osmotic, desiccation, pH, salinity or toxic-metal conditions (Decho and Lopez 1993; Schlekat et al. 1998).

15.1.2.2 Cold-specific occurrence

EPS-producing microbes are stimulated to increase their production and release of exopolysaccharides in environments of low (Roberson and Firestone 1992) or fluctuating water potential (Dudman 1977), as well as other conditions of stress (Tamaru et al. 2005; Hoagland et al. 1993). In particular, diatoms are known to produce high concentrations of EPS when cultured in subzero brine (Aletsee and Jahnke 1992) and our microscopic observations of very cold (unmelted) sea ice have confirmed the prevalence of EPS coatings around diatom cells (Krembs et al. 2002). Cold environments in which crystalline ice forms experience a temperature-driven sequence of physical and chemical changes that affect the transport and availability of water, nutrients and gasses as well as increase the concentration of various dissolved substances, sometimes to toxic levels. At the same time, biological processes involved in cellular repair are slowed by subfreezing temperature (Price and Sowers 2004), presenting a conundrum to microbes restricted to ice.

The wide range of frozen environments on this planet provide extensive habitats for specially adapted microbial communities which have to cope with seasonal and annual fluctuations in temperature, water availability and solute concentrations (Deming 2007). As importantly, individual microbes must prevent intracellular ice-crystal growth to avoid cell damage. Although the role of EPS in mitigating damage to cells in freshwater ice is virtually unexplored, the body of relevant information on sea ice is growing (Krembs and Engel 2001; Meiners et al. 2004; Mancuso-Nichols et al. 2005; Riedel et al. 2007). Recent observations that EPS both protect microbes and favorably alter the sea-ice environment (Krembs et al. 2000, 2002, 2003; Collins et al. 2007) affirm EPS as an important extracellular component of cold adaptation in ice.

15.2 Exopolymers in sea ice

15.2.1 General concepts

Temperature, salinity and solute concentrations are inseparably linked within the sea-ice system. Critical to the microbial experience within it is the physical interplay between ice and solutes as the temperature changes seasonally. Wintertime presents the most severe shifts in temperature, such that adequate inhabitable (fluid-filled) pore space, acceptable solute concentrations and fluid flow between pores become survival issues (Fig. 15.1).

15.2.1.1 Intracellular and extracellular cryoprotection

Known biological strategies for surviving freeze damage typically involve an organic means to depress the freezing point and avoid ice crystallization altogether or else to induce crystallization selectively outside of the cell. For example, sugars and in particular trehalose are often stored intracellularly to preserve a vitreous or glassy state (Angell et al. 1994). The growth of ice crystals is prevented in winter

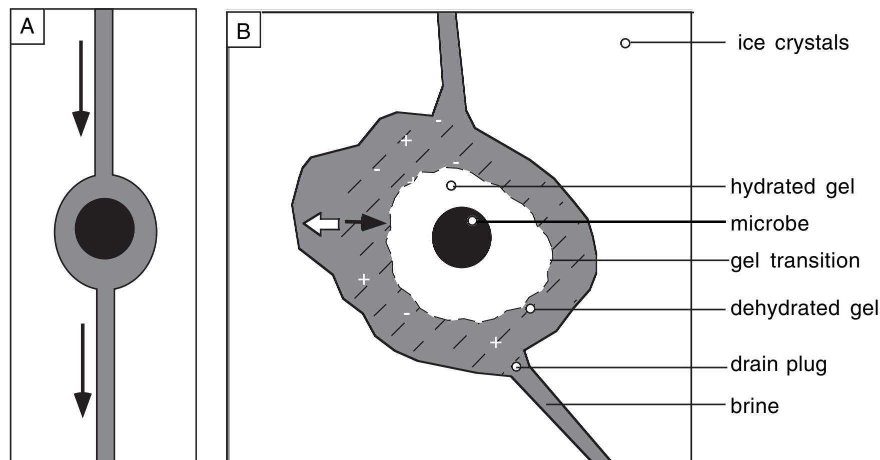

Fig. 15.1 Schematic depiction of a microbial cell in a brine vein of sea ice, without (**A**) and with (**B**) EPS present: in A) brine flows and the cell is exposed to ambient ionic strength; in B) EPS form an extracellular gel matrix that impedes the diffusive and advective transport of salt and water near the cell. Fluxes of water (*white arrow*) and ions (*black arrow*) are highest at gel periphery leading to a dehydrated, ion-rich outer envelope enclosing a more hydrated inner one. By also inhibiting crystal nucleation and growth, this gel matrix constitutes a dynamic semi-permeable barrier or protective buffer zone for the cell, one not breached by ice crystals but responsive to seasonal changes in temperature and brine salinity. (Based on Krembs et al. 2000, 2002, 2003, unpublished data)

cereal plants to −15°C via EPS that compete with the ice for interfacial liquid (Olien 1989), while some marine bacteria produce exopolymers that lead the osmotic flow of water in a direction to prevent intracellular crystallization (Kawahara et al. 1996). As temperature drops during wintertime, the inhabitable pore spaces of sea ice decrease in size due to additional water freezing out of solution. Just as dissolved sea salts become more concentrated in the remaining liquid inclusions, so do existing forms of organic matter concentrate and function as freeze-depressants (Krembs et al. 2002).

The commercial field of cryobiology provides ample evidence that sugars and synthetic polymers (such as ethylene glycol) function well as cryoprotectant additives (Michelmore and Franks 1982; Allegretto et al. 1992). Polyhydroxyl compounds (such as glycerols and sugars) are understood to be more effective in depressing the homogeneous ice-nucleation temperature than electrolytes. The addition of high molecular-weight polymers to a complex system already containing polyhydroxyl compounds decreases the freezing point even more effectively (Sutton 1991). At extremely low (eutectic) temperatures, their addition even inhibits the crystallization of salt from solution (Izutsu et al. 1995). While fully-dissolved polymeric compounds in sea-ice brines may thus result in a relatively modest (several degree) freezing point depression, naturally produced EPS gels can be expected to reduce the freezing point even further. Such gels achieve this state by lowering the amount of available free energy for ice nucleation inside the hydrated matrix or “hydrogel” and by increasing the salt concentration in the liquid phase by excluding salt ions from the gel (Hart et al. 1999, 2001).

15.2.1.2 Multi-phase dynamics

The growing ice matrix is a complex and dynamic multi-phase system. In general, when EPS accumulate at the phase boundary between ice and brine (Hung et al. 1996), they affect the diffusion of solutes and reduce the salt flux from the advancing ice surface into the pore liquid, at the same time limiting diffusive transport of water to the pore wall (Miller-Livney and Hartel 1997). Such micro-segregation (Fig. 15.1) can be expected to constrain the thermodynamic evolution of the system (Dash et al. 2006). On a larger scale, the presence of viscous EPS-enriched brine can be expected to reduce ice desalination, altering sea ice as a habitat (Krembs et al. 2003). Salt expulsion from ice depends in part on the viscosity and density of the brine (Cox and Weeks 1975) as well as the permeability of the ice on the submillimeter to centimeter scale (Wettlaufer et al. 1997). High concentrations of EPS in brine and the attendant internal phase changes (Fig. 15.1) should affect each of these parameters in directions that reduce gravitational brine drainage. Changes in viscosity density and flux of the brine will also affect, subtly or markedly, the activities of microbial residents within the pore space.

Observed effects of EPS on various properties of sea ice, though new to this decade, may prove as important as those known to occur in aquifers and sediments; e.g., to ice texture, permeability and brine drainage. The concurrent biological effects are

expected to be equally profound, with increasing evidence to support this contention (Krembs et al. 2002; Junge et al. 2006; Riedel et al. 2006, 2007; Collins et al. 2007). The rate of ice growth was previously known to affect ice-algal biomass (Cota and Smith 1991; Gleitz and Thomas 1993), since the organisms must keep pace with the advancing ice-water interface to sustain sufficient fluxes of dissolved nutrients (McMinn et al. 1999). The discovery that this colonized interface is also rich in gelatinous EPS (Krembs and Engel 2001) turned attention to the importance of the micro-segregation of ice and solutes, inorganic (Wettlaufer et al. 1997) and organic (Giannelli et al. 2001), that co-occur within an ice matrix. Above the ice-water interface, microscopic observations have indicated that brine pockets remain interconnected by fine mucus bridges (Deming 2007), which can significantly alter the thermal evolution of the pore space during temperature cycles (Krembs et al. 2000).

15.2.2 Small-scale physics and chemistry of freezing

An appreciation of the basic principles involved in seawater freezing becomes important to understanding how EPS can influence both the ice matrix and its microbial inhabitants. We examine phase transitions, supercooling and ice nucleation, ion, solute and particle rejection, and dehydration here before addressing ways in which exopolymers influence diffusion and advection, the freezing point, secondary ice nucleation and the transition to a glassy state.

15.2.2.1 Phase transition

Liquid and solid phases can coexist in equilibrium over a range of temperatures short of the eutectic point. The equilibrium freezing or melting point is the temperature at which the last tiny ice crystal disappears as a frozen solution is slowly warmed. As the temperature is lowered, the relative composition of solid and liquid changes; at the eutectic point, two solid phases of different composition (ice and precipitated salt) form simultaneously and isothermally. In binary systems such as water and NaCl, the phase transformation takes place over a temperature range rather than at a discrete temperature as in single component systems. On the microscopic scale, films of liquid remain in ice despite bulk temperatures below the freezing point (Dash et al. 2006). Such films are catalysts of crystallization and ease the transformation from solid to liquid. Their thickness is temperature-dependent, with temperature gradients in the ice inducing transport processes.

15.2.2.2 Supercooling and ice nucleation

A solution can remain in the liquid state even when cooled below the melting point, referred to as supercooling. The supercooled state is physically metastable such that highly supercooled solutions will quickly freeze if brought in contact with ice crystals. Organisms

can stabilize a supercooled state by preventing introduction of ice through the cell wall or by preventing intracellular ice nucleation (Zachariassen and Kristiansen 2000).

Ice crystal growth takes place from a nucleus, a process called ice nucleation; growth occurs at the temperature of nucleation. Homogeneous nucleation occurs when clusters of water molecules reach sufficient size to form a nucleation site that induces ice growth. The size of a water cluster is temperature-dependent; for example, 45,000 molecules at −5°C but only 70 molecules at −40°C (Vali 1995). This phenomenon determines the minimal spatial constraints for homogeneous nucleation: the smaller a cell or the denser its internal packing, the less likely homogeneous nucleation will occur intracellularly (Bigg 1953).

Heterogeneous nucleation occurs when water molecules are catalyzed by another substance (ice nucleator or nucleation agent) to form a nucleus. Hence, ice nucleation can occur at a warmer than expected temperature. Once ice crystals exist, the crystals themselves function as ice nucleators, promoting further ice growth by what is known as secondary ice nucleation. When larger ice crystals grow at the expense of smaller ones, the process is known as ice recrystallization. Some microorganisms are known for their ability, via specific proteins, to interfere with the physical processes of ice formation and recrystallization (Wowk and Fahy 2002; Raymond and Knight 2003; Kristiansen and Zachariassen 2005).

15.2.2.3 Ion, solute and particle rejection

During freezing, most molecules and particles, including bacteria (Junge et al. 2001), do not incorporate into the crystal matrix of ice but instead accumulate in the remaining liquid phase. Assuming equilibrium between ice and water in the freeze-concentrated solution, the concentration of salt in the remaining liquid can be estimated (Cox and Weeks 1975). At thermodynamic equilibrium, the solute concentration determines the bulk liquid fraction such that an increase in salt (or polymer) concentration increases the volume fraction. The total mass of solutes in the fluid phase determines the volumetric fraction of liquid in the ice matrix, and thus its inhabitable space.

The ratios of various solutes to each other in the parent solution determine the ratios in the remaining liquid phase after freezing (Assur 1958). For organisms, the absolute concentration is critical: at any given temperature, higher amounts of cell-compatible solutes like sugars dilute cell inhibitors. As the temperature drops, however, salts begin to precipitate out of solution, changing the ionic composition of the remaining liquid (Assur 1958). These constraints are altered if exopolymers are present as hydrogels (Fig. 15.1) underscoring the importance of studying microbial processes under relevant in situ conditions.

15.2.2.4 Dehydration

Bacteria live in equilibrium with external water potential (Wolfe and Bryant 1999), as do most protists. In environments of low water vapor pressure or high osmotic pressure, such as brine or sugar solutions, cells will dehydrate if they cannot

increase intracellular osmotic pressure sufficiently with cell-compatible organic solutes (Wolfe and Bryant 1999). In a freezing solution, ice crystals consisting of largely pure water molecules dehydrate the remaining liquid fraction as they grow, concentrating solutes and presenting an osmotic challenge to the microbes present.

In the absence of a cellular protective strategy, the interplay of physical events between freezing rate and dehydration can lead to structural damage and to solute concentrations that are toxic. As temperature and solute concentrations are not distributed uniformly during the cooling or warming process, the determination and prediction of temperature, solutes and phase changes is critical. Current models of mass, momentum, heat and transport phenomena during solidification (Dash et al. 2006) do not apply, however, if organic solutes are distributed non-uniformly within the ice. Non-uniformity is the case for natural sea ice. In particular, EPS hydrogels display a multitude of complex physical and chemical effects that diverge from expectation based solely on the physics of the bulk phase (Sect. 15.2.3).

15.2.3 Influence of exopolymers on small-scale processes

Exopolymers are known to alter or interfere with every small-scale physical–chemical factor described in the previous section. Their abilities to influence diffusive and advective processes, the freezing point of a solution, primary and secondary ice nucleation, and even the transition to a glassy state, as discussed below, make them powerful agents in defining the habitability of an icy environment.

15.2.3.1 Diffusion

Diffusion is a key factor for ice-crystal growth in systems of two or more phases. In sea ice, crystal growth depends on the diffusion of water towards the ice crystal and the diffusion of solutes (salts) away from the growing crystal interface (Fig. 15.2A). Solutes will not always equilibrate between phases, such that the composition of the phases in a sample may depend on the history of its preparation. Adding exopolymers to the mix can only complicate, largely via impedance (Fig. 15.2B), the diffusive patterns and rates within a freezing solution.

From the biofilm literature comes the general assumption that biofilms are porous, with pore diameters of 50–200 Å (Stoodley et al. 1997). As a consequence, hydrogels are understood to form semi-permeable membranes particularly to larger molecules. In some gels, the diffusion of small molecules (e.g., <400 Da in 1% w/w agarose gel) is almost identical in value to diffusion in water. The rate of diffusional transport in a gel is affected not only by pore size but also by the swelling character of the gel and the chemical interactions that occur between macromolecules of the gel and smaller molecules. These interactions change with the water content of the gel, which regulates the dimensions of free water within it (Sjöberg et al. 1999).

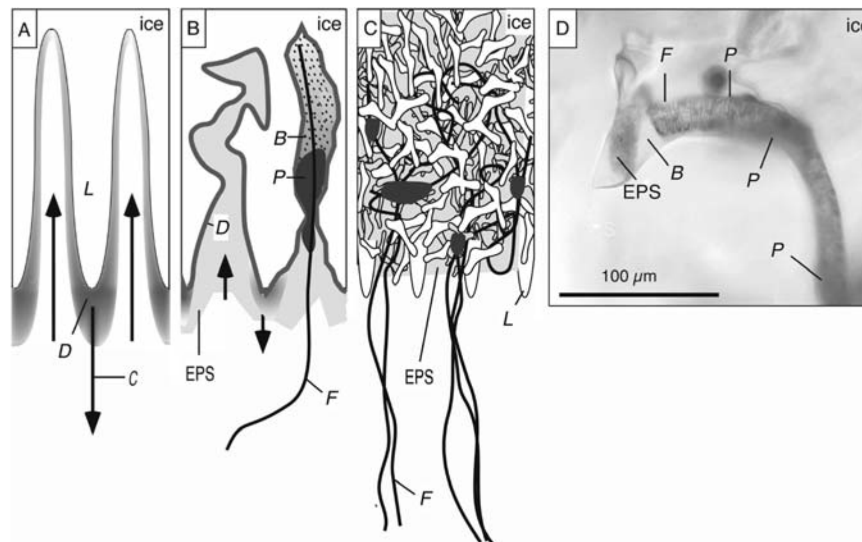

Fig. 15.2 Schematic depiction of EPS-free (**A**) and EPS-sculpted (**B,C**) sea-ice habitats and a microphotograph of stained EPS and diatom filament in sea ice at −20°C (**D**). Compare diffusional boundary layer (*D*, *dark gray areas*), brine (*B*) convection (*C*, *vertical black arrows*) and developing pore morphology (simple versus angular) in the skeletal layer (*L*) of EPS-free ice with the scenario in the presence of EPS (*light gray areas*). An EPS plug (*P*) further reduces solute diffusion and mechanically anchors a diatom filament (*F*) within increasingly angular pore space (**B**), leading to a fully sculpted ice-algal habitat (**C**). (Based on Krembs et al. 2000, 2002, 2003, unpublished data)

The osmotic effect of low molecular weight solutes within hydrogels is to increase the hydration state of the gel. A microbe in sea ice may benefit from adjustments to the composition (large versus small molecules) of their EPS buffers (Fig. 15.1B).

The chemical interactions that influence molecular transport through a gel are influenced by coulombic forces, hydrophobic interactions (Sjöberg et al. 1999), and polymer characteristics such as flexibility and charge density (Hart et al. 2001). The nature of the solvent, its pH and ionic strength are also key determinants. The self-diffusion coefficient of water in polysaccharide gels, which reflects the ability of a gel to transport water, is directly related to the degree of bound water in the gel. The reduction of acetyl groups and uronic acid in an exopolymer can reduce this self-diffusion coefficient (Hart et al. 1999).

The predominantly acidic nature of EPS under most environmental conditions imparts to exopolymers a low pK_a and ability to bind cations, selectively affecting the diffusion of anions (Hart et al. 2001). To diffuse through the pores of exopolymer networks, ions must pass through a gauntlet of hydrophilic negatively charged substituents of the polymers that orient on the channel walls where they can interact with the ions. Significant restriction of anion diffusion has been demonstrated experimentally (50–80% in gels of 3% xanthan gum), with increased polysaccharide concentration and gel thickness exacerbating the effect (Hart et al. 2001). Our

depictions of EPS gels providing semi-impermeable barriers between a microbe and the sea-ice brine it inhabits (Fig. 15.1B) and impeding salt diffusion during sea-ice formation (Fig. 15.2B) embrace these concepts. The impedance of salt diffusion also helps to explain the fractal nature of pores in EPS-rich sea ice (Fig. 15.2B) that we have observed (Fig. 15.2D; Krembs et al., unpublished).

15.2.3.2 Advection

On a larger scale, accumulations of exopolymers are known to restrict fluid flow through various porous structures, influencing both hydraulic conductivity and flow-dependent biotransformation of trace organic groundwater contaminants. In soils, the "plugging" capacity of EPS-producing bacteria has been shown to reduce porosity by 50–96% and hydraulic conductivity by 98% (Cunningham 1993; Kim 2004). In sea ice, where diatoms are responsible for the bulk of EPS present in the colonized zones (Krembs and Engel 2001; Krembs et al. 2002; Meiners et al. 2004; Riedel et al. 2007), we have projected their ability to clog pores (Fig. 15.2B) and resist being flushed from the ice (Fig. 15.2C, D).

Because the pore space of natural biofilms and gels is unlikely to be distributed homogeneously, EPS will not likely clog pores in a homogeneous manner. Heterogeneity in pore clogging facilitates the algal anchoring that we have depicted and observed (Fig. 15.3C, D), at the same time allowing for some advective nutrient exchange to continue. Locally advective fluxes in gel-laden aquatic aggregates are known to increase bacterial nutrient uptake by 60% (Logan and Hunt 1987). By analogy, the activities of bacteria observed in high numbers in large gel aggregates from sea ice may also benefit from advection (Meiners et al. 2004).

15.2.3.3 Solute segregation and selectivity

Where incompatibilities exist between solutes, the freeze-concentration process can force the formation of multiple (non-ice) phases (Heller et al. 1996). The freeze-concentrated solutes are miscible or immiscible depending on their mutual solubility. Of relevance to sea-ice EPS is that phase separation behavior generating two amorphous phases has been reported in solutions containing proteins and polysaccharides (Goff et al. 1999; Izutsu and Kojima 2000).

The freezing process can also force some combinations of solutes with steric hindrance or repulsive interactions to separate into different phases among the ice crystals (Izutsu and Kojima 2000). This freeze-induced phase separation can occur even with initially dilute solutions. Large effects on polymer miscibility are achieved with high salt concentration and low molecular weight co-solutes (Izutsu and Kojima 2000). In the case of very cold sea ice, the precipitation of ions as salt crystals could be extended to a colder temperature by the presence of EPS, retaining ionic balance in the liquid phase.

Just as high salt can affect polymer miscibility, ion composition and the selective exclusion that occurs during freezing can affect polysaccharide charge. Polymer matrices that develop highly charged zones can influence incoming macromolecules and ions. Some hydrogels highly sensitive to electrochemical gradients or pH swell or contract in response to changing values (Hirokawa et al. 1984). The presence of certain substituent groups in bacterial exopolysaccharides affects the interaction with cations, especially calcium and magnesium ions (Geddie and Sutherland 1993). Clear links between the amount of acetyl groups in a polysaccharide matrix and its ability to absorb water or permit anion diffusion have been reported (Hart et al. 2001) and might be important in regulating ion access to aggregates and cells in sea ice.

15.2.3.4 Cryogelation

In general, the addition of solutes decreases the probability of random ice nucleation and crystal growth in a freezing solution (see schematic in Wolfe and Bryant 1999), effectively decreasing the freezing point. To understand how gel-forming polysaccharides function in this way requires an appreciation of the gel hydration state and thus the structural states of water within polymer gels at subfreezing temperatures. Current conceptual models of water in polysaccharides converge on the existence of three different structural states of water in freezing solutions (Wolfe and Bryant 1999; Hayashi et al. 2002), which we call free, freeze-bound, and non-freezing water.

Free water is not influenced by polysaccharide-electrolyte molecules, such that its physical properties are the same as those of bulk water. Freeze-bound water is only weakly bound and consequently can crystallize, but the melting temperature is lower than that of the bulk water. Critical to the behavior of gels is the component of non-freezing water surrounding the macromolecules that form them. This non-freezing water is tightly bound in a polysaccharide-electrolyte system and therefore cannot crystallize.

Behavior among polysaccharide gels in ice is variable; for example, neither xanthan gum nor hyaluronic acid readily form hydrogels by usual gelation (Takahashi et al. 2000). Xanthan gum, one of the best-studied exopolysaccharides, undergoes co-operative conformational transitions when temperature is decreased or salt is added; furthermore, much stronger and more cohesive networks are formed when solutions of xanthan gum are frozen and thawed repetitively (Giannouli and Morris 2003). The use of higher xanthan gum concentrations alone does not promote cryogelation, making cryogelation a unique response to freeze-thawing. Knowing these traits can make xanthan gum a useful model exopolymer in studies of seasonally freezing seawater solutions.

15.2.3.5 Secondary ice nucleation and recrystallization

In the food industry, finding means to prevent growth of new or larger crystals from existing ones is desirable. Various polymers, particularly locus bean gum and guar

gum, are used extensively in the production of ice creams to help preserve the initial distribution of ice crystals (reduce secondary nucleation and recrystallization) during fluctuating temperatures (Goff et al. 1999). Prominent in the ice cream literature are the affects of gels on recrystallization (Regand and Goff 2003). Hydrocolloids added as stabilizers give resistance to melting and strongly affect the rate of recrystallization (Regand and Goff 2003). Some gums have been observed to form cryo-gels or entangled networks of polymers around ice crystals that become increasingly firm during repeated freeze-thaw cycles or that interfere mechanically with crystal growth (Goff et al. 1999). If such networks occur in natural sea ice, they may offer a form of cryoprotection to inhabitants, as well as a mechanism for ice-anchoring (Fig. 15.2C, D).

The mechanisms by which polymers modify the ice-solute interface in ice creams are similar to those discussed from the (unfrozen) biofilm literature, particularly with regards to diffusion. Polymer additives for ice cream can impede the diffusive flux of water to the surface of a growing ice crystal or of solutes and macromolecules away from the surface of growing ice crystals (Goff et al. 1993). They may also affect advective processes differently than molecular diffusion (Miller-Livney and Hartel 1997) or modify the ice-solution interface in other ways; for example, some polymers may adsorb directly to the surface of an ice crystal (Sutton et al. 1996), an issue of relevance to microbial retention in sea ice.

15.2.3.6 Glass transition

Vitrification is the process whereby water converts to a solid, also known as glass transition. In biological systems, the transition to a glassy or vitreous state can occur at ambient or subzero temperatures (Bryers and Drummond 1998). Important to this chapter is that vitrification occurs in the absence of deleterious ice-crystal formation (Matias et al. 2003).

In many single-component systems (such as water), the rate of cooling must be extremely high ($>10^7$ K s^{-1}) to achieve vitrification, something which does not typically occur in nature. In systems with two or more components, however, vitrification is easier to achieve (Duddu et al. 1997). The addition of solutes decreases the probability of ice nucleation and growth for two reasons. First, solutes increase viscosity, which causes motion and reorientation of water molecules into the ice structure to take longer. Second, an ice nucleus can only form if, at a particular time, a volume greater than or equal to the critical volume is free of solute molecules; at high solute concentrations, achieving solute-free critical volumes is unlikely. In the case of polymers, concentrations as low as 0.5–2%, do not appear to affect phase transitions significantly (Regand and Goff 2003). However, polymer concentrations can reach 75% by weight during freeze-concentration and then transition to glass (Streefland et al. 1998).

Inherent to the glass state is a reduction in physical and chemical processes by more than ten orders of magnitude over the aqueous state. Initial studies had suggested that vitrification would chemically stabilize molecules, but recent work has shown that some proteins can still undergo chemical deterioration at very reduced

rates (Streefland et al. 1998), while other proteins may remain catalytic (Junge et al. 2006). Water-soluble glasses of sucrose have proven to be better at molecular stabilization that those of the significantly larger polymer dextran (Streefland et al. 1998), suggesting that high molecular weight polymers retain molecular flexibility to lower temperatures. Because the glass transition temperatures of mixtures of polymers are determined by the weight ratios of their components in solution, polymers with high transition temperatures increase the glass transition temperatures of low molecular weight carbohydrates (Bakaltcheva et al. 2000). Given that polymer mixtures characterize sea ice, vitrification may be achievable in very cold winter ice (Krembs et al. 2002).

15.3 Conclusions

In focusing on small-scale processes and the ways in which EPS can influence them, we have attempted to capture the complexity of cold environments experiencing phase changes and the challenges faced by microbes inhabiting them. At the same time, it should be obvious that the production of EPS, precisely because they can alter the physical-chemical processes within a multi-phase system, represents a critical survival strategy for microbes in frozen systems. Not surprisingly, the whole genome sequence of a cold-adapted bacterium known from sea ice reveals a preponderance of genes related to production and export of widely diverse polymeric substances (Methé et al. 2005).

Perhaps the most unique aspect of exopolymer production as a cold-adaptive strategy is its extracellularity. Unlike intracellular adjustments to various forms of stress, exopolymers are released by microbes and often under circumstances when the return benefit is not immediate or even clear (Vetter et al. 1998). An important implication is that even organisms unable to produce exopolymers can benefit from pre-existing EPS in the parent solution that the freezing process then concentrates for them. The combination of active EPS producers and passive beneficiaries suggests the potential for consortial arrangements and activities in ice that can help to explain an observed diversity of metabolic processes. For example, the co-occurrence of anaerobic N_2 production and photosynthesizing oxygen-producing algae in Arctic sea ice, which should be mutually exclusive microbial activities, can be explained by EPS creating heterogeneous microzones that allow for both processes (Rysgaard and Glud 2004).

Central to the microscale scenario within a cold, naturally formed, multi-phase system are the ever-present microbes, which can be viewed as heterogeneous reaction sites embedded in hydrogels of EPS. These hydrogels must accommodate the transport of all substrates, essential nutrients, chemical signals and cellular byproducts between the surrounding fluids and organismal reaction sites. In ice, the temperature and the original chemical composition of the parent solution will drive the multiphase dynamics of the system, but the microbes, in controlling the amount and composition of EPS produced in situ, have the possibility to continuously modify their surroundings to favorable advantage. A summary of the features and processes

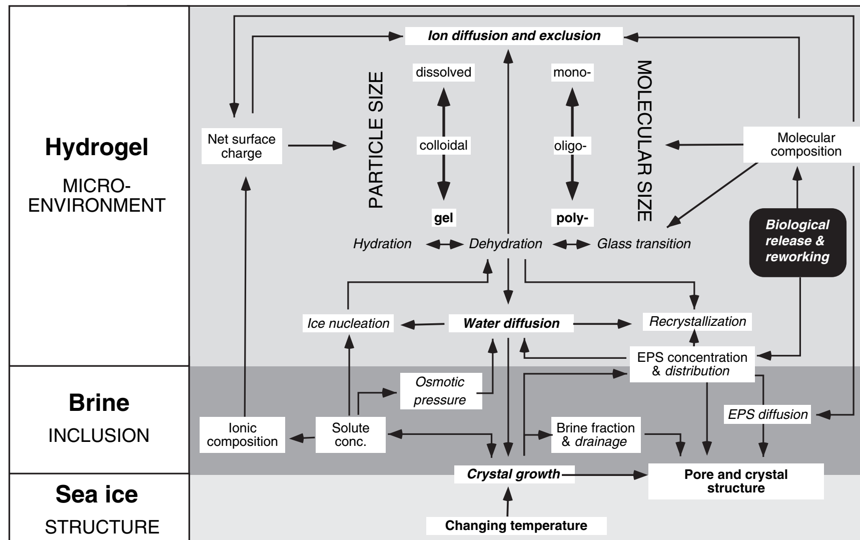

Fig. 15.3 Overview of key features and processes (*in italics*) occurring between ice, brine, and the hydrogel or EPS buffer zone (Fig. 15.1) around an active cell (not shown but *see rounded black rectangle*) as a result of changing temperature. Biology provides the complexity of EPS but temperature determines ion and water fluxes in the hydrogel, thus regulating permeability and cell exposure to (or protection from) brine and ice crystals

addressed in this chapter are brought together in a flow diagram (Fig. 15.3) where we view hydrogels as dynamic and semi-permeable gates that both protect microbes and regulate their exchanges with the brine phase of their cold environments. Future research on EPS and cold adaptation can profit from careful consideration of how these semi-permeable gates may work, in models, in laboratory experiments and in natural settings.

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Chapter 16
Genomic Analysis of Psychrophilic Prokaryotes

John P. Bowman

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16.1 Introduction

Psychrophilic bacteria and archaea are those adapted to low temperature (Morita 1975). The specificity of the adaptation falls at one extreme of a universal growth rate/temperature relationship that largely follows Arrhenius-type kinetics (Fig. 16.1). This relationship appears to be mainly dictated by the Laws of Thermodynamics that involve the way proteins (and other biological macromolecules) behave under different temperatures (Ratkowsky et al. 2005). Psychrophily is a generalized concept and essentially for the purpose of this review incorporates microorganisms that have successfully adapted to and survive well under conditions of perpetually low temperature. Many examples that fall within this classification manifest growth rate/growth temperature features that are considered "extreme" adaptations. These "true" psychrophiles are typified by an inability to grow at temperatures of 20–25°C and may require high hydrostatic pressure for growth.

Cold adaptation involves alteration of various specific features of the cellular biochemistry, in particular enzyme functionality and stability and cell membrane lipid homeoviscosity. These features are the major hallmarks of psychrophily, however,

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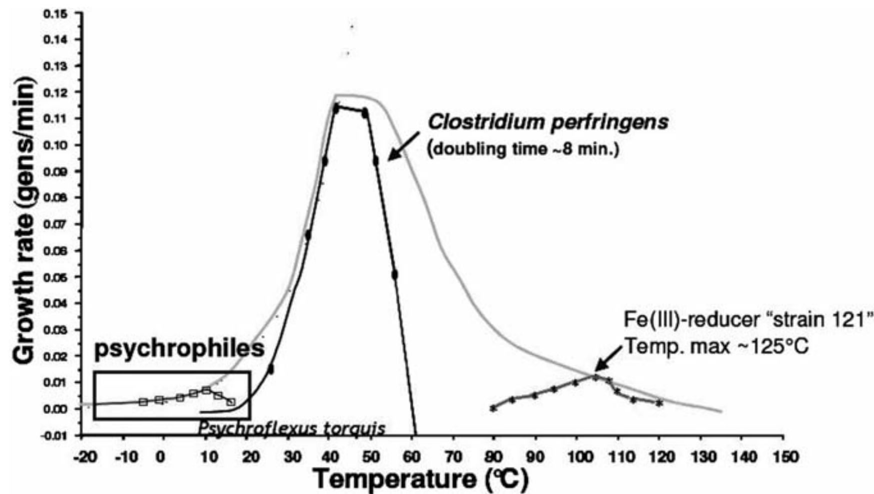

Fig. 16.1 Compilation plot showing the relationship between temperature and growth rates (the "Eppley Curve") of prokaryotes

and have been shown to be largely specific from organism to organism. Essentially cold adaptation traits do not follow set patterns and perhaps are currently in a state of evolutionary flux. Perhaps more simply the traits possessed by any given microorganism are molded closely by the environment(s) in which they exist.

Genomic science has grown tremendously since about the late 1990s, galvanized by the improvements in sequencing and sequence assembly technologies and software springing from large scale human and other eukaryote genome sequencing projects (Venter et al. 2001) and large scale metagenomic projects (Venter et al. 2004). Currently, more than 1,000 genome sequences are either complete, at draft stage (usually a shot gun clone library) or in progress (Genome Projects, National Collection of Biotechnology Information; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj). In addition, more than 30 environmental sample sequencing projects are also at various stages of completion. Amongst this veritable blizzard of data, cold-adapted bacteria and archaea are represented. A current summary of completed and draft stage species-level genomic projects is shown in Table 16. 1. Data indicated for draft genomes must be considered only as estimates. In regard to metagenomic projects, some deal with microbial communities of samples collected from permanently cold marine locations, including, submerged whale carcasses, including one off the Western Antarctic Peninsula collected from a depth of 560 m (Tringe et al. 2005), deep oceanic water masses (Sogin et al. 2006), and Arctic Ocean viromes (Angly et al. 2006).

Genomic data provides essential basic information of the genetic features of organisms. Based on gene annotation data, various levels of insight can be gleaned including cellar metabolism and functionality of a given microorganism or when the data is pooled to include broader groups. Amino acid sequences can be used to construct theoretical protein three-dimensional models to examine more closely protein

Table 16.1 Genomic data for cold-adapted prokaryotes

Species/strain	Status	Size (Mbp)	No. of rRNA operons	Protein coding genes	Coding density (%)	Physiology / ecotype	Source	References
Colwellia psychrerythraea 34-H	Complete	5.37	9	4,910	84	Facultative anaerobe; chemoheterotroph	Arctic marine sediment	Methe et al. (2005)
Desulfotalea psychrophila LSv54[T]	Complete	3.52[a]	7	3,116	85	Anaerobe; sulfate reducer	Marine sediment, Svarlbard	Rabus et al. (2004)
Methanococcoides burtonii DSM 6242[T]	Complete	2.58	3	2,273	80	Anaerobe; methanogenic	Ace Lake, Vestfold Hills, Eastern Antarctica	Saunders et al. (2003)
Pseudoalteromonas haloplanktis TAC125	Complete	3.21[c]	9	3,488	88	Aerobe; chemoheterotroph	Antarctic sea-water	Medigue et al. (2005)
Psychromonas ingrahamii 37[T]	Complete	4.56	10	3,545	77	Facultative anaerobe; chemoheterotroph	Under-ice sea-water, Alaska	Auman et al. (2006)
Photobacterium profundum SS9	Complete	4.09[ab] 2.24	14 1	3,416 2,008	82 80	Piezophile; facultative anaerobe; chemoheterotroph	Amphipod, Sulu Trench (2,551 m depth)	Campanaro et al. (2005); Vezzi et al. (2005)

(continuted)

Table 16.1 (continued)

Species/strain	Status	Size (Mbp)	No. of rRNA operons	Protein coding genes	Coding density (%)	Physiology / ecotype	Source	References
Psychrobacter arcticus 273–4[T]	Complete	2.65	4	2,120	80	Aerobe; chemo-heterotroph	Tundra permafrost, Siberia	Bakermans et al. (2006)
Psychrobacter cryohalolentis K5[T]	Complete	3.06[a]	4	2,467	83	Aerobe; chemo-heterotroph	Cryopeg, Siberia	Bakermans et al. (2006)
Shewanella frigidimarina NCIMB 400	Complete	4.85	9	4,029	84	Facultative anaerobe; chemo-heterotroph	North Sea, off Aberdeen, UK	US DOE Joint Genome Institute
Exiguobacterium sibericum 255-15[T]	Draft	3.03		3,050	87	Aerobe; chemo-heterotroph	Tundra permafrost, Siberia	Rodrigues et al. (2006)
marine actino-bacterium PHSC20C1	Draft	2.77	≥2	2,668	92	Aerobe; chemo-heterotroph	Seawater, Antarctic	Moore Foundation/ Venter Institute
Photobacterium profundum 3TCK	Draft	6.1	~15	5,549	84	Piezophile; facultative anaerobe; chemohetero-troph	Marine sediment, San Francisco Bay, USA	Companaro et al. (2005)
Polaribacter irgensii 23-P[T]	Draft	2.75	~4	2,557	86	Aerobe; chemo-heterotroph	Antarctic sea ice	Moore Foundation/ Venter Institute
Psychroflexus torquis ATCC700755[T]	Draft	4.31	3	4,026	82	Aerobic chemo-heterotroph	Antarctic sea-water	Moore Foundation/ Venter Institute

(continued)

Table 16.1 (continued)

Psychromonas sp. CNPT3	Draft	2.95	11	2,727	85	Piezophile; facultative anaerobe; chemoheterotroph	North Pacific Ocean (5800 m depth)	Moore Foundation/ Venter Institute
Moritella sp. PE36	Draft	5.16	~29	4,695	86	Piezophile; facultative anaerobe; chemoheterotroph	Pacific Ocean (3,800 m depth)	Moore Foundation/ Venter Institute
Shewanella benthica KT99	Draft	4.33	~20	4,235	82	Piezophile; facultative anaerobe; chemoheterotroph	Tonga-Kermadec Trench (9,000 m depth)	Moore Foundation/ Venter Institute
Methanogenium frigidum	Draft	~2.2		~1,800		Methanogen	Antarctic saline lakes	Saunders et. al. (2003)
Carnobacterium sp. AT7	Draft	2.44	~27	2,388	82	Piezophile; facultative anaerobe; chemoheterotroph	Aleutian Trench (2,500 m depth)	Moore Foundation/ Venter Institute

[a] Plasmids present. *Desulfotalea psychrophila* LSv54^T (large plasmid: 121.6 kb; small plasmid: 14.7 kb); *Photobacterium profundum* SS9^T(pPBPR1: 80.0 kb); *Psychrobacter cryohalolentis* K5^T (plasmid 1: 41.2 kb)

[b] The genome of *Photobacterium profundum* SS9^T consists of 2 chromosomes

[c] The genome of *Pseudoalteromonas haloplanktis* includes 2 chromosomes

functionality. The analysis of gene and derived protein sequence data can be readily used to derive evolutionary relationships and to establish molecular chronologies.

16.2 Genome features and cold temperature adaptations

The main hallmarks of cold adaptation can be defined by alterations or possession of specific cellular features. In this review, the following are discussed in relation to genomic data: protein amino acid composition and related structural features; membrane fluidity; compatible solutes; extracellular compounds; ice-active proteins; and nutrient and energy reserves. Genetic features found in the genomes of cold-adapted microorganisms have been linked to one or more of the listed features, potentially having a role to cold adaptation. However, it must be noted that some of these still remain unexplored in any detail at the present time.

16.2.1 Protein amino acid composition and link with evolution

Low temperature growth is mainly limited by loss of enzymatic functionality. Globular proteins possess a temperature range that has distinct limits (the “normal physiological range”), beyond those limits catalytic function declines as the protein structure undergoes denaturation and active site/substrate interactions become increasingly inefficient. Cold-adapted protein structures incorporate degrees of increased flexibility that occur in specific locations throughout the molecule (Hoyoux et al. 2004; Marx et al. 2007). This usually leads to enhanced thermolability of the protein structure. In a sense these adaptations represent a struggle against the thermodynamic force of entropy where at sufficiently low temperatures proteins unfold exposing internal non-polar groups to water (Privalov 1990).

Using whole genome analysis a number of studies have investigated general trends in various microorganisms in regards to amino acid composition. Certain trends can be readily correlated across the entire temperature biokinetic range (Tekaia et al. 2002). When only prokaryotes are examined the strongest negative correlations between amino acid composition and optimal growth temperature (OGT) are found with amino acids Gln, His, Ser and Thr, while a strong positive correlation between amino acid composition and OGT can be found with Gly, Glu, Pro, Arg and Val (Fig. 16.2). This suggests that polar and charged amino acid interactions and locations in protein tertiary structures are clearly influential for overall temperature stability. More extensive and specific protein modeling studies of *Colwellia psychrerythraea* 34H and *Methanogenium frigidum* based on genome data revealed polar and charged amino acids Ser, His, Gln and Thr in particular negatively correlate with OGT in both protein primary structure and in amino acids of protein surface regions (Saunders et al. 2003; Methe et al. 2005). Correspondence analysis of proteome amino acid composition data segregates species into three distinct lifestyle and phylogenetically-linked groups: eukaryotes, hyperthermophiles/thermophiles and

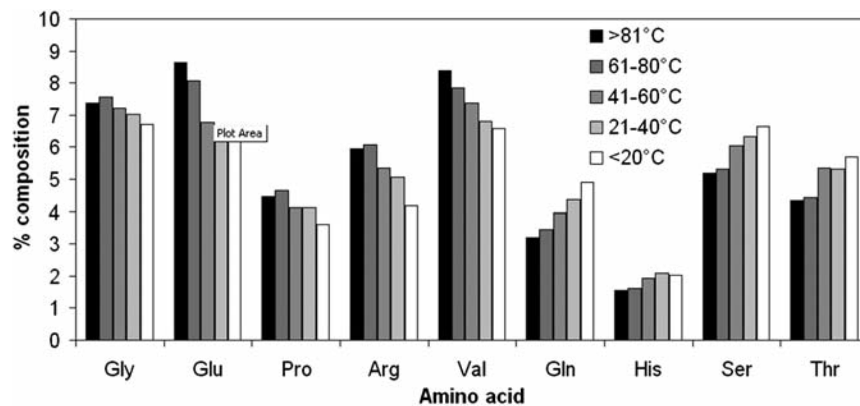

Fig. 16.2 Amino acid distribution across the prokaryote temperature biokinetic range including amino acids showing greatest changes in relation to optimal growth temperature

mesophiles/psychrophiles (Tekaia and Yeramian 2006). The most important amino acids defining these groups were His, Ser and Val, as well as polar and polar-charged amino acid pools. Based on the evolutionary model of Jordan et al. (2005) amino acids are seen as "gainers" and "losers". This is based on amino acid composition of the ancient Murchinson meteorite (Cronin and Pizzarello 1983) and the famous Miller–Urey "Spark" experiment (Miller and Urey 1959). Gainers are amino acids recruited into more recently evolved genomes, while losers are amino acids predominant in ancient phylogenetic lineages. Based on OGT comparisons of amino acid composition it was observed the main gainers were Ser, His and Cys (and Phe and Met to a lesser extent) while the main losers were Pro, Ala, Glu and Gly. Some discrepancies are plain in these studies. Val appears not to be distributed consistently in dataset comparisons since Val was regarded as a "weak gainer" (Jordan et al. 2005). However, Tekaia and Yeramian (2006) considered it to be more an important defining factor. It was observed here that Val has a strong correlation to OGT (Fig. 16.2) indicating it may be important in regards to temperature-related functionality. Ser though formed in the Miller–Urey "Spark" experiment was found to be absent from the Murchinson meteorite. An overall trend in amino acid composition data seems to support the concept of an evolutionary version of an "arrow of time" (discussed at length in Halliwell et al. 1996), where early life associated with high temperatures (Pace 1997) has been succeeded by mesophiles and psychrophiles that have recruited various specific amino acids into the genetic code. Thus, as life has expanded to new econiches across Earth and with the cooling of the Earth's surface, evolutionary trends follow a distinct directional pattern. Schwartzman (1999) summed this up by theorizing that microbial evolution during Earth's prehistory was never stochastic but rather was (and still is) quasi-deterministic constrained mainly by temperature and water availability. Thus, in adapting to extreme econiches the amino acid compositional trends related to psychrophily, e.g., polar/polar-charged amino acids Ser, Thr, Gln and His, have increased presence in proteins, while Glu and other "losers" such as Gly and Pro show declines (Fig. 16.2), essentially reflecting overall trends in evolution on Earth.

16.2.2 Membrane fluidity

Successful adaptation to low temperature entails modification to cell membrane composition in order to maintain a suitable homeoviscosity. Increased membrane viscosity reduces permeability, thus hindering a cell's capacity to absorb and transport nutrients for growth. Under low temperatures this is increasingly problematic due to reduction of enzymatic catalytic efficiency. Thus, it is not surprising that specific adaptations in this particular aspect of the cell are clearly observable. Temperature-dependent membrane modification mainly involves replacement of membrane lipids with those with reduced melting temperatures (Russell and Nichols 1999). This typically manifests as, depending on the species, reduction of fatty acid acyl chain length, increased unsaturation of fatty acid chains and increased shift from iso-branched to anteiso-branching within fatty acids. Many psychrophilic species have been found to produce omega-3 polyunsaturated fatty acids (PUFA) in their cellular membranes. These include eicospentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3) and arachidonic acid (AA, 20:4ω6). PUFA has been considered a classic hallmark feature of psychrophily and piezophily, but with increasing knowledge it has become plain it is definitely not a universal property. Some psychrophilic species do not produce PUFA, but rather improve homeoviscosity by synthesizing mainly monounsaturated fatty acids or in the case of various Gram-positive bacteria, such as *Listeria monocytogenes*, by producing mainly anteiso-branched fatty acids (Zhu et al. 2005). The same applies for cold-adapted archaea, such as *Methanococcoides burtonii*, found to possess unsaturation in archaeol phospholipid side chains. Desaturase related genes appear to be absent from the *M. burtonii* genome and thus unsaturation possibly occurs due to incomplete reduction of a precursor (Nichols et al. 2004).

Genomic studies have revealed important information on the distribution of genes coding polyketide-like PUFA synthetases (Metz et al. 2001). PUFA synthesis in bacteria is coded by a *pfa* gene operon that includes 4 genes (*pfaABCD*) (Fig. 16.3). The arrangements of these genes and position of enzymatic domains (Metz et al. 2001) have been found to be essentially conserved between PUFA-producing gammaproteobacteria, including *Photobacterium profundum* strains SS9, *Colwellia psychrerythraea* 34H, *Moritella marina* MP-1, and *Shewanella* sp SCRC 2378 (Allen and Bartlett 2002). Based on more recent genome data, this was also the case for *Psychromonas ingrahamii* 37^{T}, *Psychromonas* sp. CNPT3 and *Shewanella frigidimarina* NCIMB 400 (Fig. 16.4).

The species *Psychroflexus torquis* $ATCC700755^{T}$, a member of the phylum Bacteroidetes, unusually produces EPA and AA in significant quantities. In this species, low temperature membrane adaptation is almost entirely dictated by modulation of EPA (Nichols et al. 1997). The draft genome sequence for *P. torquis* revealed that it also possesses a *pfa*-like operon that consists of 4 genes. However, it differs in that the *pfaA* gene has been split into two separate ORFs and a *pfaB* homolog is absent (Fig. 16.3). Gene *pfaB* normally includes an acyl CoA:ACP transferase domain, but in the case of *P. torquis* this domain is present in its *pfaC*

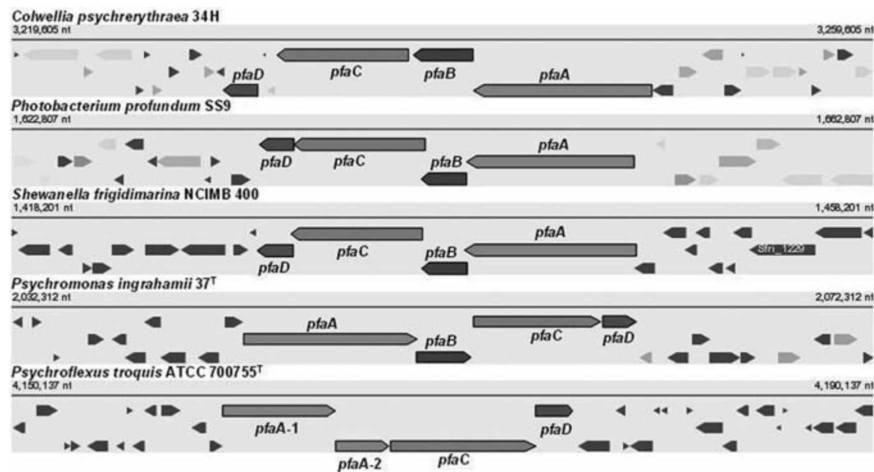

Fig. 16.3 The polyunsaturated fatty acid biosynthesis (*pfa*) operon and neighboring genes of various cold adapted bacteria, highlighting genetic rearrangements of the *pfa* operon from *Psychroflexus torquis* ATCC 700755^T

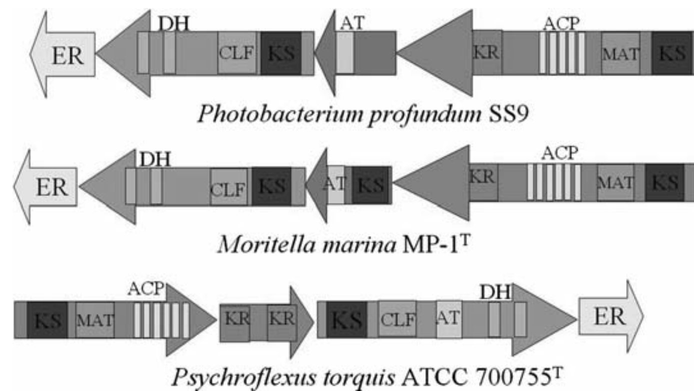

Fig. 16.4 Conserved domains in *pfa* operon genes, highlighting genetic rearrangements of the *pfa* operon from *Psychroflexus torquis* ATCC 700755^T. Abbreviations: *KS* 3-keto-acyl-acyl carrier protein (ACP) synthase; *MAT* ACP S-malonyltransferase; *ACP* acyl carrier protein domain; *KR* 3-keto-acyl-ACP reductase; *AT* acyl CoA: ACP transferase; *CLF* chain length factor; *DH* dehydratase; *ER* enoyl reductase

homolog (Fig. 16.4), suggesting possible genetic rearrangement has occurred within its *pfa* operon. The tendency of *P. torquis* to form AA, possibly owing to incomplete elongation of a precursor fatty acid, may be a consequence of this rearrangement. This is also suggested by the *P. torquis pfa* operon genes being phylogenetically linked to the proteobacterial *pfa* operon genes indicating it likely evolved from the same ancestral source (data not shown). Interestingly, the

P. torquis pfa operon is also surrounded by several transposase or remnant transposase genes. Given that no equivalent genes have been detected in other Bacteroidetes member genomes to date, this suggests a possible acquisition of the pfa operon through lateral gene transfer. Several other PUFA-producing members of the phylum Bacteroidetes have been described, some only recently. None are psychrophiles (and only grow weakly below 10°C, if at all) and all are remote phylogenetically from *P. torquis*. Species of genus *Aureispira* are very rich in arachidonic acid (Hosoya et al. 2006), while the misclassified species *Flexibacter polymorphus* produces EPA (Johns and Perry 1977). It is possible that PUFA may serve other functions beside simply cold adaptation in these microorganisms.

16.2.3 Nutrient and energy reserves

Low temperature limits microbial production as it slows nutrient uptake. The inefficiency of transport mechanisms and subsequent downstream biochemical conversions contributes to slow growth rates. One possible way efficient growth can be achieved under low temperature, especially if the temperature varies, is the possession of intracellular polymers that act as energy reserves. The most widespread carbon and energy reserves in prokaryotes include glycogen (a highly branched polymer of α 1→4-linked- and α 1→6-linked-glucose) (Roach et al. 1998), polyhydroxyalkanoates (PHA, plastic-like linear polyester polymer usually made up of β-hydroxybutyrate or β-hydroxyvalerate units) (Kadouri et al. 2005), polyphosphates (Brown and Komberg 2004), and neutral lipid globules made up of triglycerides and/or wax esters (Waltermann et al. 2007). Surveying genomic data it can be seen that the ability to store nutrient and energy reserves is largely specific at the genus or species level and correlates with overall cellular physiology (Table 16.3). *Colwellia psychrerythraea* 34H and *Photobacterium produndum* SS9 could form PHA [strain SS9 only forms polyhydroxybutyrate (PHB)]. PHB in *P. profundum* was found to act as an osmolyte and also increased in concentration (relative to amino acid compatible solutes) with increasing hydrostatic pressure (Martin et al. 2002). Saccharolytic species could store carbon as glycogen. Species with relatively specialized carbon and energy physiologies, i.e. *Desulfotalea psychrophila* and *Methanococcides burtonii*, do not seem to have the capacity to store carbon. Polyphosphate synthesis seems widespread amongst psychrophiles, as it is among bacteria in general. Low temperature resulted in accumulation of wax ester and fatty alcohol pools in psychrophilic *Psychrobacter urativorans* (previously called "*Micrococcus cryophilus*") (Russell and Volkman 1980). From genome data, a gene putatively identified as a wax ester synthase is present in *P. arcticus* but not *P. cryohalolentis*. The ability to synthesize and breakdown cyanophycin, a protein-like polymer, may serve as a nitrogen reserve in *C. psychrerythraea* 34H (Methe et al. 2005). The genes for cyanophycin breakdown were also observed in the *P. torquis* draft genome but the synthesis genes were not. Cyanophycin-related genes are not well characterized and thus may be unrecognized in many genome annotations.

Table 16.2 Compatible solute uptake and synthesis capacity of different cold adapted prokaryotes based on genomic sequence surveys

	BetT Transporter	ProVWX ABC-transporter	Opu-type ABC-transporter	Proline/ Na^+ symporter (PutP)	Choline conversion to betaine/ glycine betaine (BetAB)	Trehalose synthesis (OtsA)
*Colwellia psychrerythraea 34*H	+	+	−	+	+	−
Photobacterium profundum SS9^T	+	+	+	+	+	−
Shewanella frigidimarina NCIMB 400	+	−	−	+	+	−
Psychromonas ingrahamii 37^T	+	−	−	+	+	−
Pseudoalteromonas haloplanktis TAC125	+	−	−	+	+	−
Psychrobacter arcticus	+	−	−	−	+	−
Psychrobacter cryohalolentis	+	−	−	−	−	+
Desulfotalea psychrophila LSv54^T	−	+	−	−	−	−
Psychroflexus torquis ATCC700755^T	+	−	−	+	+	+
Polaribacter irgensii	+	−	−	+	−	−
Methanococcoides burtonii	−	−	−	−	−	−

+, Gene homologs present; −, Gene homologs absent

16.2.4 Compatible solutes

Cellular osmoprotection and to some extent cryoprotection can be achieved by uptake of polyols, sugars, amino acids, amino acid derivatives, betaine, carnitine and ectoine, and even modified peptides (Galinski 1995), depending on the organism. These compounds can be accumulated within the cytoplasm to high levels without compromising cellular functions and interact with the hydration sphere around macromolecules serving to stabilize ionic interactions in the face of osmotic shifts. From genome surveys, psychrophilic species contain several genes associated with either compatible solute uptake or synthesis of compatible solutes. Psychrophilic marine gammaproteobacteria (Table 16.2) have similar characteris-

Table 16.3 Distribution of carbon and nitrogen reserve capacity based on identifiable gene homologs present in complete and draft genome sequence data of cold adapted bacteria

Species	PHA synthesis	Glycogen synthesis	Polyphosphate synthesis	Wax esters	Cyanophycin-like nitrogen reserve
Colwellia psychrerythraea 34H	+	−	−	−	+
Desulfotalea psychrophila	−	−	+	−	−
Methanococcoides burtonii	−	−	−	−	−
Psychromonas ingrahamii 37^T	−	+	+	−	−
Pseudoalteromonas haloplanktis TAC125	−	−	−	−	−
Shewanella frigidimarina NCIMB 400	−	+	−	−	−
Photobacterium profundum SS9	+	+	+	−	−
Psychrobacter arcticus	−	−	+	+	−
Psychrobacter cryohalolentis	−	−	+	−	−
Psychroflexus torquis ATCC 700755^T	−	+	+	−	−
Polaribacter irgensii	−	+	+	−	−

+, Gene homologs present; −, gene homologs absent

tics in that they have BetT-like choline/betaine/carnitine transporters and the ability to convert choline to betaine/glycine-betaine (BetAB) (as described for *E. coli* by Lamark et al. 1991). Both *Colwellia psychrerythraea* 34H and *Photobacterium profundum* SS9 also have ABC-type transporter systems for uptake of proline/glycine betaine. PutP homologs are also present among most species, coding for Na^+/proline symporters. Though not directly involved in osmoregulation, these may have a role in uptake of proline as a compatible solute as well as for anabolism. *Desulfotalea psychrophila* LSv54^T appears to lack the ability to synthesize betaine, but may take it up using an ABC-type ProVWX transporter for proline/glycine betaine.

Compatible solute uptake appears to be important in *Psychroflexus torquis* ATCC 700755^T as it contains several BetT and PutP type transporters and multiple copies of genes coding enzymes for betaine synthesis. It also possesses genes allowing the putative conversion of serine to betaine via synthesis of lecithin, subsequent hydrolysis of lecithin by phospholipases, and dephosphorylation of phosphocholine (by a *cotS*-like homolog) to choline. *P. torquis* can also putatively accumulate trehalose as an osmo- and cryoprotectant (Elbein et al. 2003) since it possesses *otsA* and *otsB* homologs that allow conversion of glucose to trehalose. The polar seawater species *Polaribacter irgensii* lacks many of these features indicating diversity of these mechanisms amongst marine members of the Bacteroidetes.

16.2.5 Extracellular compounds

The synthesis and secretion of extracellular substances have been recently hypothesized as important mechanisms for survival and growth at extremely low temperatures (down to −20°C). Some extracellular substances may act as cryoprotectants, while others act as anti-freezes modifying ice crystal growth rates. Furthermore, secreted enzymes may be critical in overcoming mass transfer limitations imposed by low temperature on nutrient transport and uptake.

Exopolysaccharides (EPS) have been shown to have cryoprotectant properties in situ (Krembs et al. 2002). It has been shown that bacteria can stimulate EPS levels in response to lowering growth temperature. Sea ice has been noted as being very EPS-rich suggesting microorganisms there copiously produce it leading to accumulation in brine channels. The Antarctic sea-ice bacterium *Pseudoalteromonas* sp. CAM025 increased EPS yields 30-times when grown at 2–10°C compared to 20°C. Uronic acid richness also increased in EPS in CAM025 when grown at low temperature. Other analyses of the EPS from strain CAM025 indicated it was of a high molecular weight and capable of very efficient metal-ion binding. (Nichols 2006). The highly polyanionic nature of this EPS at low temperature suggests it may play a role as a ligand binding various nutrients, in particular iron and other cationic trace metals. EPS may increase the intrinsic viscosity of the region outside of cells in confined areas, such as sea-ice brine channels or within aggregated particulates in the water column (i.e. marine snow) thus altering the physical diffusion properties of the

local cellular environment. This in turn allows potentially for enhancement of substrate concentration around cells and thus overcoming cold-induced limitations of nutrient uptake and metabolism (Pomeroy and Wiebe 2001; Deming 2002). From the genome of *Colwellia psychrerythraea* 34H various genes were identified that may have roles in EPS synthesis. This species was also found to have unusually high number of genes coding extracellular enzymes that may aid the species growth at low temperature (Methe et al. 2005).

On the *Psychroflexus torquis* genome, which copiously produces a complex EPS at low temperature (Bowman et al. 1998; J. P. Bowman, unpublished data), a potential synthetic gene locus is identifiable (Fig. 16.5). This gene locus has various interesting features. The locus is located on a large genomic island flanked by numerous transposable elements, incorporating several genes not found in available genomes of other members of the phylum Bacteroidetes. This suggests the locus may have been assembled together by a series of lateral gene transfer events. The locus is also flanked by genes involved in protein secretion (*secA* homolog, tricorn protease) and a putative autotransporter adhesin suggesting the products from this putative EPS locus may have roles in adhesion and are involved in various extracellular processes. The presence of a *mazEF* toxin/antitoxin module within the locus

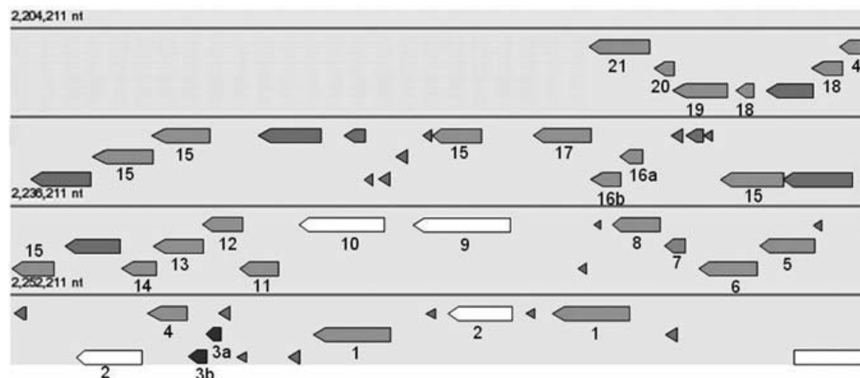

Fig. 16.5 Putative exopolysaccharide gene locus present on the genome of *Psychroflexus torquis* ATCC 700755^{T}. Predicted function of proteins coded by genes. *1* Glucose-6-P dehydrogenase; *2* predicted ATPase; *3a/3b* putative *mazEF* toxin/antitoxin module; **4** GDP-fucose synthetase; *5* UDP-glucuronate epimerase; *6* dTDP-4,6-dehydratase; *7* 23S ribosomal protein; *8* glucose-1-P thymidylyltransferase; *9* uncharacterized ABC-type transporter (fused ATPase/permease components); *10* putative aldolase/synthetase; *11* putative 2-dehydro-3-deoxyglucarate aldolase; *12* CMP-N-acetyl-neuraminate synthetase; *13* UDP-glucose-4-epimerase; *14* predicted phosphatase/hydrolase; *15* glycosyltransferase; *16a, 16b wxcM*-like genes; *17* sugar aminopeptidase – similar to *fdtA*, *fdtC* and *fdtB* of *Aneurinibacillus thermoaerophilus* coding enzymes converting dTDP-6-deoxy-D-xylohex-4-ulose to dTDP-3-acetamido-3,6-dideoxy-D-galactose (Pfoestl et al. 2003); *18* putative nucleotide diphosphate epimerase; *19* mannose-1-P guanylyltransferase; *20* mannose-6-P isomerase; *21* GDP-mannose 3″,5″ epimerase. Genes that are unlabelled code unknown hypothetical proteins

is intriguing but whether these genes have roles (such as programmed cell death) linked with EPS synthesis requires substantial research.

Antifreeze proteins (AFP) are a structurally diverse group of proteins that have the ability to modify ice crystal structure and inhibit recrystallization of ice, usually at temperatures just in the subzero range (Knight et al., 1988). *P. torquis* produced positive results in AFP assays with highest AFP production occurring in stationary growth phase cultures and when grown at −2 and 4°C (J.P. Bowman, unpublished data). In exponential growth phase and at higher growth temperature, the AFP assay results had poorer scores suggesting AFP production is growth phase and temperature dependent. Various bacteria including Antarctic lake bacteria have also been found to produce AFPs (Gilbert et al. 2005). It was considered that the ability to produce AFPs occurs because of cell damage due to formation of ice crystals. *P. torquis* when frozen (below −10°C) in marine broth or on agar plates is inactivated or killed, and even prolonged storage in marine broth (at 4°C) does not lead to cell recovery. Thus, the ability to avoid freezing effects appears to be an important adaptation to the sea-ice ecosystem. *P. torquis* was found to possess a gene coding a large putative membrane-anchored cell surface protein. The protein contains several repeat domains homologous to small AFPs of the snow mold *Typhula ishikariensis* (Hoshino et al. 2003 and unpublished). Most known AFPs have been found to be small polypeptides that are secreted into the external medium. Experiments using cell-free supernatants, not lysates, revealed only very weak AFP activity suggesting that AFP activity for *P. torquis* may be mainly cell wall or membrane associated rather than secreted. Surveys of other psychrophilic genomes did not reveal any genes similar to known AFP-type protein genes. However, this is not surprising given the sequence diversity of these proteins and general lack of knowledge of bacterial AFPs.

16.3 The environment molds the genomic traits of organisms

Prokaryotes are found in practically every location of Earth including locations considered inimical to animal life. There are limits of course as defined in Fig. 16.1; the forces of entropy confine life within certain boundaries. To survive and grow near the limits requires trade-offs usually resulting in reduced growth rate and increasing physiological specialization. The previous sections largely dealt with specific traits from a genomic perspective that provides psychrophiles the means of survival under conditions of permanent low temperature. However, from species to species these traits clearly vary considerably indicating that they are acquired independently through the processes of evolution possibly forced by the prevailing environmental conditions. This process has been hypothesized to potentially occur across a whole community, i.e. community level selection (van Elsas et al. 2003). It can only be assumed that persistent low temperature conditions results in the acquisition of traits relevant to psychrophily and eventually in some cases a

temperature specialization that is loss of the ability to grow at mesophilic temperatures. Perhaps with the constant variability within environments only a few taxa acquire true specialization having to spring from a source where psychrophilic traits are already present. Nevertheless, there seems an indication that psychrophily is widespread amongst prokaryotes and could be a recent evolutionary development (Schwartzman and Lineweaver 2004; Bowman 2005).

Much is mentioned here about the species *Psychroflexus torquis*. The reason why is that recent genomic data reveals that the species possesses a range of interesting traits that highlight its psychrophily but also suggest that it is highly adapted to its home ecosystem, sea ice. As mentioned above, *P. torquis* has an amino acid composition that is similar to other psychrophiles and modulates its cell membranes by increased biosynthesis of EPA coded for by an atypical *pfa* operon (Fig. 16.3). The species also appears to possess the ability to store carbon and energy as glycogen and polyphosphate that may be important when temperature conditions do not allow efficient transport of nutrients or nutrients are not available. The strain also forms a viscous, complex EPS that appears to have complex genetic features (Fig. 16.5). The sea-ice ecosystem is noted for varying considerably in salinity levels, ranging from low salinity due to thaw to extremely highly salinity due to salt extrusion into brine channels (Deming 2002). *P. torquis* possesses 6 *mscL/mscS* genes, coding large and small mechanosensitive channels that have been shown to be important for cell membrane integrity in the face of osmotic shifts (Yoshimura et al. 1999; Edwards et al. 2004). Compatible solute accumulation, including betaine and trehalose (Table 16.2), may allow *P. torquis* to survive when it enters areas of sea ice with high salinity though it is likely it does not grow in such locations. AFP synthesis found to occur for *P. torquis* would also play an important role in protecting the cells from ice crystal damage and may allow attachment to ice crystal surfaces. Recently, it has been found that sea ice contains phage and, owing to sea-ice brine properties, may allow increased bacterial-phage interactivity compared to seawater (Wells and Deming 2006). The phage interactions within in sea ice at low temperature may provide a mechanism for gene acquisition relevant to psychrophilic adaptations though this requires much further testing. *P. torquis* has a genome that is very rich in insertion elements, including transposases and transposase remnants, retroviral integrases and phage-like genes, suggesting a potential influence of sea-ice phages in its evolution. A number of genomic islands on the *P. torquis* genome possess genes integral in its psychrophily including the *pfa* gene cluster (Fig. 16.3) and the EPS locus (Fig. 16.5). Interestingly, the immediate relatives of *P. torquis* occur in different warmer, hypersaline ecosystems (Bowman et al. 1998; Donachie et al. 2004). It is possible that *P. torquis* is a microbe that has entirely evolved in the sea-ice ecosystem. The ancestors of *P. torquis* may have been pelagic, psychrotolerant, halotolerant microbes that acquired traits such as EPA and EPS synthesis and lost other traits including the ability to grow at temperatures above 15°C. These changes could be fueled by genomic rearrangements, loss of genes (appearance of pseudogenes) and the introduction of new genetic material through lateral gene transfer.

16.4 Conclusions

Currently, substantial genomic data has become available that enables improved understanding of the molecular biology, physiology and ecology of prokaryotes adapted to low temperature. Genomic data from individual microorganisms can provide improved understanding of biological adaptations relevant to particular low temperature ecosystems. The example used here included the species *Psychroflexus torquis*, an inhabitant of sea ice. In the end, genomic data operates as a powerful resource, although much empirical research remains to be done to fully interpret this genetic information resource and integrate this with knowledge obtained from ecological and biophysical experimental data.

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Chapter 17
Microalgae in Polar Regions: Linking Functional Genomics and Physiology with Environmental Conditions

Thomas Mock(✉) and David N. Thomas

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17.1 Introduction

Protists inhabiting polar regions have been the subject of intense interest ever since the first explorers ventured into the inhospitable seas of the Arctic and Southern Oceans (Ehrenberg 1841, 1853; Hooker 1847; Sutherland 1852). The first records of microbial biodiversity in extreme environments were made with the most basic of microscopes, and until the mid 1900s (ultimately when scientific programs in polar regions became more common) much of the work on protists remained largely descriptive and restricted to the more robust physiological experiments that could be attempted under unfavorable field conditions. Despite the fact that there have been nearly 170 years of research into algae living in the Arctic and Antarctic, it is only in the last 20 years that there has been a revolution in laboratory facilities available at remote sites, and of course the technological advances that allow collection, extraction and subsequent cultivation of organisms in home laboratories. Coupled to this, we now have sophisticated molecular tools to determine the true extent of this diversity and, in turn, we know the molecular and physiological capabilities

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that permit life to continue at the extremes of low temperature. That is not to belittle the need to still look down the microscopes as works such as Scott and Marchant (2005) eloquently demonstrate.

This review on microalgae is restricted to the Arctic and Antarctic regions, and most of the discussion will concentrate on diatoms and green algae (Fig. 17.1, see color plate) living in sea ice, lake waters and snow, because most physiological and molecular studies have been conducted with species from these two groups. However, there are other seasonally ice-covered sub-polar regions, such as the Baltic Sea and Sea of Okhotsk where much of our understanding about cryogenic adaptations and microbial ecology is being taken forward (see Granskog et al. 2006). Both in polar and sub-polar systems, a huge diversity of microalgal species exists that for the purposes of this review are split into either psychrophiles, organisms with an optimal growth temperature at or below 15°C, and a maximum growth temperature below 20°C, or psychrotrophs, organisms with the ability to grow at temperatures below 15°C, but exhibiting maximum growth rates at temperature optima above 18°C (Deming 2002).

Permanently low temperatures combined with strong seasonality of solar irradiance are the most important environmental factors for evolution and life of polar photosynthetic organisms. Despite this fact, a wide range of phylogenetic groups of algae have successfully adapted to these extreme environmental conditions although the polar regions are a geologically young habitat in the Earth System. The Antarctic continent, with the formation of a permanently cold-water ocean (Southern Ocean), was formed ca. 25 million years ago whereas the Arctic Ocean formed ca. 6 million years ago (Beil and Thiede 1990; Hansom and Gordon 1998; Fogg 1998). However, most of the algal groups are older than 25 million years (Kooistra and Medlin 1996). Thus, the formation of polar environmental conditions was a major radiation event where new species developed that were able to grow under these extreme conditions.

17.2 Environmental conditions

17.2.1 Light

The strong seasonality of solar irradiance is the major factor that influences the availability of light for photoautotrophic organisms at high latitudes. However, snow and ice thickness very much determine how much of the light is able to penetrate to regions where photosynthetic organisms are living (Eicken 1992). It cannot be forgotten that despite high latitude regions being commonly thought of as light limited systems. In fact, during summer periods irradiances on snow and ice surfaces can be extremely high, with high doses of harmful ultraviolet radiation being a commonly reported stress factor (reviewed by Brierley and Thomas 2002). Therefore, a wide range of photoadaption is exhibited and is a prerequisite for photoautotrophic organisms living at high latitudes (Kirst and Wiencke 1995). Snow algae, which are mainly chlorophytes, grow on and within snow and ice surfaces and may therefore

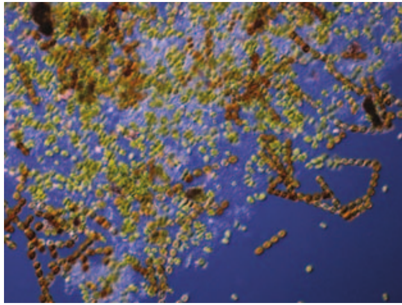

Fig. 17.1 Polar marine microalgal community composed of a green alga (*Chlamydomonas sp.*) and a chain-forming diatom (*Melosira arctica*). Image courtesy by Brian Eddie, Arizona State University, USA

Fig. 17.6 Ice floe (upside down, about 80 cm thick) with dense populations of pennate diatoms at the sea-ice water interface (indicated by brown color that is caused by their main light harvesting pigment fucoxanthin)

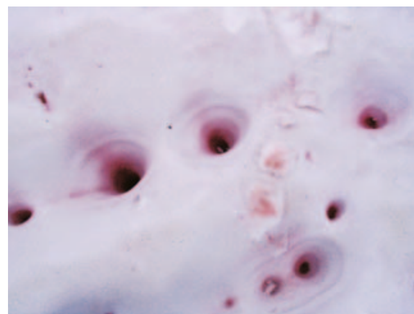

Fig. 17.8 Watermelon snow pits superimposed with an organg bootprint. The coloration is caused by *Chlamydomonas nivalis*. Wikipedia image

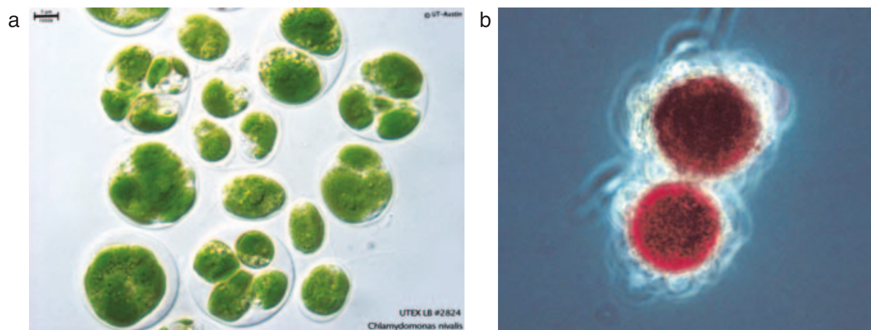

Fig. 17.9 **a** Vegetative stages of *Chlamydomonas nivalis*. Some of the single cells show two flagella. Picture from UTEX Image Bank: (http://www.bio.utexas.edu/research/utex/photogallery/c/Chlamydomonas%20nivalis%20LB%202824.htm); **b** *Chlamydomonas nivalis* aplanospores filled with the red pigment astaxanthin and with attached particles. Image courtesy by Brian Duval and Lynn Rothschild

be exposed to high doses of UV radiation. This is of course true for the microalgal assemblages of glacial lakes, cryoconite holes on glacial systems and seasonally formed melt features, both in terrestrial systems and on the surfaces of pack ice (Hodson et al. 2005; Mindl et al. 2007; Säwström et al. 2002; Vincent et al. 2000).

In contrast, microalgae growing inside or under sea ice as well as in or under the permanent ice covers of the Antarctic dry valley lakes are photosynthetically active in a light environment almost without UV radiation and less than 1% of incident photosynthetically active radiation (PAR). Beyond the aquatic systems, hypoliths—mostly cyanobacteria and chlorophytes—grow on the underside of stones and rocks in periglacial systems, where they utilize irradiances far less than >0.1% of the incident light for photosynthesis (Cockell and Stokes 2004). Far beyond these habitat-specific light irradiance differences, all photoautotrophes in high latitude regions must have the physiological ability to survive several months of darkness.

17.2.2 Seawater

The polar oceans are the major habitat of microalgae in terms of biomass and biodiversity. Most of these cold water masses are characterized by seasonal surface-freezing (Fig. 17.2, see color plate) and strong vertical mixing due to katabatic polar winds, convection at frontal zones or deep-water formation (Tomczak and Godfrey 2003). However, the central Arctic Ocean and the Southern Ocean are not similar regarding their physical and chemical conditions. The Arctic Ocean is a central ocean surrounded by landmasses with a permanent cover of multi-year sea ice around the North Pole. The Southern Ocean surrounds the Antarctic continent with a series of circumpolar fronts and different water masses with distinct physical and chemical characteristics occurring between these fronts (Tomczak and Godfrey 2003).

Most of the sea ice in the Southern Ocean is seasonal with an advance and retreat of 16 million km^2 in sea ice around the continent within one year (Fig. 17.2, see color plate). Multi-year sea ice only occurs close to the continent in inlets or bays or in major ocean gyre systems in the Weddell and Ross Seas (Comiso 2003). Antarctic sea ice has a mean thickness of about 0.55 m. In contrast, the mean thickness of Arctic sea ice is approximately 3 m, due to 50% of the Arctic pack ice being multiyear ice lasting between 2 and 11 years (Dieckmann and Hellmer 2003). The latitudinal influence of sea ice is greater in the Arctic covering a region that extends from 90°N to 44°N, whereas in the Southern Ocean the region is only 75°S to 55°S, although at the maximum extents Arctic ice covers an area of 16 million km^2 and Antarctic sea ice an area of 19 million km^2 (Comiso 2003).

The Southern Ocean has the highest inventory of unused macronutrients in the World Ocean (Fig. 17.3, see color plate) and is the most important province for the export and burial of biogenic silica from diatoms (Smetacek 1998; Smetacek and Nicol 2005). The discovery of high macronutrient concentrations and relatively low phytoplankton concentrations in the Southern Ocean led to the concept of the

"Antarctic Paradox" that was subsequently referred to as high nitrogen–low chlorophyll region (HNLC). Micronutrients such as iron are considered to be the reason for this Antarctic Paradox (iron is a requirement for proteins involved in photosynthetic carbon assimilation), and several international large-scale iron fertilization experiments confirmed this hypothesis (reviewed by Boyd et al. 2007). Thus, the supply of iron to Southern Ocean phytoplankton resulted in marked increases in both carbon fixation and nitrate utilization rates. However, it is assumed that many offshore species do have a lower requirement for iron and are therefore well adapted to these conditions.

In contrast, the Arctic Ocean is relatively rich in micronutrients such as iron because of terrigenous sources of micronutrients, primarily via river runoff, and also dust and sediments deposited in shallow coastal water masses. Thus, macronutrients are more important in limiting phytoplankton biomass in the Arctic Ocean compared to the Southern Ocean. However, the most important factors regulating the large-scale distribution of phytoplankton production and biomass in the Arctic Ocean is probably the surface ice cover and the depth of the surface mixed layer, and thus the availability of light (Sakshaug and Slagstad 1991). The distribution of macronutrients in the Arctic Ocean is very heterogenous between basins resulting in significant regional differences in primary production dynamics (Wheeler et al. 1997; Dittmar and Kattner 2003; Jones et al. 2003).

Beside the dominance of diatoms in both polar oceans, Prymesiophytes such as *Phaeocystis* spp. and *Emiliania* spp. are the second most abundant algal group. They even may form large blooms under more stable conditions and therefore outgrow bloom-forming diatoms (e.g., Smith et al. 1991; Merico et al. 2003). Dinoflagellates, Chlorophytes, Prasinophytes and other algal groups are underrepresented in polar oceans (Kopczynska et al. 1986; Smetacek et al. 2002). However, a recent study by Lovejoy et al. (2006), who used 18S rRNA clone gene libraries, indicated a high diversity of microbial eukaryotes in the Arctic Ocean. This is either indicative of a large number of endemic species or a high number of undersampled taxa. Nevertheless, the dominance of diatoms in polar oceans makes this group ecologically the most important among polar microalgae (Lizotte 2003a, 2003b). Diatoms in general are estimated to contribute to at least 50% of the global marine primary production (Nelson et al. 1995).

Due to the presence of glaciers and permafrost, photosynthetic biomass on land in polar regions is negligible compared to that found in the ocean. Consequently, polar diatoms are of interest not only because of their important role as the main food source for the whole polar food web (terrestrial and aquatic), but also because of their ability to thrive in this extreme ecosystem.

17.2.3 Sea ice

Sea ice being one of the most extreme and largest habitats in polar oceans is important in structuring the whole polar ecosystem (Eicken 1992; Brierley and Thomas 2002;

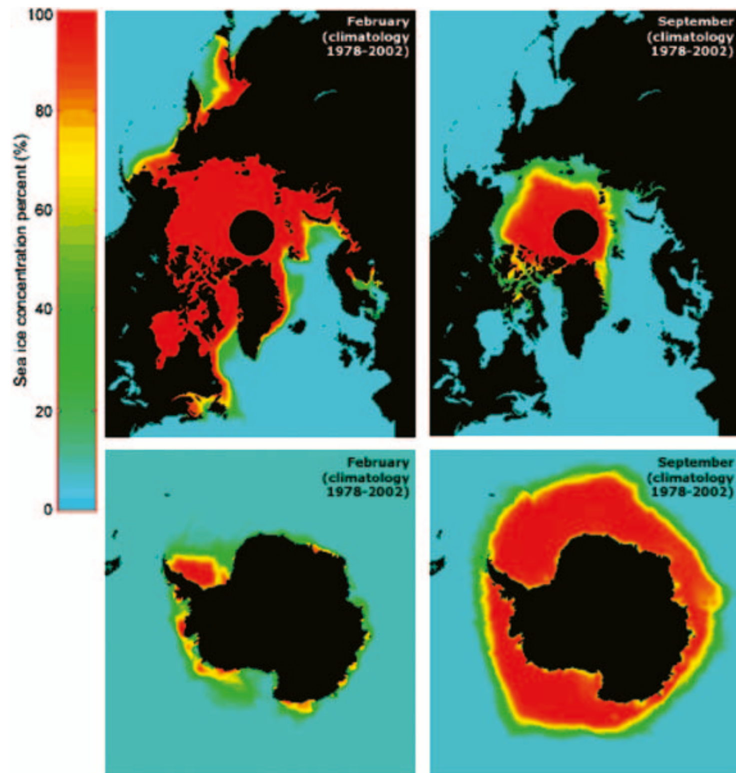

Fig. 17.2 Arctic and Antarctic sea-ice concentration climatology from 1978–2002, at the approximate seasonal maximum and minimum levels. Image provided by National Snow and Ice Data Center, University of Colorado, Boulder, USA

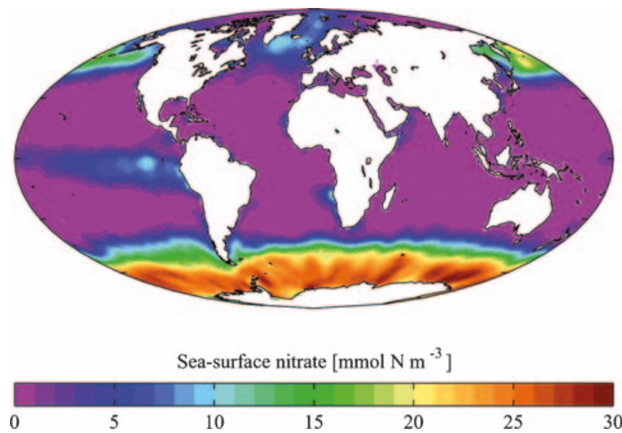

Fig. 17.3 Map of High Nutrient–Low Chlorophyll (HNLC) regions around the world. Measurement in map is of nitrate, with the *scale* as a gradient of color pictured on the bottom (http://www.atmosphere.mpg.de/media/archive/1058.gif)

Arrigo and Thomas 2004). At its maximum, it covers 13% of the Earth's surface (Comiso 2003). The physical characteristics pertinent to the biology living in sea ice have been reviewed by Eicken (2003): Sea ice, in contrast to freshwater ice, is not solid, but is comprised of a system of brine channels (Fig. 17.4) that provide a habitat characterized by low temperature (ca. −2 to −20°C), high salinity (35–200 psu), high pH (up to 11) and low irradiances that can be below 1 µmol photons m^{-2} s^{-1} at the sea ice–water interface (Eicken 1992; Gleitz et al. 1995; Kirst and Wiencke 1995).

Sea water, typically containing about 34 g of dissolved salts and ions (mostly sodium, chloride, sulphate, magnesium, calcium and potassium) per liter, does not begin to freeze until temperatures drop below −1.86°C (28.65°F). At this temperature, ice crystals begin to form and rise to the surface. These initial ice crystals (termed frazil ice) vary in shape, from plates to needles, and size, from ≤ millimeter to a centimeter in length. The crystals consolidate by wind and water motion within hours to form loosely aggregated discs (termed pancakes). After a few days of growth by accumulation of more and more ice crystals that form in the upper water column, pancakes can be several meters across and up to 50 cm thick. They freeze together and after one or two days a closed ice cover has formed (termed pack ice). As temperatures continue to decrease this pack ice thickens, not necessarily by the accumulation of more ice crystals, but by the growth of columnar ice at the ice-water interface. This type of ice is formed by the vertical elongation of frazil ice crystals. The proportion of frazil ice to columnar ice depends largely on the turbulence of the water in which it was formed. The more turbulent the water, the more frazil ice is usually formed. Antarctic sea ice thus contains up to 80% frazil ice as it is formed under more turbulent conditions. In the Arctic, sea ice is formed under more calm conditions containing up to 80% columnar ice (Eicken 2003) (Fig. 17.5, Arctic and Antarctic ice, polarization).

When ice is formed from seawater, salt ions and air in the water cannot be incorporated into the ice crystals and are therefore concentrated as salty brine either into inclusions of pockets and channels (Fig. 17.4) or released into the water below the ice. Thus, sea ice is a solid matrix penetrated by a labyrinth of channels and pores that contain highly concentrated brine and air bubbles (Fig. 17.4). Brine channels vary in size from a few micrometers through several millimeters in diameter and are the main habitat for all microorganisms in sea ice (reviewed by: Brierley and Thomas 2002; Deming 2002; Lizotte 2003a; Mock and Thomas 2005; Mock and Junge 2007). Their volume and the concentration of salt in them is directly proportional to temperature (Fig. 17.5) (Eicken 2003; Weissenberger et al. 1992; Krembs et al. 2000). When temperatures decrease, brine volume decreases and salt content increases. Thus, the colder ice contains brine channels with highly salty brines and overall fewer, smaller and less interconnected channels than warmer ice. Since ice at the sea ice–air interface is usually colder than ice in contact with the underlying water, a temperature gradient exists through the ice, resulting in a gradient in brine salinity and the overall volume of brine in sea ice as well. A host of protists and zooplankton have been recorded from sea ice (Horner 1985; Palmisano and Garrison 1993; Lizotte, 2003a; Schnack-Schiel 2003; Werner 2006), although among the photoautotrophs the most studied are the diatoms. All organisms living

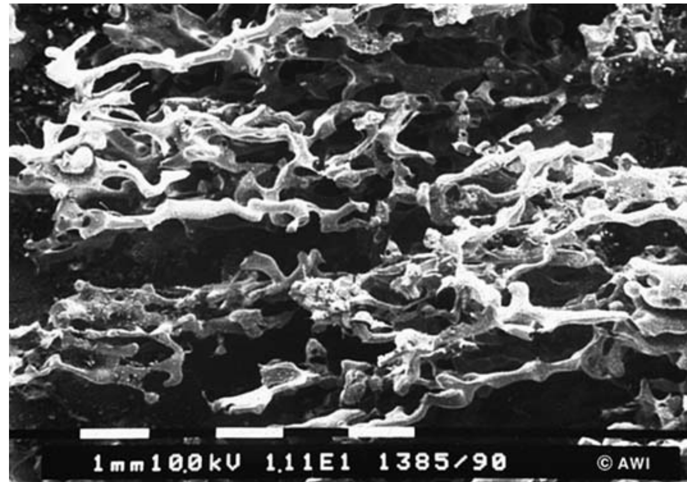

Fig. 17.4 Brine channel system in columnar sea ice made visible by filling the system with epoxy resin under a vacuum. Picture by Alfred-Wegener Institute for Polar and Marine Research, Bremerhaven, Germany, based on the work of J. Weissenberger et al. (1992)

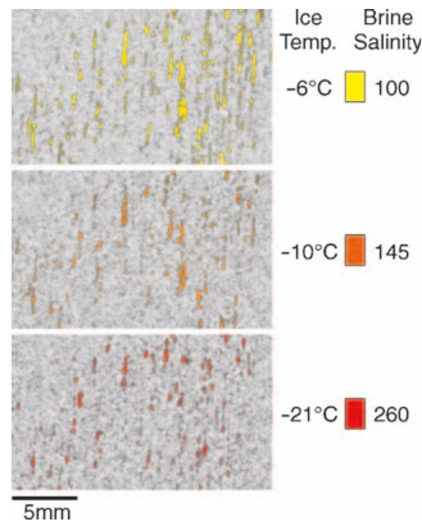

Fig. 17.5 Magnetic resonance images of the same piece of ice shows how the pore space and size of the brine channels and pockets reduces with decreasing temperature, with corresponding increase in salinity of brines contained within the pores. Image after Thomas and Dieckmann (2002) based on work of Eicken et al. (2000)

within the sea-ice matrix have to have plastic physiologies to cope with these ever-changing physical and chemical conditions of their environment, which are dominated by temperature and salinity changes.

Microalgae are mainly introduced into the ice as it is forming. They get caught between ice crystals or simply stick to them as crystals rise through the water when it freezes in fall. During the formation of consolidated ice, diatoms become trapped

within brine channels. Pennate diatoms are the most conspicuous organisms in sea ice along with other microalgae (e.g., dinoflagellates, flagellates), heterotrophic protists (e.g., ciliates) and bacteria (Brierley and Thomas 2002; Thomas and Dieckmann 2002; Lizotte 2003a). These micrometer-sized algae, with their main light harvesting pigment being fucoxanthin, can reach such concentrations in sea ice that they discolor the ice visibly brown (Fig. 17.6, see color plate). The time for acclimation to the new conditions in sea ice is not very long since daylight hours are continually decreasing as winter approaches. Nevertheless, diatoms, especially at the ice–water interface, where conditions are most similar to the water below the ice, are often able to photoacclimate rapidly and can accumulate to high biomass even before the winter begins (Gleitz and Thomas 1993). Sea-ice diatoms are very efficient in using solar irradiance and are able to grow at irradiance levels below 1µmol photons m^{-2} s^{-1} (Mock and Gradinger 1999). Light levels are minimal during polar winters, not only due to short days or complete darkness, but also due to snow cover on top of the ice that is a very efficient reflector of solar irradiance (Eicken 2003).

Sea ice is mostly an ephemeral feature since, after its formation and consolidation, the majority of it melts resulting in the release of all organisms within to the underlying water. Increase in solar irradiance is the most important factor that causes the ice to melt. A common feature and a sign for the beginning of the ice melt is the formation of melt ponds on the surface of the ice (Fig. 17.7). They are more common in the Arctic than Antarctic. One reason for fewer melt ponds in the Antarctic is that more heat is derived from underlying water and melting from above is less significant

Fig. 17.7 Melt ponds on top of Arctic sea ice. This picture is 100m across. (http://www.arcticice.org/close100 m.htm)

(Haas 2003). When melting continues due to increasing water temperatures and solar irradiance on top of the ice, the ice gets thinner and more porous. Large pores and brine channels that are filled with seawater characterize warm ice and the ice itself has very little strength and is easily broken up. However, not all the ice that is formed in fall actually melts during the next summer. If it survives the summer, refreezing occurs during the following winter that makes the ice even thicker. The longevity of the ice depends on the geographical location, on the wind and on ocean currents. Sea ice of northern Greenland and the Canadian archipelago can be up to 15 years old with an average thickness of 6—8 m (Haas 2003). Such differences in physical properties of the ice also result in differences in the abundance, activity and composition of the microbial communities within sea ice.

Despite the high diversity of autotrophs within sea ice, which also include Prasinophytes, autotrophic dinoflagellates and ciliates, two small pennate diatoms, *Fragilariopsis cylindrus* (Grunow) Krieger and *F. curta* (Van Heurck) Hustedt, and the prymnesiophyte *Phaeocystis antarctica* Karsten are the dominant species in blooms in the Antarctic sea ice zone (Leventer 1998; Lizotte 2001). Gleitz et al. (1998) found that at high diatom standing stocks species diversity decreases. This has also been reported by Gleitz and Thomas (1993), who showed that, as first-year sea ice grew and high algal standing stocks established, the assemblages were dominated by only a very few small diatom species. Taking into consideration the findings of other studies, Gleitz and Thomas (1993) suggested that pore and channel size was the major factor in the preferential accumulation of a few smaller species within sea ice. However, Gleitz et al. (1998) subsequently concluded that it was the physiological capacity of these species to maintain high growth rates in the spring and summer, in connection with their life history cycles, that may be the key to the prominence of so few diatom species in the ice.

Phaeocystis species are more usually found in sea-ice habitats not constrained by the brine channel systems, such as surface ponds, rotten summer sea ice or freeboard/infiltration layers. Especially in the latter, these are situations where the contraints of salinity, temperature and low light do not inhibit primary production as they do in interior ice assemblages, thereby enabling high standing stocks (including diatoms) to accumulate (Haas et al. 2001; Kennedy et al. 2002; Kattner et al. 2004).

Dense dinoflagellate and chrysophyte assemblages can develop in the upper sea-ice interior and high rates of primary production have been measured at these sites, especially in spring when the upper sea ice temperature is low and brine salinities are high (Stoecker et al. 1997, 1998, 2000). These algal assemblages are often poorly defined, but they may make an important additional contribution to total sea-ice primary production.

Even psychrophilic, halotolerant *Chlamydomonas* spp. have been isolated from sea ice in both the Arctic and Antarctic (Krembs and Engel 2001; Ikävalko and Gradinger 1997; Hsiao 1983; Brian Eddie, Arizona State University, unpublished data). Whilst diatoms have received most of the research attention in sea ice work, other groups of organisms such as *Chlamydomonas* species will increasingly attract effort, especially since similar organisms are routinely isolated from saline and freshwater lakes in Arctic and Antarctic sites.

17.2.4 Snow

In regions where snow persists during the summer, such as at high altitude and in the high-latitude polar regions, its color may change from white to red, pink, green, yellow or orange. The largest patches are often red and therefore called "blood snow" or "watermelon snow" (Fig. 17.8, see color plate). These macroscopic expressions are based on massive growth of unicellular psychrophilic green algae. The most up-to-date review of these algae is given by Hoham and Duval (2001). Most snow algae belong to the genera *Chlamydomonas* and *Chloromonas* (Chlorophyta, Volvocales), and they are most active in spring and summer. The beginning of snow melt in spring provides liquid water between ice crystals that is essential for the vegetative stages. The snow has to be neither too cold nor too dry, such as freshly fallen snow. Green flagellated stages are often observed within this wet snow (Fig. 17.9a, see color plate). They are able to move within the snow layer to reach optimal depths for their light and temperature requirements. They can form massive blooms and color the snow green if enough nutrients are available, as is commonly found close to bird colonies or nutrient-rich streams or ponds.

During summer and fall, they have to acclimate to extreme temperature regimes, high irradiance and UV radiation and low nutrient levels. For instance, in high-altitude regions (above 2,500 m), UV radiation can be very high and spherical integrated photosynthetic active radiation (PAR) can often reach from 4,500 to occasionally 6,000 µmol photons $m^{-2}s^{-1}$. If less liquid water becomes available, and therefore also nutrients, most flagellated stages turn into immotile hypnoblast stages (Fig. 17.9b, see color plate) because this form is the most resistant to environmental changes. The transformation into hypnoblasts is characterized by a massive incorporation of reserve material, including sugars, lipids, and by formation of esterified extraplastidal secondary carotenoides. Most recent studies have shown that the cells mainly form oxycarotenoides and in particular astaxanthin, which has a red color and therefore gives the "blood snow" its name (Muller et al. 1998). These hypnocygotes and other resting cells have thick cell walls and sometimes mucilaginous envelopes (Muller et al 1998). They can survive dry and warm periods in a dormant state, and tolerate high pressure such as under thick snow. They also tolerate freezing in ice blocks at temperatures down to −35°C during winter. However, some of these resting stages can remain photosynthetically active even under very high photon flux densities because of well-protected photosystems by secondary carotenoids (Remias et al. 2005).

17.2.5 Rock surfaces

Most of the Antarctic continent is covered with a several-kilometer-thick layer of meteoric ice. However, parts of this continent are ice free, such as the McMurdo Dry Valleys in southern Victoria Land (Fig. 17.10). With ca. 4,500 km^2, this is the

largest ice-free area on this continent. Precipitation in this region is below 10 cm per year, which makes it to one of the driest deserts on Earth with air temperature ranging from +5 to −55°C (Priscu 1998).

Periglacial activity, the freezing and thawing of ground water, often sorts rocks and stones into defined patterns in polar deserts, regions of permafrost and high-altitude stone rubble fields. The sorting of the stones results in a high degree of spatial heterogeneity. In turn, these results in regions of the stone field where light penetrating the stones is sufficient to support photosynthetic carbon assimilation by the hypoliths inhabiting the underside of the stones (Cockell and Stokes 2004; Cannone et al. 2004).

The patterning of stone fields influenced by periglacial activity is often polygonal, and in both the Arctic and Antarctic rocks at the edges of the polygons support well-developed assemblage of photosynthetic organisms, whereas in the centre of the polygons colonization by hypoliths is significantly reduced (Cockell and Stokes 2004). These cyanobacteria and unicellular algae are growing, even thriving, in an extreme environment, where temperatures sink below −30°C, water is minimal, and light conditions are reduced to virtually nothing. Likewise, there are microalgae associated with cryptoendolithic layers growing within sandstones in regions such as the Dry Valleys. Typically, these communities have layers of fungi and cyanobacteria, but microalgae such as *Hemichloris antarctica* are frequently found in the lowest bands of endolithic systems where the irradiance can be as low as 0.05 µmol quanta m^{-2} s^{-1} (Johnston and Vestal 1991; Friedmann et al. 1993).

17.2.6 Permanently ice covered lakes

Besides short-term glacier melt events, most liquid water, and therefore accumulation of organisms, is available in the perennial ice-covered lakes that are characteristic for the Dry Valleys and also regions such as the Vestford Hills. However, the source of lake waters is also assumed to be glacier meltwater that penetrates the lakes without melting the surface ice cover (Priscu 1995). A sensitive balance of freeze–thaw cycles is assumed to keep the lakes permanently covered with ice but also accessible for water in- and outflow from underneath (McKay et al. 2006). There are numerous permanently covered lake systems in the McMurdo Dry Valleys, but the best studied are Lake Bonney, Hoare, and Fryxell which are located within the Taylor Valley (Fig. 17.10).

The major food-web components in all lake systems are unicellular eukaryotes and prokaryotes. The main groups are green algae, diatoms, ciliates, rotifers, heterotrophic nanoflagellates, bacteria and viruses. Autotrophic phytoplankton plays an essential role in the food web by production of organic carbon. However, photosynthesis is strongly limited by the availability of solar irradiance. Measured irradiance below the ice cover of Lake Bonney never exceeds 50 µmol photons m^{-2} s^{-1} and the wavelength of maximum transmission through the water column is in the range 480–520 nm with longer wavelengths (>600 nm) being diminished. This light is only

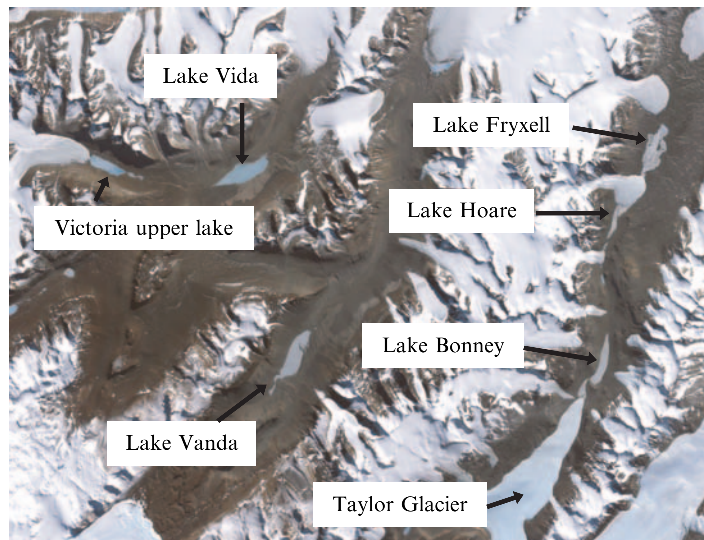

Fig. 17.10 Satellite image (NASA, USA) from McMurdo Dry Valleys. Best studied lakes are Lake Bonney, Hoare, and Fryxell that are located within the Taylor Valley

available from late September through mid-March and this seasonality is the trigger for spring phytoplankton growth in these non-turbulent waters. Vertical stratification is very pronounced in most of the lakes that is sometimes accompanied with strong gradients in salinity and nutrients. Vertical stratification is also pronounced for many phytoplankton species. The water layer immediately beneath the ice cover in Lake Bonney is dominated by the cryptomonad *Chroomonas* sp. and *Chlamydomonas intermedia* whereas *Chlamydomonas raudensis* is confined to the deep saline and low-irradiance layers of the photic zone (Morgan-Kiss et al. 2006).

17.3 Adaptation of microalgae at high latitudes

17.3.1 Diatoms (Bacillariophyceae)

Psychrophilic diatoms are one of the most abundant groups of phytoplankton in polar oceans. This is mainly due to the presence of higher silicate concentrations in these waters and to their successful adaptation to strong vertical mixing in polar waters, strong seasonality in solar irradiance, freezing temperatures, and extremes of salinity (Boyd 2002; Fiala and Oriol 1990; Cota 1985; Mock and Valentin 2004; Ryan et al. 2004; Ralph et al. 2005). Due to their importance as primary producers, many physiological studies with polar diatoms were related either to growth and its

dependency on nutrients or temperature or to regulation of photosynthesis under typical polar conditions. Only recently, functional genomics were applied to these diatoms to begin uncovering the molecular basis of growth and, thus, adaptation to polar conditions (Mock and Valentin 2004; Mock et al. 2006). This section aims to provide a comprehensive overview of new data regarding physiological and in particular molecular adaptation for this important group of polar algae.

Maximum growth rates for many polar diatoms are in the range of 0.25–0.75 divisions per day, that is 2- to 3-fold slower than growth at temperatures above 10°C (Sommer 1989). Many of these diatoms are psychrophilic and not able to live at warmer temperatures (above ca. 15°C) indicative of the presence of specific molecular adaptations that enable these diatoms to grow under freezing temperatures.

17.3.1.1 Functional genomics

The first approaches to uncover the gene repertoire of a polar diatom was done with *Fragilariopsis cylindrus*, an indicator species for cold water at both poles (Quillfeldt 2004). Two expressed sequence tag (EST) libraries, one under freezing temperatures (Mock et al. 2006) and another under increased salinity (Krell 2006) were constructed. To date, 2,485 *F. cylindrus* EST sequences have been generated: 996 from the cold-stress library and 1,691 from the salt-stress library. All ESTs are deposited at the dbEST-databank at NCBI. Furthermore, about 200 gene-specific oligonucleotides (70mers) for functional gene-array experiments are available (Mock and Valentin 2004). All EST-sequences were compared against the genome of *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, and 11 additional algae and plant databanks were consulted to annotate those sequences that were not found in the genomes of both diatoms. Nevertheless, only fewer than 50% of all sequences displayed similarity to known sequences in these databanks and to both diatom genomes even when using a comparatively high e-value of $\leq 10^{-4}$ (Mock et al. 2006).

In the cold-stress library, the most abundant functional categories were related to translation, post-translational modification of proteins and transport of amino acids and peptides by ABC-transporters. Some of these ABC-transporters displayed homology to bacterial permeases, whereas others appeared to be involved in translational control or post-translational processes. However, most of them have no assigned function at this point.

The presence of six different DNA/RNA helicases in the cold-stress library indicated that DNA and RNA coiling and uncoiling are important under freezing temperatures. Minimizing the likely formation of secondary structures and duplexes of mRNAs under low temperature stress is necessary to initiate translation. However, protein domains of DNA/RNA helicases are also the eighth most abundant protein domain in the genome of *T. pseudonana* and therefore more evidence is necessary to conclude that these enzymes are essential to cope with freezing temperatures (Armbrust et al. 2004). The most abundant sequences in this library in terms of their redundancy were either sequences that were related to energy generation

(e.g., fucoxanthin-chlorophyll a, c binding proteins) or completely unknown sequences (Mock et al. 2006).

In the salt-stress library, the most abundant functional categories of sequences were related to post-translational modification of proteins [e.g., heat-shock proteins (hsps)] and ion-transport (Krell 2006). Most of them were hsps and different ionic transporter genes reflecting the requirement to re-establish homeostasis under salt stress. Several sequences of different kinds of V-type H^+-ATPases and antiporters for various ions such as sodium, potassium and calcium were found in this library. V-type H^+-ATPases are of great importance in establishing an electrochemical proton gradient across the tonoplast to drive sodium sequestration into the vacuole (Shi et al. 2003). One important organic osmolyte under salt stress in diatoms is the amino acid proline. Many genes involved in proline synthesis were found in the salt-stress-EST library indicating that this pathway was active under the experimental conditions (Krell 2006). The gene coding for pyrroline-5-carboxylate reductase (P5CR, catalyzing the final step in proline synthesis) could be identified among the most abundant sequences in the salt-stress library, which indirectly indicates that this gene was at least moderately expressed under salt stress and therefore important for salt acclimation (Krell 2006).

One of the most interesting discoveries in the salt-stress library was a gene involved in antifreeze processes. The protein sequence (translated from the nucleotide sequence) of this gene showed high sequence similarity to an isoform protein of the snow mold *Typhula ishikariensis* (Janech et al. 2006) (Fig. 17.11). The *T. ishikariensis* protein has been shown to reduce the freezing point of seawater by approximately 0.1°C (Hoshino et al. 2003). However, this is not a strong freezing point depression when compared to the antifreeze ability from numerous antifreeze proteins identified from fish, insects, plants and bacteria. Thus, they might represent a new class of ice-binding proteins (IBPs). Using a proteomics approach and another polar diatom, *Navicula glaciei*, the corresponding protein was also discovered (Janech et al. 2006). This protein is probably unique in polar diatoms since none of the temperate diatoms tested so far had this class of genes, and the two whole genomes available for diatoms (temperate) did not contain the gene (Janech et al. 2006).

Furthermore, in contrast to polar diatoms with IBPs, temperate diatoms were not able to survive when subjected to freeze–thaw cycles (Janech et al. 2006). Using nearly pure IBPs, 2-D polyacrylamide gel electrophoresis yielded a spot of approximately 25 kDa with a pI of about 5.0. Tandem-mass spectrometry-sequencing of the band yielded four peptide sequences. These sequences were found to be similar to several antifreeze isoforms of the snow mold *T. ishikariensis*. The amplification of the *Navicula* gene revealed strong similarity to the gene from *F. cylindrus* and the corresponding *T. ishikariensis* genes (Fig. 17.11). The N-terminal sequences of the identified IBPs of *N. glaciei, F. cylindrus* IBPs and each of the *T. ishikariensis* antifreeze isoforms are most likely signal peptides and have low probabilities of being mitochondrial- or chloroplast-targeting peptides (Fig. 17.11).

These IBPs also show some similarity (between 43 and 58% amino acid sequence identity) to hypothetical proteins from Gram-negative bacteria such as

Cytophaga hutchonsonii and *Shewanella denitrificans*, species that have frequently been isolated from Arctic and Antarctic sea ice (Junge et al. 2002). The former species belongs to the Cytophaga–Flavobacterium–bacteroides (CFB) phylum group—a bacterial group that appears to be especially important in well-established sea-ice algal assemblages (Bowman et al. 1997) and the coldest (wintertime) sea ice (Junge et al. 2004) (Fig. 17.11). Several psychrophilic species of *Shewanella* have been isolated from sea ice, often in association with algal assemblages (Bowman et al. 1997). Whether polar sea-ice bacteria also express IBPs remains to be explored. The sequence similarity observed to bacterial and fungal IBPs also invites questions about the origin of these genes (Fig. 17.11).

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Navicula         ----MMFLAKTVTLLVALVASSVAAEQ-SAVDLGTAGDFAVLSKAGVSTT 45
Fragilariopsis   MMNLNLFLISAAAMVSVASASTALPPSPPAVNLGTAEDFVILAKAGVTNV 50
Typhula          -----MFSASSLLAVIALAVSSVSAAGPSAVPLGTAGNYVILASTGVSTV 45
Cytophaga        TGIKDAAGAALPSNVTWSFTTGANASVLAVVNLRTAVNYVLLAKTAINNN 146
Ferroplasma      VSPASPVTVNGAAITVNVSFTKLAPVSISPVNLGTAGNYAILAKTGISNT 129

Navicula         GPTEVTGDIGTSPIASTALTGFALIKDSSNTFSTSSLVTGKIYAADYTAP 95
Fragilariopsis   PGGAITGDIGVSPIAASAMTGFDLVMDSSNEFSTSTEITGKAYAPDYMSP 100
Typhula          PQSVITGAVGVSPGTAASLTGFSLILSGTGTFSTSSQVTGQLTGADYGTP 95
Cytophaga        PTSAVTGAIGLSPAATSYITGFSLT-NATG-YATSSQVTGHIFAADMVSP 194
Ferroplasma      GTTSIVGNIGVSPASSTYITGLSLTMNSSGQFSTSSMVTGNVYAATYASP 179

Navicula         TPSKMTTAISDMSTAFTDAAGR-----------SDPDFLELGAGSIEGET 134
Fragilariopsis   TGTKLTTAVSDMLTAYNDAAARPVTGGPFGNSLSGETYTNLGAGEIGGLT 150
Typhula          TPSILTTAIGDMGTAYINAATR-----------SGPDFLEIYTGALGGTT 134
Cytophaga        TSSNLTTAINDMQTAYTDAAGR-----------KTPDYVELGTGNIGGKT 233
Ferroplasma      TPSTLTTAVGDMQTAYTNAAGR-----------TNPNYVNLGAGDLNGMT 218

Navicula         LVAGLYKWGTDVSFTS-SLVFDGSATDVWILQVAKDFIVGNGAQMYLTGT 183
Fragilariopsis   LTRGVYTYDINVSITSGKVTFHGGADDVFIIKTSKSVLQAANTEVVLTGG 200
Typhula          LLPGLYKWTSSVGASA-DFTISGTSTDTWIFQIDGTLDVATGKQITLVGG 183
Cytophaga        LQPGLYKWTSSVSVPS-DVTISGGANDVWIFQISGNLSLSAGAKITLSGG 282
Ferroplasma      LVPGLYKWGTGVSIST-SITLTGNSSSVWIFQISGGLTFGNGAHIILSGG 267

Navicula         AKAENIFIQVSGAVNIGTTAHVEGNILSATAIALQTGSSLNGKALSQTAI 233
Fragilariopsis   AQAKNIFWSVAQEVNVGAGAHMEGILLVKTAVKFITGSSFVGRVLSATAV 250
Typhula          AQAKNIIWVVAGAVNIEVGAKFEGTILAKTAVTFKTGSSLNGRILAQTAV 233
Cytophaga        AQAKNIFWQVAGTVTAGTTSHIEGVILSKTGITFNTGASLKGRALAQTAI 332
Ferroplasma      AQPQNIFWQVASGATIGTGATFYGTILSQTAITIATGSSMTGLALAQTAV 317

Navicula         TLDSVTIVS----------------------------------------- 242
Fragilariopsis   TLQSAAITAPATSAPTTRRGPRGLQVA----------------------- 277
Typhula          ALQSATIVEK---------------------------------------- 243
Cytophaga        ILDGNTVTQP---------------------------------------- 342
Ferroplasma      TLQSDTITAPLEPQSITAAMYGVTFTEAGLPSGTQWNVTLNGVLLSSTVP 367
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Fig. 17.11 ClustalW alignment of ice-binding proteins from *Navicula glaciei* (Acc. No. DQ062566), *Fragilariopsis cylindrus* (CN212299) and *Typhula ishikariensis* (AB109745), and hypothetical proteins from *Cytophaga hutchinsonii* (ZP_00309837) and *Ferroplasma acidarmanus* (ZP_500309837). Predicted signal peptides are underlined. Gaps have been inserted to improve alignment. Conserved residues are shaded. The N-terminal sequence of *Cytophaga* protein and the N-and C-terminal sequences of *Ferroplasma* protein are truncated. Residue numbers are shown at right. Alignment is taken from Janech et al. (2006)

Horizontal gene transfer from fungi is one possibility, because basidiomycotic fungi (which include *Typhula*), are also known to inhabit sea ice and are believed to have arisen hundreds of millions of years before diatoms appeared. Horizontal gene transfer from members of the CFB-group of bacteria is another possibility because of their known association with algal assemblages in sea ice and their relation to species with IBP-like genes. In other organisms, antifreezes appear to have arisen from a variety of proteins with other functions, although some retain the original functions (Cheng 1998). Overall, the evolution of IBPs remains to be further explored.

17.3.1.2 Molecular physiology

The presence of genes in a genome only indicates the potential for physiological adaptation, but knowledge of the expression and regulation of genes and their respective proteins leads to an actual understanding of how these diatoms cope with the extreme polar conditions. Expression analysis can be done by focusing on single genes (e.g., northern blots or quantitative PCR) or by using gene arrays (Fig. 17.12) that are either composed of known genes (gene-specific arrays) and or the whole genome sequence (tiling arrays). In a recent study (Mock and Valentin 2004), about 200 oligonucleotides (70mers) were obtained for gene-only arrays based on both EST libraries from *Fragilariopsis cylindrus*. Nylon-membrane-based macro-arrays were designed to investigate the expression of genes involved in photosynthesis and

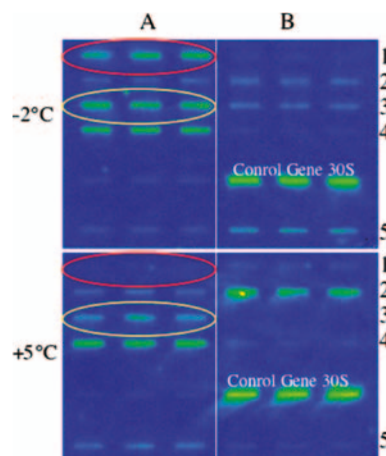

Fig. 17.12 Two Macro-Arrays that show gene expression for selected genes from *Fragilariopsis cylindrus* at +5°C and –1.8°C. Gene specific oligonucleotides (70mers) were spotted in triplicate. Dark gray circle shows induction of gene expression and light gray circle shows upregulation of gene expression under the freezing point of sea water. Genes that were selected: *A1* ABC-transporter no. 1; *B1*: ABC-transporter no. 2; *A2* polyketide-synthetase (PKS); *B2* high-light-inducible protein (HLIP); *A3* Delta-5-desaturase; *B3* ice-binding protein; *A4* unknown protein no.1; *B4* unknown protein no. 2; *A5* Sigma factor protein; *B5* fucoxanthin-chlorophyll a,c binding protein (FCP)

cold acclimation of *F. cylindrus*. Short-, mid- and long-term acclimation to the freezing point of seawater was investigated to differentiate acclimation between fast-changing environmental conditions (representing sea-ice formation in fall) and long-term acclimation (e.g., to different geographic locations and therefore different temperature regimes).

One of the most dramatic environmental changes in polar marine sea ice habitats is the freezing of seawater and the melting of the ice. The inclusion of organisms into newly-formed sea ice represents a strong selective pressure. Only those organisms that are capable to acclimate to the relatively fast-changing conditions of temperature, irradiance and salinity can survive. To date, only one experiment has been conducted using a polar diatom, *Fragilariopsis cylindrus*, to investigate gene expression changes from non-freezing to freezing temperatures in order to obtain molecular information of the acclimation potential (Mock and Valentin 2004). Macro-arrays composed of genes from different pathways were specifically designed for these experiments. The short-term response to freezing temperatures, which simulates the incorporation into newly formed sea ice during fall, was characterized by down-regulation of genes encoding proteins for photosystem II (psbA and psbC) and carbon fixation (RUBISCO large subunit, rbcL) regardless of light intensity used (3 and 35 µmol photons $m^{-2}s^{-1}$). However, under higher irradiance (35 µmol photons $m^{-2}s^{-1}$) up-regulation of genes encoding chaperons (hsp 70) and genes for plastid protein synthesis and turnover (elongation factor EfTs, ribosomal rpS4 and plastidial ftsH protease) was observed. Freezing accompanied with a reduction in irradiance (from 35 to 3 µmol photons $m^{-2}s^{-1}$) showed a typical response to low-light acclimation by up-regulation of genes encoding specific fucoxanthin-chlorophyll a,c binding proteins (fcps) without signs of a cold stress response. Fcps are a diverse gene family composed of genes involved in light harvesting as well as dissipation of light (see Sect. 17.2). Up-regulation of stress response genes and genes for protein turnover only under higher light intensities and decreasing temperatures indicates that a decrease in temperature at such light intensities mimics a further increase in light that could be more stressful than the actual decrease in temperature was by itself. This phenomenon is probably part of a cold-shock response that is also known from temperate plants when they get exposed to lower temperatures (Allen and Ort 2001).

In contrast to temperate plants and diatoms, though, psychrophilic plants and diatoms are able to acclimate to higher irradiances under low temperatures (Ralph et al. 2005; Streb et al. 1998; Mock and Hoch 2005; Morgan-Kiss et al. 2006). Long-term acclimation experiments to higher irradiances at freezing temperatures, when compared to the same light intensity but higher temperatures (+5°C), revealed that cells kept at lower temperatures showed a typical response known from high-light acclimation: higher non-photochemical quenching, up-regulation of the gene psbA and up-regulation of high-light fcps that are involved in energy dissipation (Mock and Hoch 2005). A reduction in expression of other photosynthesis-related genes (such as rbcL) was not observed after several months under freezing conditions indicating that long-term acclimation had been achieved. Temperature effects that are less dependent on adjustments of the energy flow under freezing temperatures could also be identified by gene expression analysis.

For this study, genes were selected that either were abundant in the EST libraries (e.g., ABC transporters), or were important for general acclimation to freezing temperatures (e.g., IBP, fatty-acid desaturase) (Fig. 17.12). Three unknown but abundant genes (in EST libraries) were also selected to see whether at least one of them is up-regulated under freezing temperatures. Expression of these genes was investigated at +5°C and 9 days after reducing the temperatures to −1.8°C (Fig. 17.12). Up-regulation of a gene encoding a delta5-desaturase under freezing temperatures indicated the necessity for production of polyunsaturated fatty acis (PUFAs) to maintain membrane fluidity at lower temperatures. Delta-5 desaturases produce omega3-fatty acids such as EPA (20:5 n-3), one of the most abundant fatty acid in diatoms and the main fatty acid in the galactolipids MGDG and DGDG. Thus, it can be assumed that more EPA is necessary under freezing temperatures to keep the thylakoid membrane fluid for electron transport or other membrane-bound processes. In contrast, a delta-12 desaturase gene also known for producing PUFAs was not up-regulated in temperate cyanobacteria (Nishida and Murata 1996). This indicates a different mechanism of gene regulation for this enzyme in psychrophilic diatoms.

Strong up-regulation of a gene that encodes an IBP (ca. 50-fold) was also measured under freezing temperature supporting the hypothesis that these proteins are of great importance not only under salt stress but also under freezing temperatures to protect the cells from injury by growing ice crystals (Fig. 17.12). One uncharacterized ABC-transporter gene was strongly up-regulated at −1.8°C. However, the family of ABC-transporters is composed of genes with very diverse functions (see above) so that there is no direct evidence, based on gene expression only, that this gene has actually something to do with transporting molecules. One of the unknown genes being 2-fold up-regulated under freezing temperature indicated some importance for acclimation to low temperatures. In contrast, another unknown gene was down-regulated at −1.8°C, while the third gene didn't change its expression at both temperatures but was relatively strongly expressed under both temperatures.

Overall, these initial results indicate that psychrophilic diatoms have specific genes to cope with freeze-thaw cycles that are also known from other psychrophilic organisms, but are not present in temperate diatoms. However, it remains to be seen whether temperate diatoms also have a unique set of genes that is specific to them.

17.3.2 Green algae (Chlorophyceae)

Most polar green algae live in freshwater ecosystems such as snow, permanently ice-covered lakes or more ephemeral habitats like creeks or melt ponds on top of snow or sea ice. Most species belong either to the genera *Chlamydomonas, Chloromonas* or *Chlorella*, and many of them are very motile due to the presence of flagella. Ecologically important species that are physiologically and molecularly well characterized are *Chlamydomonas raudensis* and *Chlamydomonas nivalis. C. raudensis* is an abundant species in permanently ice-covered lakes and the clone UWO241 have been studied for decades (see review by Morgan-Kiss et al. 2006).

C. nivalis is a dominant representative of the snow-algae community and also intensively studied (Williams et al. 2003). Therefore, this discussion will mainly focus on these species but will also include important findings from other high latitude green algae. Not many genomic or proteomic data are available yet for polar green algae. The following paragraph has therefore the emphasis on molecular physiology.

17.3.2.1 Molecular physiology

Maximum growth rates of polar green algae are comparable to those from polar diatoms. They range from 0.2–0.4 day^{-1} (Tang et al. 1997). Temperatures above 18°C are mostly lethal to these algae. C. *raudensis* has its maximum photosynthetic rates at +8°C, which decline steadily with increasing temperatures (Morgan-Kiss et al. 2006). This indicates maximal efficiency in converting light into photosynthetic energy at low temperatures. Quality of light also plays an important role and *C. raudensis* is not able to grow under red light (Morgan-Kiss et al. 2005). This inability to grow under red light is probably a consequence of being almost never exposed to a longer wavelength spectrum in the natural habitat of permanently ice-covered lakes where the ice cover absorbs all longer wavelengths of solar irradiance (Fritsen and Priscu 1999; Morgan-Kiss et al. 2006) Only green and blue light passes the ice cover and can therefore be used for photosynthesis. However, the majority of this light is reflected on the white surface of ice and scattered while passing the ice. Thus, the environment below the ice is characterized by low intensities enriched in blue-green wavelengths (Lizotte and Priscu 1992).

Many physiological and molecular investigations have been conducted with *C. raudensis* to find the reasons of successful photoadaptation under these extreme conditions. A comparison with the temperate *C. reinhardtii* partly uncovered the mechanisms of photoadaptation in *C. raudensis* (Morgan-Kiss et al. 2005, 2006): In contrast to the temperate *C. reinhardtii*, the psychrophile has lost its ability to live under high light but increased its efficiency of light harvesting under low light in the blue-green spectrum. This adaptation can be seen in structural changes of the photosynthetic apparatus (Fig. 17.13). For instance, *C. raudensis* has an unusually high ratio of photosystem II to I, and significantly higher levels of light harvesting II complexes than its temperate counterpart *C. reinhardtii*. These changes are probably an adaptive advantage under constant exposure to blue light of low photon flux densities because the light harvesting apparatus of photosystem II (PSII) utilizes chlorophyll b and short-wavelength-absorbing chlorophyll a to absorb light predominantly in the blue region.

Interestingly, most marine algae (e.g., red algae, diatoms), which are also living in a blue-green light environment because of optical properties of the seawater, also show a high ratio of PSII to PSI due to chromatic regulation (e.g., Fujita 2001). However, most of them, and even psychrophilic diatoms, have the physiological ability to grow under high irradiance levels. Another interesting similarity between diatoms (psychrophilic and temperate) and this psychrophilic green alga is the biochemistry and architecture of the thylakoid membrane. Diatoms, as well as

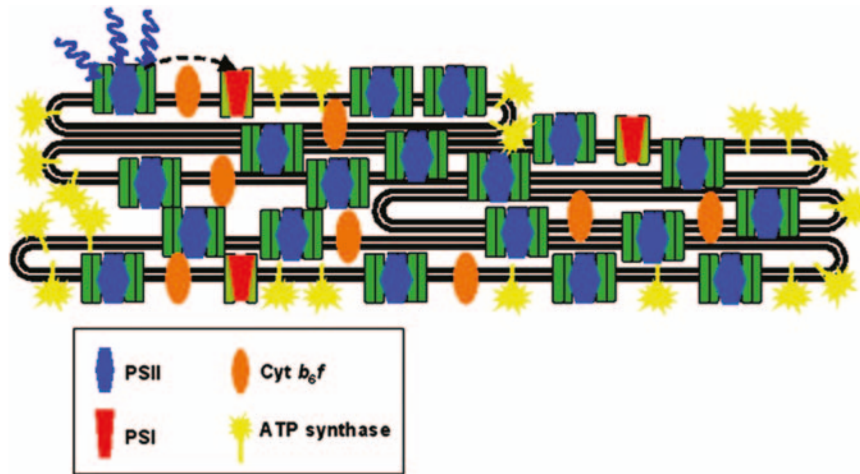

Fig. 17.13 Model for organization of thylakoid pigment-protein complexes of the electron transport chain in the psychrophilic *Chlamydomonas raudensis* UWO 241. In the natural, extremely stable light environment of extreme shade and predominantly blue-green wavelengths, the majority of available light would be preferentially absorbed by PSII. Adaptation in *C. raudensis* to this light environment has led to an unusually high PSII/PSI stoichiometry and highly efficient energy transfer from LHCII to PSII. Conversely, PSI and associated light-harvesting complexes are both structurally and functionally downregulated. Given the severe reduction in light-harvesting capacity of PSI, it is proposed that PSI centers are largely excited via a spillover energy transfer mechanism from PSII (dotted line). Photosynthetic membranes may be arranged as loose stacks rather than distinct granal and stromal regions to promote energy spillover between the photosystems. Picture from Morgan-Kiss et al. (2006)

C. raudensis, have high concentrations of poly-unsaturated fatty acids in their thylakoid lipids classes and their thylakoid membranes are not organized in grana and stroma (Mock and Kroon 2002a, 2002b; Morgan-Kiss et al. 2006). This possibly means that looser membrane stacks in *C. raudensis* and homogeneously folded membranes in diatom plastids promote energy spillover between photosystems and therefore light energy transfer between photosystems (Morgan-Kiss et al. 2006). Living in a blue-green light-dominated environment possibly caused a similar kind of photosynthetic adaptation in two completely different groups of algae.

The snow alga *C. nivalis* is exposed to the full spectrum of solar irradiance (UV-C to infrared) and must therefore have a completely different photosynthesis performance compared to the low light adapted *C. raudensis* (Remias et al. 2005). The most striking difference between photosynthesis of both psychrophilic green algae is that *C. nivalis* does not seem to be inhibited by high solar irradiances. Even an exposure of cells to photon flux densities of 1,800 µmol photons $m^{-2}s^{-1}$ for 40 minutes at 1.5°C did not inhibit net photosynthesis (Remias et al. 2005). This extreme photosynthetic performance is only possible by a change in the life cycle. A combination of factors may trigger the formation of immotile red hypnoblast

stages that are most resistant to environmental changes (Muller et al. 1998; Remias et al. 2005). The transformation into hypnoblasts is characterized by a massive incorporation of sugars and lipids, and by the formation of esterified extraplastidal secondary carotenoids (Hoham and Duval 2001). The most important carotenoid is astaxanthin which is located in cytoplasmatic lipid globuli (Muller et al. 1998; Remias et al. 2005), and this pigment is assumed to be responsible for the high photostability and therefore the absence of photoinhibition under strong solar irradiance on top of snow (Remias et al. 2005). Mature hypnoblasts can contain about 20 times more astaxanthin than chlorophyll a, where the astaxanthin is possibly acting as a filter to reduce the irradiance that would otherwise be damaging to the photosynthetic activity inside the plastids.

High solar irradiance is not the only harsh condition on top of snow. Drought due to freezing of water is another main stress factor of the hypnoblast stages of *Chlamydomonas nivalis*. Like cacti in the desert, these stages have very rigid cell walls as the outer boundary to an extreme environment (Muller et al. 1998; Remias et al. 2005). Sometimes cells secrete carbohydrates to produce a visible mucilage sheet around them (Muller et al. 1998). These carbohydrates are not only attractive to bacteria that use them as a substrate but they also trap particles transported into the snow by wind. These particle-covered cells increase the absorption of solar irradiance and therefore the production of heat. This heat might cause melting of surrounding snow crystals and therefore provide liquid water to the cells (Takeuchi 2002). Such small spots of melt events around warm bodies (e.g., rock debris, cells) are called cryoconite holes (Takeuchi 2002). However, these adhering particles may also shade and thus protect *C. nivalis* against high irradiance. This is not universal and hypnoblasts from *C. nivalis*, for example, never show such attached structures.

Only recently have molecular approaches been used to uncover the genetic basis of cold adaptation in high-latitude psychrophilic green algae (Morgan-Kiss et al. 2006; Kan et al. 2006; Rigano et al. 2006; Odom et al. 2004). However, no genome sequence from a psychrophilic green algae is available yet, nor to our knowledge is either planned to be sequenced, but EST projects are planned (Thomas Leya, personal communication). The genome sequence from the temperate *Chlamydomonas reinhardtii* has been available for a couple of years (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) and would provide an excellent basis for a comparative analysis with ESTs from, e.g., the psychrophile *C. nivalis*. Thus, most of the molecular studies with psychrophilic green algae are related to specific genes and in particular to cold-adapted enzymes.

Chemical reactions are influenced by temperature according to the relationship described by Arrhenius. In general, a 10°C reduction in growth temperature causes biochemical reaction rates to decline two to three times. However, doubling times of psychrophilic algae can be comparable to mesophilic algae (Sommer 1989) which means that rates of enzyme catalyzed reactions must be optimized to low temperatures in these organisms (Feller and Gerday 2003). Studies with the enzyme nitrate reductase (NR), for instance, showed that these enzymes from psychrophilic algae possess structural modifications that make them more cold adapted, being more catalytically efficient at lower temperatures but at the same time less thermally

stable, than NRs from mesophilic species (Rigano et al. 2006). In contrast to NR, the temperature maximum for carboxylase activity of ribulose-1–5-bisphosphate carboxylase/oxygenase (Rubisco), one of the most critical enzymes for inorganic carbon fixation in photoautotrophes, was not altered in some psychrophilic green algae and the specific activity at low temperatures was actually lower in the psychrophilic if compared to the mesophilic Rubisco (Devos et al. 1998). Decreased catalytic efficiency of these Rubiscos under low temperature seems to be at least partly compensated by an increased cellular concentration of the protein.

Besides cold adaptation of cellular enzymes, structural parts such as the cytoskeleton of the cell also have to be adapted to low temperatures in order to conduct mitosis, meiosis, secretion and cell motility. Willem et al. (1999) showed that alpha-tubulin from two *Chloromonas* spp. had five amino acid substitutions compared to the mesophilic *Chlamydomonas reinhardtii*. Two of these substitutions occurred in the region of interdimer contacts that could therefore positively influence microtubule assembly under low temperatures. Detrich et al. (1989) also showed that brain tubulins from an Antarctic fish are able to polymerize at temperatures close to 0°C whereas the assemble of microtubules from purified mammalian brain tubulins occurred only at much higher temperatures.

17.4 Conclusions

These examples from polar diatoms and green algae clearly give evidence of a psychrophilic nature in these algae. However, comparative approaches on a broader level (proteomics and genomics) are clearly needed to identify novel yet unknown genes in these organisms that are unique to them and responsible for their adaptation to low temperature or other more habitat specific stresses. The genome sequencing project with *Fragilariopsis cylindrus* is the first step in that direction, and a comparative analysis to genomes from non-polar diatoms (*T. pseudonana* and *P. tricornutum*) will probably shed light on unique adaptations that are necessary to thrive under polar conditions. Another genome project with a psychrophilic green alga (e.g., *C. raudensis*) would be of great value for a similar comparison to the temperate green alga *C. reinhardtii* for which the genome sequence is already available. To our knowledge there is no genome-wide expression study (e.g., EST library) yet published for a polar green alga. However, a proteome study with a psychrophilic *Chlamydomonas* sp. under control temperature and below freezing point identified two proteins that were only synthesized under freezing conditions (Kan et al. 2006): an isopropylmalate/homocitrate/citramalate synthase, which is involved in transport and metabolism of amino acids, and a gluthation-S-transferase that scavenges radical oxygen that is produced under intense photosynthesis, such as under high irradiance on top of the snow. More studies like that are needed to identify novel candidate genes and proteins (similar to the novel ice-binding proteins in diatoms) in psychrophilic green algae. Another genome sequence from a polar green alga would also be helpful in identifying whether core genes and

specific modification on the amino acid level are unique to both psychrophilic algal groups. These genomic data will definitely provide novel hypotheses on how polar algae are adapted to their environment and therefore also on how changes of the environment (e.g., due to global warming) will effect the diversity of these species and thus biogeochemical cycles in polar aquatic systems.

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Chapter 18
Metagenomics: Microbial Community Genomes Revealed

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18.1 Introduction

The rapidly expanding field of metagenomics has revolutionized the ability to analyze microbial communities by providing access to 'true' microbial diversity. This field has a broad range of applications in the areas of biodiversity, systems biology and biotechnology (Handelsman et al. 2002). As most microbial communities in soil, sediment or aquatic environments are highly complex, consisting of hundreds or even thousands of species of which only a few have been cultured, the approaches collectively described as metagenomics, community genomics or environmental genomics have been developed to help to unlock this thus far hidden diversity. Metagenomics is therefore the application of modern genomic tools used to analyze

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the collective genomes of whole microbial communities (the metagenome) in an environmental sample, thereby bypassing the need for isolation or cultivation (Béjà et al. 2000; Riesenfeld et al. 2004b). Even though the tip of the microbial ice-berg has barely been scratched, this approach is rapidly increasing our knowledge of microbial genetic and functional diversity through gene/pathway discovery either by sequencing or activity-based screening strategies (Handelsman 2005).

On a broader scale, environmental community genomics studies have the potential to provide new ecological and biogeochemical insights (Béjà et al. 2000; Allen and Banfield 2005; DeLong 2005) and to produce novel compounds for therapeutic and biotechnological applications (Short 1997). For reviews, see Schloss and Handelsman (2003), Ferrer et al. (2005) and Lorentz and Eck (2005). The ability to sequence and assemble large volumes of genomic DNA extracted directly from the environment has revolutionized the studies of uncultivated microorganisms (Handelsman 2005). As more metagenomes are sequenced the capacity to compare metagenomic data from different ecological niches expands. Ultimately, the association of comprehensive metagenomic sequence data with environmental data offers tremendous potential for understanding the complex functions of microbial ecosystems. This chapter provides a brief outline of the different approaches and applications of metagenomics, and discusses some of the more recent results from community genomic studies.

18.2 Tapping into hidden diversity

The direct cloning of environmental DNA and subsequent metagenomic analysis provides a unique route for accessing genomic information from uncultured microorganisms. Some of the diverse environments that have been examined using these methods include soil (Rondon et al. 2000; Tringe et al. 2005), marine sediments (Breitbart et al. 2004; Hallam et al. 2004; Angly et al. 2006), marine waters (Stein et al. 1996; Béjà et al. 2000; Venter et al. 2004; DeLong et al. 2006), Antarctic waters (Lopez-Garcia et al. 2004; Moreira et al. 2004; Grzymski et al. 2006; Moreira et al. 2006), different mines (Tyson et al. 2004; Baker et al. 2006; Edwards et al. 2006), whale carcasses (Tringe et al. 2005), and phosphorous removal sludge (Martin et al. 2006). For recent reviews, see DeLong (2005), Handelsman (2005) and other articles in the special issue "Focus on Metagenomics", as well as other articles (Riesenfeld et al. 2004a; Lorentz and Eck 2005; Deutschbauer et al. 2006; Green and Keller 2006; Sjöling et al. 2006).

With metagenomic approaches, the entire genome complement of any sample is accessible, together with individual components: single genes, operons, cassettes, pathways and extrachromosomal units. Most importantly, no a priori knowledge of sequence information is required and a large number of different screening/identification strategies may be applied.

Metagenomic libraries are created by direct cloning of DNA extracted from an environmental sample in a suitable vector (e.g., plasmid, phage, fosmid, cosmid or bacterial artificial chromosome, BAC), which is then transformed into a suitable

host. For reviews, see Riesenfeld et al. (2004b), Daniel (2005), DeLong (2005) and Sjöling et al. (2006). Various methods to isolate and purify high quality environmental DNA suitable for metagenomic cloning have been used and are discussed in articles by Quaiser et al. (2002), Gabor et al. (2003) and Robe et al. (2003). The efficiency of DNA extraction strongly influences how effectively the library represents the sample community, and the quality of DNA and the choice of screening method guides the selection of extraction method. Typically, in order to obtain large DNA fragments of high quality suitable for cloning into fosmid or BAC vectors, the DNA is gently extracted by in situ lysis of dispersed cells. More harsh extraction methods, which may be highly efficient, result in smaller fragments of sheared DNA suitable for cloning into plasmids.

The cloning of larger DNA fragments (20–150 kb), which allows the characterization of large contiguous regions of individual genomes, is an advantage if the aim is to obtain whole gene clusters encoding complete pathways or indigenous promoter regions. Large fragments may also contain a phylogenetic marker gene (e.g., genes for 16S rRNA, RecA, elongation factors, heat shock protein 70) within the same fragment as the genes of interest, which facilitates the linkage of identity to function (see Sect. 18.4.2). Smaller genome fragments (1–20 kb) may be more efficiently expressed in vitro and are more suitable for whole genome shot-gun sequencing of community DNA preparations. DNA from soil or sediment can be difficult to purify due to the presence of humic compounds (polyphenols) that tend to co-purify with the DNA, restricting the cloning efficiency, particularly in BAC vectors (Handelsman et al. 2002). Additional purification steps are commonly used to obtain pure DNA from such samples (Quaiser et al. 2002). These difficulties in obtaining high yield and quality DNA have, in some cases, been solved by using enrichment cultures where selective media are employed to target specific groups of organisms or specific biological activities, for instance particular enzymatic activities or bioremediation capacities (Healy et al. 1995; Voget et al. 2003; Uchiyama et al. 2005). Metagenomic libraries of metabolically active populations can be constructed from stable nitrogen and carbon (stable isotope probing) or bromodeoxyuridine (BrdU)-labelled DNA, which is then used for cloning (Urbach et al. 1999; Wellington et al. 2003). Cloned DNA fragments are analyzed using either sequence-based or functional screening approaches depending on the purpose of the metagenomic study. Both methodologies are subject to technical limitations, as discussed by Daniel (2004).

18.3 Approaches in metagenomic analysis

18.3.1 Functional screening

In functional screening protocols, genes are heterologously expressed and the gene products are identified by screening assays designed to detect a specific function, e.g., enzymatic or antibiotic activity, (for reviews, see Schloss and Handelsman

2003 and Riesenfeld et al. 2004b). Screening for function has the advantage that it secures a full-length gene or gene cluster and has the potential to detect completely novel genes encoding new bioactive compounds or new enzymes. Another advantage of expression screening is that genes that encode enzymes with very low identities to known sequences can be isolated, where sequences may be so divergent from any known protein genes that identification based on sequence homology would not have been successful (Rees et al. 2003). However, one of the limitations of expression screening is the dependence on a compatible expression system (transcription, translation, folding, secretion) by a heterologous host, which is generally *Escherichia coli* (Daniel 2004).

Heterologous expression screening has resulted in the recovery of several enzymes, e.g., agarase (Voget et al. 2003), alcohol oxidoreductase (Knietsch et al. 2003), amidase (Gabor et al. 2004), amylase (Rondon et al. 2000; Richardson et al. 2002; Voget et al. 2003), esterase/lipase (Henne et al. 2000; Rondon et al. 2000; Rhee et al. 2005; Lee et al. 2006b; Hårdeman and Sjöling 2007), hydroxybutyrate dehydrogenase (Henne et al. 1999), xylanase (Lee et al. 2006a), and a limited number of bioactive compounds, e.g., antibiotics (MacNeil et al. 2001; Gillespie et al. 2002; Brady and Clardy 2004). For more detailed reviews, see Riesenfeld et al. (2004b), Daniel (2005), Lorentz and Eck (2005) and Sjöling et al. (2006). However, the rate of obtaining positive 'hits' is typically low for most of the enzymes screened to date (Sjöling et al. 2006) due to limitations in heterologous expression screening (Daniel 2005) and the relative low metagenomic coverage of most published expression libraries (Cowan et al. 2005). Conversely, high-throughput screening systems (HTS), for the discovery of stable enzyme from extreme environments, and biopanning, a technique that allows for the enrichment of specific sequences, have both been very successfully applied by biotechnological companies such as Diversa Corporation (Gray et al. 2003).

A variation of functional screening is the selective enrichment for the activity of interest by substrate-induced gene expression (SIGEX; Uchiyama et al. 2005). An expression vector incorporating the gene for green fluorescent protein (*gfp*) is used. If a metagenomic gene is functionally expressed upon addition of substrate, *gfp* is co-expressed and fluorescent activated cell sorting can be used to select for clones containing target genes, e.g., catabolic genes (Uchiyama et al. 2005).

In principle, the screening of metagenomic expression libraries for eukaryotic genes should be largely ineffective due to the presence of introns in most eukaryotic sequences. This limitation can be overcome by the preparation of metagenomic cDNA libraries, after the reverse transcription of extracted/purified mRNA. However, metagenomic cDNA libraries will always be less comprehensive than genomic DNA libraries since they can never represent non-expressed genes and there are severe technical difficulties in the efficient isolation of metagenomic mRNA (Sjöling et al. 2006). Nevertheless, eukaryotic open reading frames have been successfully identified in environmental cDNA libraries, demonstrating that these technologies can be used to exploit uncultured eukaryotic genomes (Grant et al. 2006).

18.3.2 Sequence-based screening

Sequence-dependent metagenomic approaches can be broadly divided into two sub-groups of methods. PCR-dependent methods are generally focused on identification of small sequence units, typically single genes. Such methods rely heavily of the use of gene-specific consensus primers. Alternatively, direct sequencing of metagenomic clone libraries exploits a range of methods, including end-sequencing and random sequencing,

PCR-amplification and hybridization methods are based on the DNA sequence consensus, and are principally used for detecting genes of interest: phylogenetically informative genes, transcriptionally active genes or other biologically interesting genes (Stein et al. 1996; Béjà et al. 2000; Rondon et al. 2000; Liles et al. 2003; Sogin et al. 2006). A sequence-based approach is not limited by potential incompatibility between host and cloned genes. Another advantage is the possibility of identifying a phylogenetic marker gene on the same fragment as a gene or gene cluster of interest, making it possible to link function with identity (Béjà et al. 2000), a possibility which increases proportionally with the size of the cloned insert.

The most powerful approach in metagenomics is the application of whole genome shot-gun sequencing (WGS), or, more recently, the cost effective ultra-high-throughput pyrosequencing, to entire microbial communities (Tyson et al. 2004; Venter et al. 2004; Angly et al. 2006; Edwards et al. 2006; Woyke et al. 2006). Such projects have become feasible with the relatively recent advent of automated, high-throughput sequencing offered by sequencing centres, such as US Joint Genome Institute and 454 Life Science/Roche Diagnostics, and powerful algorithms for sequence assembly and annotation (Markowitz et al. 2006). To be effective, community genome sequencing requires an enormous cloning and sequencing effort. Shot-gun sequencing relies on the preparation of comprehensive metagenome fragment libraries. An 'average' environmental genome may house in excess of 1,000 prokaryotic genomes (Curtis et al. 2002), even ignoring lower eukaryotes, viruses, etc. For effective coverage of an estimated 5 Gbp metagenome (assuming an average microbial genome at 5 Mbp) in excess of 10 Gbp of unique sequence must be binned, assembled and annotated. The obvious strength of this approach is the unprecedented access to the full gene complement and the theoretical ability to assembly complete genomes, particularly of predominant community members (Tyson et al. 2004; Chen and Pachter 2005; DeLong et al. 2006). Projects sequencing partial or near complete communities include the Sargasso Sea bacterioplanktonic fraction (Venter et al. 2004), acid mine drainage biofilm (Tyson et al. 2004), Minnesota mine fluids (Edwards et al. 2006), Minnesota farm soil and deep sea whale carcasses (Tringe et al. 2005), deep sea sediment (Hallam et al. 2004), ocean bacterioplankton (DeLong et al. 2006), marine oligochaete symbionts (Woyke et al. 2006), human gut communities (Gill et al. 2006), enhanced biological phosphorus removal sludge communities in laboratory-scale enrichment culture (Martin et al. 2006), viruses in marine sediment (Breitbart et al. 2004), and human feces (Breitbart et al. 2003). Many more metagenomic sequencing projects are under way.

Sequence-based metagenomic studies are also possible without the cloning step, if it is replaced by whole genome amplification (i.e. GenomiPhi procedure), as shown by recent studies (Abulencia et al. 2006; Angly et al. 2006; Edwards et al. 2006; Yokouchi et al. 2006), further discussed in Sect. 18.6.

18.4 Metagenomics: for what purposes?

The development of metagenomic technologies allows fundamental questions relating to the structure and function of microbial communities to be posed. These include, but are not restricted to: Who is there? What is the functional role of each member of a population? What is the metabolic capacity of the community? How is one community different from another? What are the ecological and evolutionary roles of the community? What novel bioactive compounds produced by the members of the community can be accessed?

18.4.1 Assessing phylogenetic diversity and population structure

Metagenomics greatly facilitates the identification of novel species. A phylogenetically diverse environmental sample, such as soil, sediment or water, may contain several thousands of different prokaryotes and viruses, containing several Gbp of genomic DNA. Through new metagenomic approaches, whole communities of both simple and more complex structure may be analyzed for total gene content, from which phylogenetic diversity, population structure and, to some extent, trophic interactions can be determined (Venter et al. 2004; Tringe et al. 2005; Angly et al. 2006; DeLong et al. 2006; Edwards et al. 2006; Whitaker and Banfield 2006). The ambitious whole genome shot-gun sequencing of bacterioplankton in the oligotrophic Sargasso Sea by Venter and co-workers generated 1.6 billion bases of nucleotide sequence, identifying over 1,800 species (defined by rRNA, EFTu, EFG, HSP70, RecA and RpoB genes), of which 140 were previously unknown (Venter et al. 2004). Furthermore, the analysis of large genomic fragments cloned directly from the environment has also led to the identification of uncultured novel organisms and at least parts of their genomes (Béjà et al. 2000). The true diversity of viruses in the environment is still largely unknown. However, recent surveys by Rohwer and co-workers showed the vast diversity of viromes in different environments (see Angly et al. 2006 and references therein). Understanding the forces generating viral or prokaryotic diversity and 'species' clusters, and how to address the "species problem" is still a challenge, but metagenomics may provide some insight by establishing clearer genetic relationships and evolutionary pathways within and between major groups of microorganisms (W.F. Doolittle, Algorithmic Biology Conference 2006).

18.4.2 Linking novel functional roles to phylogeny

Metagenomics makes it possible to link functional genes of uncultured organisms to organism identity. This can be achieved through the identification of a phylogenetic marker gene and a functional gene on the same DNA fragment, or where sequenced genes can be assembled together in reconstructed genomes from whole metagenome sequencing. The former was nicely demonstrated through the discovery of a whole new class of marine phototrophy, based on proteorhodopsin in a bacterioplankton metagenome (Béjà et al. 2000). This photoreceptor in bacteria harvests light energy for proton pumping coupled to carbon cycling. Further metagenomic mass sequencing of bacterioplankton revealed that proteorhodopsin is widespread and exists in about 13% of the bacteria at the ocean's surface, indicating an important role in global carbon transformation (Venter et al. 2004). Consequently, further information of important functional roles through metagenomics may provide a deeper perspective of the physiological roles of environmental microorganisms (DeLong 2005).

18.4.3 Assessing metabolic pathways, ecology and evolution

Fundamental issues of microbial ecology, which include microbial population interactions, metabolic processes, biogeochemical activities and genetic adaptation of cultured and uncultured organisms, are difficult to address. Metagenomics provides tools for understanding the interactions between different organisms and their immediate environment and for linking microbial communities to their ecological functions and roles (Allen and Banfield 2005). For instance, the almost complete genome of the anaerobic ammonia oxidizer *Kuenenia stuttgartiensis* from a bioreactor community was assembled using an environmental genomics approach, allowing the models for anammox catabolism and CO_2 fixation to be substantially refined (Strous et al. 2006). Genome sequence data from metagenomics may also help to design selective isolation strategies to cultivate organisms based on the presence, absence or distribution of metabolic genes and pathways (Allen and Banfield 2005; Angly et al. 2006; DeLong et al. 2006; Edwards et al. 2006).

The comparative metagenomic analysis of the genomic content across entire microbial communities, such as those populating environmental gradients, should provide extensive data on the genetic basis for adaptation and may resolve differences in the genetic, biochemical and metabolic capacities of communities adapted to different environmental parameters. Comparative metagenomics has the potential to reveal previously unrecognized biological interactions and new habitat-specific functions and correlations that may otherwise have been missed in single genomic studies (Allen and Banfield 2005; DeLong 2005; Edwards et al. 2006; Woyke et al. 2006).

Moreover, community genome sequence information can be used to design probes for gene expression profiling using micro-arrays, or to identify proteins

using "meta"proteomics (Allen and Banfield 2005; Ram et al. 2005). Where genomic data analysis is integrated with gene expression studies, this will facilitate the assignment of key functions to specific members of populations and communities (Allen and Banfield 2005).

Metagenomics may also contribute to the assessment of evolutionary processes, including selection and adaptation, based on the analysis of nucleotide substitutions within species and horizontal gene transfer events between unrelated taxa (Tringe et al. 2005). In particular, comparative metagenomics may eventually reveal the evolutionary processes that lead to genome diversification and speciation (Allen and Banfield 2005; Tringe et al. 2005; Edwards et al. 2006).

18.4.4 Sequencing whole genomes of communities

Whole genome sequencing (shot-gun or pyrosequencing) of an entire microbial community, or major portions of it, represents an increasingly used approach in metagenomics, e.g., bacterioplankton in the Sargasso Sea (Venter et al. 2004), an acid mine biofilm (Tyson et al. 2004), whale fall and Minnesota farm soil (Tringe et al. 2005), and oceanic viruses (Angly et al. 2006). The Sargasso Sea dataset represented a ground-breaking metagenomic survey because of its size and the novel functional diversity it revealed. Not only were a very large number of phylotypes identified, but more than 1.2 million unknown genes were detected. More detailed analysis of the assembled sequence gave some dramatic insights: for example Archaeal ammonium monooxygenase genes were identified (previously, only members of the domain Bacteria were implicated in oceanic nitrification) as well as the extensive distribution of proteorhodopsin (782 new variants of this gene), demonstrating the potentially enormous photosynthetic capability of marine microorganisms (Venter et al. 2004; DeLong 2005). Several complete plasmids and bacteriophage were identified but only two complete microbial genomes were reconstructed. Although in this latter respect the Sargasso Sea metagenome sequencing project was very incomplete, the range and significance of the data obtained demonstrates both the potential and the limitations of mass sequencing of complex diverse assemblages (Venter et al. 2004).

With the selection of low complexity communities, coupled with an adequate level of sequence coverage, it is reasonable to expect to obtain largely complete genome sequences of dominant organisms, even if coverage of large numbers of low frequency species is comparatively poor. This has been demonstrated by the sequencing of a simple acid mine biofilm, dominated by only three bacterial and two archaeal lineages (Tyson et al. 2004). Sequencing of 75 Mbp was sufficient to assemble two near-complete genomes of *Ferroplasma* II and *Leptospirillum* II and partial assembly of the three additional genomes. The metagenomic information enabled the assignment of biochemical roles of the different organisms of the self-sustaining acidic biofilm. Thus *Ferroplasma* I and II were identified as dominant heterotrophs with numerous sugar and amino acid transporters, while only one of

the organisms, *Leptospirrilum* III, was shown to be able to fix nitrogen, making it a keystone species in the ecosystem (Tyson et al. 2004).

18.4.5 Bioprospecting

The global commercial pharmaceutical (particularly antibiotic) market, but also the enzyme market, have developed from the exploitation of genes and genomes through culture-dependent access to microorganisms. The vast genetic diversity, evolved through adaptation of organisms for the survival in every environmental niche, represents a potential source of novel agricultural, pharmaceutical and industrial products. With tools such as metagenomics, a unique route of tapping into the uncultured majority of microorganisms is offered, providing the likely prospect of discovering many truly novel natural gene products (Sjöling et al. 2006). Several enzymes and bioactive compounds have been detected through functional screening that may have commercial applications (Daniel 2004; Lorentz and Eck 2005; Sjöling et al. 2006). Hydrolases (protease, amylase, xylanase, cellulase, lipase, esterase, phytase, nitrilase), oxido-reductases (glucose oxidase), transferases (glucosyl transferases, DNA polymerase) or catabolic enzymes for environmental clean-up purposes are targeted by companies such as Diversa Corporation (Gray et al. 2003). Other commercial start-up companies within this market sector have begun to establish themselves, e.g.; Cubist Pharmaceuticals, Genencor International, BRAIN AG, Aventis Pharmaceuticals, Prokarya and Vicuron Pharmaceuticals, and patents have been filed, although at this point few metagenomically-derived products are commercially available (Sjöling et al. 2006).

The combination of metagenomic bioprospecting with genetic engineering by DNA evolution, gene shuffling and gene targeting provide valuable tools for discovering enzymes with desired properties. However, requirements such as the yield of recombinant enzyme production will have to be met for industrial needs in process engineering (Antranikian et al. 2005).

18.5 Metagenomics of low temperature environments

The low-temperature environments represent an enormous untapped resource. Given that the largest proportion of the Earth's biomass exists in low temperature environments, largely marine (Siddiqui and Cavicchioli 2006), this biomass represents a resource for identification of new species, new processes and new genes (DeLong 2005).

To date, the overwhelming majority of metagenomic enzyme discovery has been made from samples of temperate soil (Lorentz and Eck 2005). However, low temperature active enzymes have at least biotechnological value since many biotechnological processes (e.g., dairy and fruit juice processing) rely on efficient

bio-catalysts that operate around ambient temperature (Siddiqui and Cavicchioli 2006). Enzymes from low temperature active isolates have been shown to possess properties suitable for such applications, most especially high catalytic activity at low temperatures. These catalytic properties derive from low activation enthalpies, as a result of high protein flexibility around the active site. However, the consequence is that cold-active enzymes typically show low stability at higher temperatures. For detailed reviews of low temperature active enzymes and their applications, see Chapters 13, 14 and 20 in this book, and Margesin and Schinner (1999), Cavicchioli et al. (2002), Feller (2003), Antranikian et al. (2005), D'Amico et al. (2006) and Siddiqui and Cavicchioli (2006).

Thus far, few low-temperature active enzymes have been isolated and characterized by metagenomic expression screening. These include novel nitrilases from the deep sea and polar regions (Robertson et al. 2004), beta-lactamases, important in resistance of antibiotics, from cold-seep sediments (Song et al. 2005) and a cold-active xylanase of waste water (Lee et al. 2006a). A novel lipase of Baltic Sea marine sediment was shown to be both low-temperature active and thermo-sensitive (Hårdeman and Sjöling 2007). In this study, protein regions possibly implicated for low-temperature adaptation were identified by protein structure modelling (Sjöling, unpublished).

Due to their diversity and low level of current exploitation, low-temperature environments are predicted to provide a rich resource for future identification of commercially interesting cold-active compounds, including enzymes, other functional proteins (e.g., cryoprotectants), lipids and small molecules (Podar and Reysenbach 2006).

Metagenomics will undoubtedly contribute to an ecological and evolutionary perspective of low-temperature life (species diversity, molecular adaptations, metabolic pathways). Possibly as a consequence of convenience of handling water samples, most metagenomic analysis of low temperature environments have been made using marine samples. Early studies sequenced large DNA fragments of marine bacteria and archaea from BAC or fosmid libraries (Stein et al. 1996). For example, a DNA polymerase was identified in the genome of an uncultivated archaeon associated with a marine sponge (Schleper et al. 1997). Similarly, the phylogenetic diversity and horizontal gene transfer processes of crenarchaeota in deep Antarctic waters were revealed via large DNA fragment sequencing (Lopez-Garcia et al. 2004), and genome fragment analysis of Antarctic bacterioplankton revealed a novel deltaproteobacterial group (Moreira et al. 2004, 2006). Hallam and co-workers sequenced marine sediment fosmid clones and showed that an uncultured archaeon contained the gene complement required to oxidize methane (Hallam et al. 2004). DeLong and co-workers sequenced fosmid clones (approximately 30 kb) containing genomic fragments of marine microbial communities isolated from various ocean depths from globally distributed sites, in an attempt to correlate microbial diversity to nutrient status, salinity, temperature and light availability in the ocean. Analyses of the depth distribution of microbial genes suggested depth-variable community trends in carbon and energy metabolism (DeLong et al. 2006). Rohwer and co-workers recently employed whole community sequencing for comparative metagenomic analyses in order to investigate the microbial diversity of two different

low-temperature deep mine communities (approximately 11°C at 700 m below surface; Edwards et al. 2006). Biogeochemical analyses and comparative metagenomics showed that the different communities performed distinct biochemistry on available substrates. For example, carbon utilization, iron acquisition, nitrogen assimilation and respiratory pathways were found to be different between the two communities. Results from this study indicate that comparative metagenomics may be a tool to identify genes, pathways and subsystems common in a particular environment (Edwards et al. 2006).

A recent study of the metagenome of symbionts of a marine benthic oligochaete, by researchers at DOE JGI and collaborators, provided an insight into how resources (available electron acceptors and donors) were shared between the symbionts and the worm, and how different metabolic pathways are used by the symbionts to generate energy as the worm shuttled between oxic and deeper anoxic sediment (Woyke et al. 2006).

While most extreme low-temperature environments are those of polar regions, few detailed metagenomic studies of these habitats have yet been reported. Metagenomic sequencing of six fosmid clones of Antarctic bacterioplankton living at water temperatures of <2°C yielded numerous open reading frames identified as putative protein sequences, including putative proteins involved in cellular roles, such as protein folding, protein synthesis, DNA metabolism, transcriptional regulation, and DNA binding (Grzymski et al. 2006). The authors also performed a comparative analysis of translated metagenomic sequence data. In comparison with mesophilic sequence data, the Antarctic sequences showed a reduction in salt-bridge-forming residues (arginine, glutamic acid, and aspartic acid), reduced proline contents and a reduction in stabilizing hydrophobic clusters. Stretches of disordered amino acids were significantly longer in the Antarctic sequences than in the mesophilic sequences (Grzymski et al. 2006).

Viruses are important in controlling bacterial numbers and affect microbial evolution. Recent analysis of the viral metagenome in different oceanic regions, including arctic marine waters, supported the idea that viruses are widely dispersed and that local environmental conditions enriched for certain viral types through selective pressure (Angly et al. 2006).

While several metagenomic sequence studies have been made on samples collected from low temperature environments, few have focused on the aspect of low temperature adaptation as a major objective of the analysis. Future metagenomic studies are therefore expected to provide specific information on genetic and metabolic adaptation to low temperature environments.

18.6 Novel applications

18.6.1 Comparative community genomics and pyrosequencing

New approaches for the analysis of microbial community genome data continue to be developed (DeLong 2005). Potentially, one of the most powerful and informative methods is comparative metagenomics; the analyses of the total microbial community

genome pool of one environment compared to that of another. This approach is not limited to the comparison of just two metagenomes, but given sufficient sequence data and computational power, could be extended to multi-metagenome analysis, thereby giving access to issues of micro- and macro-heterogenity, temporal change, short- and long-term evolutionary processes, incremental changes in environmental parameters, and much more.

Tringe et al. (2005) compared sequences from more complex environments such as agricultural soil in Minnesota (estimated to contain 3,000 ribotypes) and decomposing deep sea whale carcass samples (estimated to 25–150 ribotypes) (Tringe et al. 2005). Although up to 100 Mbp were sequenced in each study, insufficient sequence coverage was obtained to fully assemble any microbial genomes. This is not surprising, since 100 Mbp of sequence corresponds to little more than 20 complete bacterial genomes. Instead, the authors used the unassembled and partially assembled sequence fragments, so-called environmental gene tags or EGTs, to compare gene distribution to metagenomic sequence data from acid mine drain and the Sargasso Sea samples. By comparisons on four different hierarchical levels (individual genes, operons (neighbouring genes), higher-order cellular processes (KEGG pathways) and functional roles (broad functional categories from COG), both similarities and clear distinctions between the samples from the different environments were identified (Tringe et al. 2005). Furthermore, the gene complement of the analyzed communities could be used as a fingerprint, where comparison between the communities was gene-centric, rather than genome-centric (Tringe et al. 2005). Specific physiological traits of the community, such as the primary energy sources used and the dominant biosynthetic pathways present, could be defined. For example, the soil communities contained a higher frequency of genes associated with starch and sucrose metabolism and polyketide synthesis. The latter was taken as evidence for the prevalence of taxa possessing defence mechanisms (such as Actinomycetes). In contrast, the whale carcass communities were over-represented in genes associated with bacterial chemotaxis. The ability of cells to physically translate to resource rich habitats might well be taken as an adaptive response to existence in primarily oligotrophic environments (Tringe et al. 2005).

The development of a new sequencing technique, pyrosequencing, by Drs. M. Uhlén and P. Nyrén at the Royal Institute of Technology in Stockholm (Ronaghi et al. 1998) has presented a fundamentally different route for rapid high-through-put sequencing. By combining pyrosequencing with emulsion-based PCR and mass scaling (Margulies et al. 2005), a rapid and cost-effective sequencing alternative is offered by the company 454 Life Sciences/Roche Diagnostics (GS 20). More recently, pyrosequencing has successfully been applied to metagenomic studies (Angly et al. 2006; Edwards et al. 2006). The low cost, high yield of pyrosequencing and the possibility of circumventing a cloning step are advantages, see next section. On the other hand, biases from whole genome amplification and the problem of short sequence read length, up to 300 bp, are limitations. However, in the near future increasing sequence lengths of 500 bp or more can be expected. In a study of the microbial ecology of a deep mine in Minnesota, 70 Mb of sequence data from two different (oxidized and reduced), but physically close, sites were obtained and used

in comparative analysis using the curated SEED database (http://theseed.uchigago.edu/FIG/index.cgi). Respiration pathways, nitrogen assimilation, iron acquisition and sulfur metabolism were differentiated between the two communities (Edwards et al. 2006). Comparison with other available metagenomes of acid mine drainage (Tyson et al. 2004) and Minnesota farm soil (Tringe et al. 2005) showed, not unexpectedly, organismal differences that were reflected in the individual biochemistries of the samples, i.e. protein synthesis, production of secondary metabolites, fatty acid metabolism and membrane transport (Edwards et al. 2006).

Another recent advance which may facilitate metagenomics is whole genome amplification. This procedure allows the amplification of very low amounts of linear genomic DNA (using Phi29 DNA polymerase with random primers), circumventing the metagenomic direct cloning procedure and thus eliminating the problems that are associated with this step of metagenomics, for example the requirement of at least microgram scales of DNA and counter selection against potentially toxic genes (see Edwards et al. 2006 and references therein). In this way, samples with very low cell densities can be analyzed. However, whole genome amplification may introduce an amplification bias depending on for example template accessibility and priming efficiency (Abulencia et al. 2006; Yokouchi et al. 2006).

18.6.2 Metatranscriptomics and metaproteomics

The metagenomic complement may reflect the functional capacity of a community, but it gives no information on the actual metabolic activity of individual cells or of the community as a whole. In contrast, studies of gene expression, at either the RNA or protein level, can provide a critical insight into metabolic activities under different environmental conditions. In this context, community data obtained by metagenomic methods facilitate both mRNA expression micro-array analysis and "meta" proteomics studies of whole communities (Ram et al. 2005). In addition, genes that are expressed under any given condition in the environment can be selected through reverse-transcriptase PCR of metagenomic mRNA, taking into account the limitations inherent in this process, as for eukaryotic cDNA library construction (Wilson et al. 1999). As the technology for metatranscriptomic analysis matures, it is probable that it will both complement and strengthen metagenomic analysis.

18.7 Dealing with high volumes of metagenomic sequencing

The information generated from metagenomic sequencing is huge, making the task of assembly and annotation a challenge, especially as these procedures are in early stages of development. Each sequencing centre (Joint Genome Institute, the Venter Institute, 454 Life Science sequencing) follow their specific assembly and annotation

process (Markowitz et al. 2006). Traditional assembly and annotation algorithms are less suitable for metagenomic whole genome shot-gun or pyrosequencing data due to immense wealth of short fragmented sequences (Chen and Pachter 2005). In response, new standardized ways of assembly, annotation (Tringe et al. 2005), management and archiving are being developed (Integrated Microbial Genomes/ Management IMG/M; Markowitz et al. 2006). Bioinformatics strategies have been developed for phylogenetic classification of metagenomic sequence fragments based on a self-organizing map (SOM; Abe et al. 2005) of tetranucleotide frequencies as they vary significantly among species genomes (Teeling et al. 2004; Web server TETRA). Other tools developed are databases (i.e. Megx.net for marine metagenomes; Lombardot et al. 2006) and computer programs, for example, MetaGene, for identifying prokaryotic genes from environmental genome shotgun sequences (Noguchi et al. 2006; MetaClust, (references in Woyke et al. (2006), MEGAN (MEtaGenome Analyzer, http://www-ab.informatik.uni-tuebingen.de/software/megan/welcome.html) or via CAMERA (Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis) that allows analysis of large metagenomic datasets. Many more tools are expected to be developed for the management and analyses of environmental genomics sequence data.

18.8 Conclusions

Despite its recent development as a technology, metagenomics has proved to be a unique and extremely powerful tool for understanding the genetic diversity and functional capacity of microbial communities. Cheaper and faster sequencing and assembly methods have spurred the production of increasingly complex datasets while the development of expression screening techniques has facilitated the detection of novel gene products. In addition to widening access to new products for biotechnological purposes, metagenome analysis can contribute to our understanding of both large scale (macro-ecological) and small-scale (molecular) processes. For example, comparisons of homologous genes and proteins from the metagenomes of different thermal environments should lead to a better understanding of the catalytic mechanisms and molecular adaptations of psychrotrophs (Ferrer et al. 2005).

Many hurdles remain to be resolved. For example, the linkage between phylotypic signal and organismal function remains one of the primary developmental objectives of the field of metagenomics. In addition, the difficulties in efficiently expressing active proteins in heterologous hosts remain a substantial limitation in the scope of this technology that aims to access new regions of sequence space.

The metagenomic analysis of low-temperature microbial communities, (diversity, community structure and identification of novel gene products) has only begun and potential of this technology to expand our understanding of these systems is extremely high.

The rapid development of high throughput DNA sequencing and powerful bioinformatic analyses will enable comparative studies that will give further insights

into the processes driving microbial genome dynamics, the mechanisms of gene mobility, and adaptation and evolutionary processes. The analysis of horizontal gene pool mediators (phages, plasmids, transposons) is vital in order to further understand the spread of functional abilities and fitness within communities for evolution and adaptation to a changing environment (Whitley et al. 2005; Angly et al. 2006; Deutschbauer et al. 2006). Current research on genome plasticity and fluidity has enabled the identification of pathogenicity islands (secretion systems, genes for toxins, invasins, adhesins) in the genomes of environmental bacterial isolates (Hagström, personal communication) that has furthered our understanding of the spread and evolution of pathogenicity and resistance.

Additional metagenomic sequencing, together with analysis of existing community phylogenetic data, will facilitate the modelling of microbial interactions and ecological processes in different communities (DeLong et al. 2006). Importantly, it has been demonstrated that by combining comparative metagenomics and biogeochemical analysis, the microbiology of different environments can be correlated with in situ chemistry to define ecological niches (Edwards et al. 2006) and to understand the physicochemical factors which drive microbial community development, stability and evolution.

Finally, it is widely predicted that, due to the huge advances in high throughput sequencing and the associated reduction in bulk sequencing costs, the volume of accessible environmental sequence data will rise dramatically. The challenge of molecular ecologists in the next decade (and beyond) will be to use this data to answer some of the fundamental and unanswered questions relating to the function and importance of microorganisms and microbial communities in global processes. We will be required not only to model but also to validate hypotheses on such critical issues as regulation of habitat-specific processes (regulation of population and community specific metabolism and biogeochemical transformations), interactions within and between populations, and interactions with the wider environment, (DeLong 2005; Markowitz et al. 2006).

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Chapter 19
Proteomic Studies of Psychrophilic Microorganisms

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19.1 Introduction

Research on psychrophilic microorganisms has entered the post-genomic era [in this chapter, psychrophilic and psychrotolerant (or psychrotrophic) microorganisms are not distinguished, and the term "psychrophilic microorganisms" is used throughout]. As of December 2006, complete genomic DNA sequences are available for the following eight psychrophilic microorganisms according to Genomes OnLine Database (http://www.genomesonline.org/): *Desulfotalea psychrophila* LSv54 (Rabus et al. 2004), *Photobacterium profundum* SS9 (Vezzi et al. 2005), *Psychrobacter articus* 273-4 (unpublished), *Colwellia psychrerythraea* 34H (Methe et al. 2005), *Pseudoalteromonas haloplanktis* TAC125 (Medigue et al. 2005), *Methanococcoides burtonii* DSM6242 (unpublished), *Psychrobacter cryohalolentis* K5 (unpublished), and *Psychromonas ingrahamii* 37 (unpublished). In addition, genome projects on many other psychrophilic microorganisms, including *Flavobacterium psychrophilum*, *Polaribacter irgensii* 23-P, *Psychroflexus torquis* ATCC 700755, *Psychromonas* sp. CNPT3, *Shewanella livingstonensis* Ac10, *Shewanella violacea* DSS12, and *Vibrio salmonicida* LFI1238, are in progress. These sequence data provide a framework for fundamental and application studies of psychrophilic microorganisms. With the aid

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of these data, it is possible to make global identification of proteins expressed under a particular growth condition. In this chapter, proteomic studies of psychrophilic microorganisms, which will give us an important clue to elucidate their cold-adaptation mechanism, are reviewed.

19.2 Global identification of proteins produced at low temperatures

Living organisms, including psychrophilic microorganisms, produce different sets of proteins depending on environmental temperatures. Because proteins that are inducibly produced at low temperatures are supposed to play important roles at low temperatures, many studies have been carried out to identify cold-inducible proteins to gain insight into cold-adaptation mechanisms of psychrophilic microorganisms.

19.2.1 Identification of cold-inducible proteins without whole genome sequence information

Although identification of cold-inducible proteins has been greatly facilitated by whole genome sequence data, identification of several cold-inducible proteins were reported before whole genome sequence data were available. One example is the identification of cold-inducible proteins from *Arthrobacter globiformis* SI55 (Berger et al. 1996, 1997). By comparing proteins of this bacterium grown at different temperatures, Berger and coworkers found that CapA, a homolog of CspA from *Escherichia coli*, is inducibly produced at low temperatures. CspA from *E. coli* binds to single-stranded nucleic acids and functions as an RNA chaperone. CapA was produced very rapidly following cold shock, but unlike its mesophilic counterparts, it was still expressed during prolonged growth at low temperatures. Growth resumption following a temperature downshift correlated with CapA expression. Based on these findings, it was suggested that low-temperature acclimation is conditioned mainly by the ability of cells to restore an active translational machinery after cold shock.

Another example is the identification of peptidyl-prolyl *cis-trans* isomerase as a cold-inducible protein of *Shewanella* sp. SIB1 (Suzuki et al. 2004). This bacterium grows most rapidly at 20°C and can grow at temperatures as low as 0°C. Total soluble proteins extracted from the cells grown at 4°C and 20°C were analyzed by two-dimensional gel electrophoresis, and it was found that the amount of an FKBP family member protein with peptidyl-prolyl *cis-trans* isomerase activity increased at 4°C compared to that at 20°C. The results suggest that this protein facilitates protein folding when the bacterium grows at low temperatures.

Cold-inducible proteins have also been determined in other psychrophilic microorganisms such as *Pseudomonas fragi* and *Rhizobium leguminosarum* (Michel et al. 1997; Hebraud and Potier 1999; Drouin et al. 2000). Although these works identified several cold-inducible proteins of psychrophilic

microorganisms, the number of cold-inducible proteins identified has greatly expanded by proteomic studies based on the whole genome sequence information as described below.

19.2.2 Methanococcoides burtonii

M. burtonii is a flagellated, motile methanogen isolated from permanently cold (1–2°C), methane-saturated waters from the bottom of Ace Lake, Antarctica. Cold-inducible proteins that are predicted to be important for cold adaptation of this archaeon were examined by comparing two-dimensional electrophoresis profiles for cells grown to late log phase at low temperature (4°C) and T_{opt} (23°C) (Goodchild et al. 2004). Cold-inducible proteins were also identified by isotope-coded affinity tag chromatography and liquid chromatography-mass spectrometry (Goodchild et al. 2005). Proteins identified in these studies are listed in Table 19.1.

These studies revealed that key aspects of cold adaptation of this archaeon relate to transcription, protein folding and metabolism. In particular, specific roles for RNA polymerase subunit E, a response regulator and peptidyl-prolyl *cis-trans* isomerase were suggested. RNA polymerase subunit E was unique to growth at 4°C. This protein may fulfill a specific role in regulating the transcription of genes involved in low-temperature growth or in facilitating transcription at low temperatures in general. A response regulator consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain was also unique to growth at 4°C. The increased abundance of the response regulator reinforces the finding that cold adaptation involves transcriptional regulation, and that it may involve a temperature-responsive two-component regulatory system similar to those found in bacteria. Peptidyl-prolyl *cis-trans* isomerase was one of the most abundant proteins with higher (3.1-fold) spot intensity at 4°C and probably plays an important role in protein folding at low temperatures.

Proteins with increased spot intensities at 4°C included the 40 kDa subunit of $F_{420}H_2$ dehydrogenase involved in methanogenesis. $F_{420}H_2$ dehydrogenase is a membrane-bound proton pump, which generates a proton gradient that drives most cellular processes including ATP synthesis. In methylotrophic methanogens, ATP can also be produced by a sodium motive force. However, at low temperatures, a proton motive force is easier to maintain than a sodium motive force. Higher level of $F_{420}H_2$ dehydrogenase at 4°C probably leads to the generation of a proton motive force, which is preferred in *M. burtonii* during low-temperature growth.

19.2.3 Bacillus psychrosaccharolyticus

B. psychrosaccharolyticus is a facultative anaerobic sugar-digesting bacterium found in soil and lowland marshes. It grows well in glucose broth under anaerobic conditions. It grows at 0°C, but does not grow at temperatures above 30°C. Spores are produced at 0°C and germinate at 0°C. Proteomic analysis was conducted for this bacterium to identify abundantly expressed proteins and cold stress response

Table 19.1 Proteins of psychrophilic microorganisms inducibly produced at low temperatures[a]

Micro-organisms	*M. burtonii*[b]	*B. psychrosaccharolyticus*	*P. cryohalolentis* K5	*P. articus* 273–4	*S. livingstonensis* Ac10
Temperature	4°C/23°C[c]	0°C or 15°C/30°C[d]	−4°C/16°C or −4°C/4°C[e]	4°C/22°C or 4°C-S/22°C-S[f]	4°C/18°C[g]
Transcription	RNA polymerase subunit E (>1.8), Response regulator (> 2.7), TATA-box binding protein (1.7)	Transcription elongation factor	Antitermination factor (NusA) (3.37[h])	DNA-directed RNA polymerase, 30–40 kDa subunit: RNA polymerase α chain, bacterial and organelle (2.6)	DNA-directed RNA polymerase α subunit (RpoA) (26), Transcription elongation factor (GreA) (86)
Translation	SSU ribosomal protein (2.0), Peptidyl-prolyl *cis-trans* isomerase (3.1)	SSU ribosomal protein S30P, Chaperonin HSP10	Ctc form of ribosomal protein L25 (RplY) (1.33), Ribosomal protein S2 (RpsB) (2.18[h]), Cold shock protein (CspA) (>25), GTP-binding protein or elongation factor Tu (TypA) (2.00 & >6.6[i]), Elongation factor Ts (Tsf) (>8.9)	Ribosomal protein S15 (3.2[j]), tRNA synthetases, class II (G, H, P and S): seryl-tRNA synthetase class IIa: aminoacyl-tRNA synthetase, class II (2.0), Translation elongation factor Tu (EF-Tu) (3.8), Putative chaperonin HSP60 family (2.8), Peptidyl-prolyl *cis-trans* isomerase, cyclophilin type (2.2), Putative chaperonin HSP10 (unique)	Cold shock protein (CspA) (577), GTPase-translation elongation factor (TufB) (33), Translation elongation factor P/ Translation initiation factor (Efp) (39), FKBP type peptidyl-prolyl *cis-trans* isomerase (Trigger factor) (Tig) (5.6), Lysyl-tRNA synthase class II (LysU) (120)

(continted)

Table 19.1 (continued)

Metabolism	Methyl coenzyme M reductase, α subunit (2.3), Methyl coenzyme M reductase, β subunit (2.0), Methyl coenzyme M reductase, γ subunit (2.0), Trimethylamine methyltransferase (>3.3 & 4.2[i]), $F_{420}H_2$ dehydrogenase 40 kDa subunit (3.6), Methylcobalamine: CoM methyltransferase (2.0), Monomethylamine methyltransferase (2.0), Pyridoxine biosynthesis protein (2.6), Riboflavin biosyntesis protein (2.5), Dihydroorotate dehydrogenase (3.7), 3-Isopropylmalate dehydratase (>2.1), Adenosylhomocysteinase (>6.7), Glutamate dehydrogenase (5.9), Isopropylmalate synthase (2.1), Glyceraldehyde 3-phosphate dehydrogenase (2.4)	Glyceraldehyde 3-phosphate dehydrogenase, Vegetative catalase 1, Catalase, Electron transfer flavoprotein α subunit, ATP synthase (subunit β), Pyrimidine nucleoside phosphorylase, Deoxyribose-phosphate aldolase, Succinyl-CoA synthetase (α subunit)	Malate/lactate dehydrogenase (Mdh) (3.34[h]), Isocitrate lyase (AceA) (2.78), Acetate kinase (AckA) (2.11), F_1/F_0 ATP synthase β subunit, H^+/Na^+ translocating (AtpF) (>17), NH_3-dependent (glutamine-hydrolyzing) $NAD(^+)$ synthetase (NadE) (2.70), Glycine dehydrogenase (2.37), Shikimate 5-dehydrogenase (AroE) (>10), Amino transferase (>7.1), Xanthine dehydrogenase (XdhA) (>8.4), Pyridine nucleotide transhydrogenase (2.00)	d-3-Phosphoglycerate dehydrogenase (2.1[j]), Aminotransferase (2.1[j]), Fumarate hydratase class II (fumarase) (4.5), Possible acetone carboxylase γ subunit (3.1), δ-Aminolevulinic acid dehydratase (3.4), Nucleoside diphosphate kinase (2.3), Aldehyde dehydrogenase family protein (2.7)	Inorganic pyrophosphatase/ exopolyphosphatase (Ppx1) (34), Phosphoribosylamine-glycine ligase (PurD) (45), Deoxyribose-phosphate aldolase (DeoC) (62), Electron transfer flavoprotein α-subunit (FixB) (80), NADPH: flavin oxidoreductase (NemA) (52), Pyridoxal phosphate biosynthesis protein (PdxJ) (55), Predicted extracellular nuclease (202), Predicted carboxypeptidase (31)

(continued)

Table 19.1. (continued)

Micro-organisms	*M. burtonii*[b]	*B. psychrosaccharolyticus*	*P. cryohalolentis* K5	*P. articus* 273–4	*S. livingstonensis* Ac10
Transport		ABC transporter-ATP-binding protein	ATPase of ABC transporters with duplicated ATPase domains (Uup) (2.78), ABC-type Fe^{3+} transporter, periplasmic component (AfuA) (2.38[h]), TRAP-T family transporter, substrate binding subunit (DctP) (1.41[h]), Outer membrane efflux system (TolC) (>12), ABC lipoprotein exporter, ATPase (LolD) (>8.9), Outer membrane receptor for Fe^{3+} dicitrate, TonB dependent (FecA) (>7.5)		Porin (OmpC) (3.6), Outer membrane protein A (OmpA) (611)
Cell division					Cell division GTPase (FtsZ) (39)
Motility					Flagellar basal body and hook protein (FlgE) (26), Flagellin and related hook-associated protein (FlgL) (32)
Others		General stress protein 17M	Chemotaxis protein histidine kinase (CheA) (2.04), Hydroperoxide detoxification protein (OsmC) (>14)	Putative PhoH-like protein, predicted ATPase (3.0), Putative cold-shock protein (unique)	6Fe-6S prismane cluster-containing protein (33), Predicted outer membrane protein (51), Nucleotide-binding outer membrane protein (Tsx) (63), Transposase (143)
References	Goodchild et al. (2004, 2005)	Seo et al. (2004)	Bakermans et al. (2007)	Zheng et al. (2007)	Kawamoto et al. (2007)

(continued)

Table 19.1 (continued)

[a] Relative abundance at indicated temperatures is shown in parenthesis. The value is not available for *B. psychrosaccharolyticus* in the literature

[b] TATA-box binding protein and monomethylamine methyltransferase were identified by using isotope coded affinity tag chromatography and liquid chromatography-mass spectrometry (Goodchild et al. 2005). All the other proteins were identified by comparing two-dimensional electrophoresis profiles (Goodchild et al. 2004)

[c] Cells were grown to late log phase at indicated temperatures

[d] Cells were grown to mid-log phase at 30°C and further incubated at indicated temperatures for 1 h.

[e] Cells were grown at indicated temperatures until OD_{600} reached 0.22

[f] Cells were acclimatized to indicated temperatures by cultivation in 1/2 tryptic soy broth with or without 5% NaCl. "-S" indicates that cells were grown with 5% NaCl

[g] Cells were grown to early stationary phase at indicated temperatures

[h] Relative abundance for −4°C/4°C

[i] Two spots appeared on the 2-D PAGE gel

[j] Relative abundance for 4°C-S/22°C-S

proteins (Seo et al. 2004). The cells were grown to mid-log phase at 30°C and further incubated at 0°C, 15°C and 30°C for 1 h. Proteins extracted from the cells were analyzed by two-dimensional electrophoresis. Proteins inducibly synthesized by temperature downshift are listed in Table 19.1. Cold-induced proteins in *B. psychrosaccharolyticus* were similar to the proteins expressed for the cold shock response in *B. subtilis* (Brigulla et al. 2003). Cold-induced proteins included chaperonin HSP10. It was suggested that recomposition of proteins involved in metabolic functions and stress response is a major factor for psychrophilicity of *B. psychrosaccharolyticus*.

19.2.4 Psychrobacter cryohalolentis *K5*

P. cryohalolentis K5 is a Gram-negative, non-motile bacterium isolated from Siberian permafrost. It grows at temperatures from −10 to 30°C. Patterns of protein abundance were examined for the cells grown at 16, 4, and −4°C (Bakermans et al. 2007). It was found that growth temperature substantially reprogrammed the proteome: about 31% of the proteins at each growth temperature responded to temperature. Proteins whose relative abundance increased at −4°C are listed in Table 19.1.

Two ribosomal proteins (S2 and the Ctc form of L25) were inducibly synthesized at −4°C, suggesting that these proteins specifically contribute to ribosomal function at low temperatures. S2 and Ctc are among the eight ribosomal proteins (of 53 total) that are transcribed individually in *P. cryohalolentis* K5, suggesting that they are regulated independently of the "core" of ribosomal proteins. Two elongation factors (EF-Ts and TypA) and CspA, which probably exhibits RNA chaperone activity, were also induced at low temperatures. Induction of these proteins involved in translation suggests that modulation of translation machinery is required for the growth of this bacterium at subzero temperatures.

Six transport-related proteins, AfuA, FecA, LolD, TolC, DctP, and Uup, were up-regulated at low temperatures. These proteins are probably required to counteract lower rates of diffusion and transport across the membrane at low temperatures. Induction of the lipoprotein transporter, LolD, suggests an increased need for lipoproteins for maintaining fluidity of the membrane or activity of membrane proteins at low temperatures. The observation that two of the four iron transporters of this bacterium, AfuA and FecA, were up-regulated at low temperatures suggests that this bacterium employs cold-adapted alleles to ensure adequate transport of iron into the cell.

Two enzymes of the glyoxylate cycle, malate dehydrogenase and isocitrate lyase, were up-regulated at −4°C. Acetate kinase, which is involved in introduction of acetate into the glyoxylate cycle, was also up-regulated at low temperatures. Acetate was the only carbon and energy source provided in these experiments, and these enzymes probably contribute to efficient assimilation of the carbon source and generation of energy. This speculation is supported by the fact that a possible acetate transporter, DctP, is inducibly produced at −4°C.

Another cold-inducible protein, OsmC, is supposed to detoxify organic hydroperoxides that are produced during aerobic respiration. Oxidative stress increases at low temperatures because the concentration of oxygen radicals is higher at low temperatures due to higher solubility and slower consumption of oxygen. It is likely that OsmC counteracts higher oxidative stress at low temperatures.

19.2.5 Psychrobacter articus *273-4*

P. articus 273-4 was isolated from a 20,000- to 40,000-year-old Siberian permafrost core, which is characterized by low temperature, low water activity, poor nutrition, and high salinity. It survives at −10°C with optimal growth rate at 22°C. To explore how it survives in the permafrost environment, proteins in four samples of cells cultured at 4 and 22°C in media with and without 5% sodium chloride (saline medium and nonsaline medium, respectively) were profiled and comparatively studied by two-dimensional HPLC and MS (Zheng et al. 2007). Proteins up-regulated at 4°C are summarized in Table 19.1. These proteins are supposed to increase the resistance of cells to the cold environment.

When nonsaline medium was used, putative chaperonin HSP10 and putative cold-shock protein were detected only in the cells grown at 4°C in nonsaline medium. In addition, HSP60 and peptidyl-prolyl *cis-trans* isomerase (cyclophilin type) were inducibly produced at 4°C in nonsaline medium. HSP60, together with HSP10, prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions. The overexpression of HSP60 is consistent with the increase of HSP10 at 4°C. Peptidyl-prolyl *cis-trans* isomerase (cyclophilin type) probably accelerates protein folding at 4°C. tRNA synthetase and EF-Tu were also up-regulated at 4°C in nonsaline medium. tRNA synthetase promotes tRNA synthesis, and EF-Tu promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. These results suggest that the modulation of protein expression at the translational level is an important mechanism involved in cellular response to low temperature.

Ribosomal proteins S3, S4, S6, S15, L2, L7/L12, L15, and L28 were observed down-regulated more than two-fold at 4°C in nonsaline medium. The decrease of ribosomal proteins at low temperature suggests that protein synthesis is decreased at low temperature to conserve energy.

It is noticeable that proteins that are significantly regulated due to temperature effects in saline medium differ from those in nonsaline medium by the amount and/or the direction of regulation. For example, HSP60 and peptidyl-prolyl *cis-trans* isomerase (cyclophilin) type were down-regulated more than two-fold in saline medium at 4°C, whereas they were up-regulated more than two-fold in nonsaline medium at 4°C. Down-regulation of these general stress-related proteins at 4°C in saline medium suggests that 4°C is favored over 22°C in saline medium by this bacterium. The phenomenon may be related to the chemical properties of

salt in solution. It is known that the ionic strength and the ion activity of salt in the solution decrease with the decline of the temperature, which results in lower external osmotic pressure exposed on cells in saline media at 4°C than 22°C. High salt concentration probably exposes less stress on cells at 4°C than at 22°C.

19.2.6 **Shewanella livingstonensis *Ac10***

S. livingstonensis Ac10 is a cold-adapted bacterium isolated from Antarctic seawater. The cells grow most rapidly at 18°C, but can also grow well at 4°C. The doubling time of this strain is 2.5 h at 18°C and 8.8 h at 4°C in LB medium. To elucidate the cold-adaptation mechanism of this bacterium, we conducted proteomic analysis based on the draft genome sequence (Kawamoto et al., 2007). The cells were grown at 4°C and 18°C, and soluble and membrane proteins were analyzed by two-dimensional gel electrophoresis. Proteins whose relative abundance increased at 4°C more than two-fold were identified by peptide mass fingerprinting (Table 19.1).

Two transcription-related proteins, RpoA and GreA, were up-regulated at 4°C. RpoA is a subunit of RNA polymerase playing an important role in assembly of RNA polymerase and promoter recognition. GreA induces cleavage and removal of the 3′ proximal dinucleotide from the nascent RNA to control transcriptional fidelity. Since the secondary structure of mRNA is greatly influenced by temperature, different set of proteins and/or different amount of proteins are probably required to ensure efficient and accurate transcription at different temperatures. CspA, which is supposed to function as an RNA chaperone, was also inducibly synthesized at 4°C, suggesting that this protein is required to make the secondary structure of mRNA suitable for translation. Cold-inducible Tig probably contributes to efficient translation at low temperatures. Tig is a ribosome-associated chaperone with peptidyl-prolyl *cis-trans* isomerase activity, which facilitates proper folding of newly synthesized proteins. It is composed of three domains, an N-terminal domain, which mediates association with the large ribosomal subunit, a central substrate binding domain with homology to FKBP proteins showing the peptidyl-prolyl *cis-trans* isomerase activity, and a C-terminal domain of unknown function. As described in Sect. 19.2.1, peptidyl-prolyl *cis-trans* isomerase (termed FKBP22) is inducibly produced at low temperatures in *Shewanella* sp. SIB1. Although Tig of *S. livingstonensis* Ac10 is similar to FKBP22 in that it is supposed to have the peptidyl-prolyl *cis-trans* isomerase activity, it is different from FKBP22 in that Tig has two additional domains, N-terminal and C-terminal domains, that are not found in FKBP22.

Two putative outer membrane porin homologs, OmpA and OmpC, were inducibly produced at 4°C. Homologs of these proteins from other bacteria have been shown to form channels for hydrophilic solutes and play important roles in uptake of nutrients. Up-regulation of these proteins probably counteracts low diffusion rate of solutes at low temperatures and enables efficient uptake of nutrients.

The amounts of hook-related proteins of flagella, FlgE and FlgL, were increased at low temperatures, suggesting that *S. livingstonensis* Ac10 modulates its motility by altering the expression of these proteins. Consistent with this speculation, we found that the cells are more motile at 4°C than at 18°C. The physiological significance of higher motility of this strain at low temperatures remains to be clarified.

19.3 Conclusions

Proteomic studies have been performed for several psychrophilic microorganisms. A set of cold-inducible proteins produced by one psychrophilic microorganism were shown to be different from those produced by another (Table 19.1). The results suggest that different psychrophilic microorganisms take a different strategy to cope with cold environments. Nevertheless, some of those strategies appear to be common in several psychrophilic microorganisms. For example, an RNA chaperone, CspA, is inducibly produced at low temperatures by several microorganisms including *A. globiformis* SI55, *P. cryohalolentis* K5, and *S. livingstonensis* Ac10. Another example is peptidyl-prolyl *cis-trans* isomerase, which facilitates protein folding. Cold induction of this protein was observed for several psychrophilic microorganisms including *Shewanella* sp. SIB1, *M. burtonii*, *P. articus* 273–4, and *S. livingstonensis* Ac10, although the type of peptidyl-prolyl *cis-trans* isomerase inducibly produced at low temperatures is different in different psychrophilic microorganisms: FKBP-type protein, cyclophilin-type protein, and Tig-type protein are produced by these strains. Isomerization of peptidyl-prolyl bonds for proper protein folding is probably a crucial process for many microorganisms to survive at low temperatures. Modulation of RNA polymerase and ribosome is also supposed to be important for the cells to grow at low temperatures because subunits of these complexes are up-regulated at low temperatures in several psychrophilic microorganisms. More distinctive features of proteomes of psychrophilic microorganisms will be clarified by proteomic studies of many other psychrophilic microorganisms whose whole genomic DNA sequences are available and by advanced proteomic studies to identify proteins whose cellular amount is small and separation from other proteins is difficult by conventional methods. Proteomic studies will undoubtedly provide us with more clues to understand how psychrophilic microorganisms adapt to cold environments in the near future, and the information will also be useful for engineering of these microorganisms for biotechnology.

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Part IV
Biotechnological Aspects

Chapter 20
Biotechnological Aspects of Cold-Adapted Enzymes

Adrienne L. Huston

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20.1 Introduction

Beginning with the development of basic tools by our hominid predecessors, humans have continually searched for and utilized novel materials from the natural environment to survive and thrive. Today, our knowledge of the surrounding world extends to the molecular scale as we enter the age of genomics and systems biology, enabling previously unimaginable insight into processes that promise application in the agricultural, energy, food, medical, structural material and textile industries. As environmental concerns arise, biological tools are increasingly replacing harsh chemical and physical means of processing materials and they even harbor promise for creating cost-effective sustainable energy sources. It is imperative that we continue investigating ways in which natural products can offer economical alternatives to traditional industrial processes. Due to the growing and wide-spread use of enzymes in a variety of industrial applications, this review aims to build on previous works (Brenchley 1996; Ohgiya et al. 1999; Gerday et al. 2000; Allen et al. 2001; Cavicchioli et al. 2002) by illustrating

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recent advances and potential opportunities for the biotechnological application of cold-adapted enzymes.

20.1.1 Mining the cold biosphere for biomolecules of biotechnological interest

An estimated 75% of the Earth's biosphere is located in perennially cold (<5°C) environments, including little-explored deep-sea, polar and alpine regions, shallow subterranean systems (i.e. caves), and the upper atmosphere. Organisms that successfully inhabit these environments, including representatives from the archaea (Cavicchioli 2006), bacteria (Deming 2002), and eukarya (Peck 2002) in addition to viruses (Wells and Deming 2006), must possess adaptations that enable growth and activity at low temperature. Indeed, native consortia of cold-adapted bacteria have been observed to catalyze reaction rates comparable to their mesophilic counterparts at warmer temperatures (Pomeroy and Wiebe 2001), and some psychrophilic strains isolated in laboratory environments have been observed to possess doubling times at 4°C approaching those of *Escherichia coli* at 37°C (Feller and Gerday 2003). In order to overcome the detrimental effects of low temperature, cold-adapted organisms have developed numerous strategies to maintain sufficient rates of metabolic activity and survive in environments considered harsh from an anthropocentric point of view. Such adaptive strategies observed thus far include alterations in membrane fluidity (Russell 1997), expression of cold-shock and cold-acclimation proteins involved in transcription and translational processes (Michel et al. 1997), antifreeze/ice-nucleating proteins (Muryoi et al. 2004), production of compatible solutes and exopolysaccharides (Nichols et al. 2005), and enzymes capable of catalyzing chemical reactions at low temperature (Gerday et al. 1997). The necessary cold-adaptation of all aspects of ectothermic organisms suggest that a wide variety of biomolecules may find application in existing and future biotechnological processes, such as the production of polyunsaturated fatty acids for aquaculture (Russell 1997), the use of ice-nucleating proteins for food processing (Li and Lee 1995), and the application of cold-active enzymes for their various beneficial properties.

Despite the great potential that cold environments hold for revealing diverse products and processes which may have countless industrial applications, our knowledge of the ecology, physiology, metabolism, enzymology and genetics of cold-adapted organisms remains limited. The traditional method of investigating novel biocatalysts from microorganisms inhabiting extreme environments relies upon cultivation and isolation in the laboratory using an organic-rich medium and subsequent screening for desired products. Since only a small portion (<1%) of viable microorganisms in a given environment are estimated to be recovered by traditional cultivation techniques (Giovannoni et al. 1990), existing culture collections greatly under-represent the genetic diversity and biotechnological potential of cold-adapted organisms. Fortunately, this state of affairs is changing with rapid advances in technology. New culturing methods, such as the High Throughput

Culturing™ technology developed by Diversa in San Diego, are enabling the cultivation and isolation of a greater proportion of the microbial community living in extreme environments. Furthermore, the emerging field of metagenomics enables the direct extraction and cloning of large environmental DNA fragments, thus bypassing the need to cultivate microorganisms to isolate novel genes encoding enzymes and other biomolecules from microbial communities (Lorenz et al. 2002). High-through-put sequencing techniques and advances in high performance computational power reaching petascale capabilities are enabling the comparison of whole genomes, mining of environmental metagenomic DNA, and prediction of molecular interactions over shorter timescales. This facilitates the identification of gene products that may have industrial applications. When coordinated with functional genomics studies (gene and protein expression studies using proteomics and microarrays) followed by screening, these advances are providing a systems approach to understanding the mechanisms of cold adaptation at both molecular and cellular levels. Because cold, stable regions are so vast (covering an estimated 72% of the Earth's surface), they span many different environments where organisms have adapted to varying levels of pressure, salinity, pH, *uv* irradiation, redox chemistry and water availability. In addition to clarifying the adaptations to low temperature, metagenomic studies in these regions, particularly those focusing on sea ice (Cavicchioli et al. 2002; Deming 2002), hold great promise for increasing our knowledge of natural diversity and adaptation strategies to multiple stressors while revealing novel products of biotechnological interest.

20.1.2 Cold-adapted enzymes

The ability of organisms to synthesize enzymes capable of catalyzing reactions at temperatures approaching the freezing point of water is no small feat. Low temperatures lead to exponential decreases in chemical reaction rates and also tend to increase the compactness of proteins, thus interfering with the conformational movements necessary for catalysis (Rasmussen et al. 1992). Cold-adapted enzymes have generally been observed to possess high specific activity or catalytic efficiency at low and moderate temperatures relative to enzymes derived from mesophilic or thermophilic organisms. In some cases, cold-adapted enzymes have been observed to catalyze reactions at rates up to one order of magnitude higher than those observed for mesophilic counterparts at low and even moderate temperatures (Fig. 20.1; D'Amico et al. 2006). In naturally-evolved enzymes, low temperature activity has generally been associated with low conformational stability as seen by the shift of the apparent temperature optima to lower temperatures and low stability versus chemical denaturants (see Chap. 13). These observations suggested a necessary inverse relationship between low-temperature activity and structural stability and stimulated the investigation of structural factors responsible for these characteristics. Comparative investigations of numerous protein models and crystal structures revealed that cold-adapted enzymes tend to exhibit an attenuation of the strength and number of structural factors known to stabilize protein molecules. Observed amino acid substitutions leading to these characteristics include

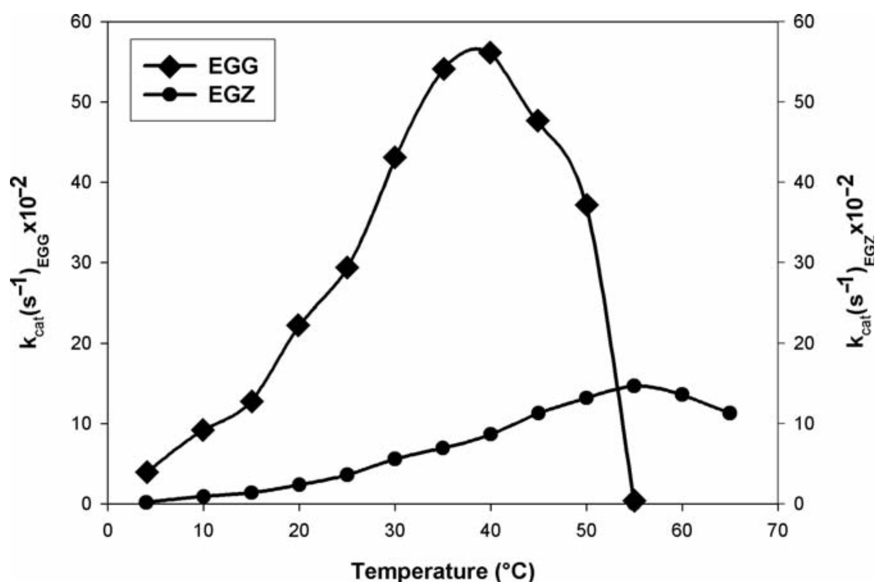

Fig. 20.1 Thermodependence of activity for the cold-adapted cellulase produced by *Pseudoalteromonas haloplanktis* (*EGG*) and its mesophilic homolog produced by *Erwinia chrysanthemi* (*EGZ*); after D'Amico et al.(2006)

decreases in proline residues, aromatic interactions and ion pairs, a re-distribution of residues that facilitate interaction with the solvent, decreased hydrophobic packing (resulting in an entropy-driven effect upon unfolding), and a weaker binding of stabilizing cofactors, to name a few (for a review, see Feller and Gerday 2003).

More recent studies, however, suggest the relationship between low temperature enzymatic activity and conformational stability is more complicated than initially believed based on comparative activity and structural studies alone. Biophysical studies enabling thermodynamic investigations of protein unfolding have revealed that some cold-adapted proteins do indeed exhibit a decrease in global stability relative to mesophilic homologs (D'Amico et al. 2003a), whereas others display decreased stability only in localized areas necessary for substrate accommodation and catalysis (Bentahir et al. 2000; Lonhienne et al. 2001; Zecchinon et al. 2005). These results suggest that localized areas of flexibility and rigidity may be compatible with, if not important for, maintaining activity at low temperatures in certain enzymes. Localized rigidity may even increase the low-temperature activity through a reduction of the entropy of the native state (Lonhienne et al. 2000). Furthermore, site-directed and random mutagenesis experiments performed on cold-adapted enzymes have indicated that low temperature catalytic activity is not necessarily associated with conformational instability (Table 20.1). At present, there appears to be no specific strategy for low-temperature enzymatic adaptation;

Table 20.1 Mutagenesis experiments performed on cold-adapted enzymes indicating that low-temperature activity is not always linked with low structural stability

Enzyme	Organism	Mutagenesis technique	Changes relative to wild-type	Reference
Subtilisin	Antarctic *Bacillus* sp.	Site directed	1 amino acid change at the calcium binding site: 2-fold greater activity and stability	Narinx et al. (1997)
Subtilisin	Antarctic *Bacillus* sp.	Random, saturation mutagenesis and shuffling	7 amino acid change: 3-fold activity increase at 10°C; 500-fold greater stability at 60°C	Miyazaki et al. (2000)
Citrate synthase	*Arthrobacter* sp. DS2-3R	Site directed	Loop insertion: reduced activity T_{opt}; increased stability	Gerike et al. (2001)
Ribonuclease HI	*Shewanella* sp SIB1	Site directed	1 amino acid change: increased both the low-temperature activity and stability	Ohtani et al. (2001)
α-amylase	*Pseudoalteromonas haloplanktis*	Site directed	Several mutants with 1–5 amino acid changes: several maintained low temperature activity when stabilized	D'Amico et al. (2001, 2003b)
Family 42 β-galactosidase	Antarctic *Plannococcus* sp.	Directed evolution	4 amino acid change: 1.5-fold activity increase at 18°C; no change in stability	Panasik (2002)
Chitinase	Antarctic *Arthrobacter* sp. TAD20	Site directed	Point mutations: no direct relationship between stability and low-temperature activity	Mavromatis et al. (2003)
Serine alkaline protease	Antarctic *Shewanella* sp.	Site directed	1 amino acid change: increase in catalytic efficiency and stability	Kulakova et al. (2003)
Family 2 β-galactosidase	Antarctic *Arthrobacter* sp. SB	Site directed and saturation	2 amino acid change: 2.5-fold activity increase at 15°C; no change in stability	Coker and Brenchely (2006)

each protein family may adopt its own set of amino acid substitutions depending upon the selection pressure and substrate catalyzed. It is, furthermore, unclear whether the observed trade-offs in stability and activity of natural cold-adapted enzymes is a result of necessary structural alterations (such as enhanced flexibility in certain areas of a protein's structure to counteract increasing compactness of proteins at low temperature), or a result of random genetic drift and a lack of selective pressure for stable enzymes in perennially-cold environments (Arnold et al. 2001). Regardless, the observation that low-temperature catalytic activity can be separated from structural instability in laboratory-evolved enzymes greatly increases the potential cold-adapted enzymes may hold for industrial applications.

20.2 Industrial enzymes: history and recent advances

The modern history of industrial enzymes dates back to 1874 when the Danish chemist Christian Hansen manufactured an enzyme of relatively high purity for industrial purposes by extracting chymosin from the stomach of calves for the manufacture of cheese. Since then, the estimated global industrial enzyme market has increased substantially, to a current value of USD 2.1 billion (Novozymes 2005), and is expected to grow by 10–15% annually. Chemical catalysts and transformation processes used in industries have traditionally required high temperatures, pressures, or harsh chemical additives to drive reactions. These practices lead to high energy consumption as well as harmful by-products that may have deleterious environmental impacts. Enzymes offer numerous advantages over chemical processes in that they are highly specific in their catalytic activity, exhibit fast reaction rates, operate under mild conditions of temperature and pH, are derived from renewable resources, and are biodegradable. The industrial enzyme market has traditionally been divided into three sectors. Technical enzymes, representing an estimated 65% of sales, are used in the detergent, leather, personal care, pulp and paper, starch, textile, molecular biology, biotransformation, biosynthesis, and, more recently, fuel ethanol industries. Food enzymes, representing about 25% of the market, are used in the baking, brewing, dairy, fats, oils, juice, and wine industries. Feed enzymes comprise about 10% of the market and degrade substances such as phytate, starch, and protein to enhance the digestibility of animal feed (Cherry and Fidanstef 2003).

Although environmentally-friendly, enzymes must be able to compete economically with inexpensive traditional chemical processes. Originally, commercial enzymes were developed from their naturally-producing organisms. This limited the diversity of enzymes that were produced to culturable organisms alone, and resulted in complex mixtures of secreted products at low yields. Currently, over 90% of industrial enzymes are produced recombinantly in fungal or bacterial hosts, such as *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Escherichia coli* (Krishna 2002). The large recent increases in the industrial enzyme market (e.g., an increase in market value of USD 1.5 billion in 2000 to 2.1 billion currently), are in part attributed to developments in recombinant DNA fermentation technologies that enable cost-effective production.

Advances in genetic engineering furthermore have allowed the fine-tuning, development and economical production of improved enzymes that possess industrially-relevant characteristics.

Several techniques can tailor enzymes for specific applications by introducing mutations in a controlled and measurable manner. Rational protein design using site-directed mutagenesis techniques was the earliest approach to protein engineering, and is still widely used if the structural basis of a desired characteristic is known. However, when attempting to engineer certain attributes whose structural bases are currently not understood, such as conformational stability, directed evolution techniques hold greater potential. Directed evolution involves random mutagenesis of a gene encoding a target protein followed by a selection or screening step of the resulting mutant library for protein variants exhibiting the desired property. Random mutagenesis techniques utilized thus far include error-prone PCR, cassette mutagenesis, saturation mutagenesis, DNA shuffling, random-priming recombination, and staggered extension process (StEP recombination) to name a few (Arnold et al. 2001). Mutant genes obtained from the first round may be used as templates for subsequent cycles of mutation and screening. Thus, directed evolution is an iterative process resulting in mutants with the optimal combination of beneficial mutations for a desired characteristic, and is finding wide-spread application in the development of industrially-useful enzymes.

20.3 Industrial potential of cold-adapted enzymes

The scientific interest in cold-adapted enzymes has increased substantially in recent years, as evidenced by the growing number of examples recently isolated and characterized from psychrophilic and psychrotolerant organisms. In much the same way that enzymatic catalysts have replaced numerous traditional chemical processes, cold-active enzymes can be exploited in a variety of industrial applications to good effect. They are beneficial not only for their enhanced selectivity and stereospecificity, but because they can also catalyze numerous reactions at low and moderate temperatures (<40°C) more efficiently and with fewer undesired chemical reactions that may occur at high temperatures, thereby decreasing the overall energy expenditures and processing costs associated with heating steps (Cavicchioli et al. 2002). Their thermal lability can also result in their facile and rapid inactivation by a mild heat treatment (Margesin et al. 2002). Such a characteristic may prove beneficial for preserving product quality in the food industry or for sequential multi-step processes such as those involved in molecular biology. Furthermore, by enhancing our fundamental knowledge of the molecular basis of enzymatic activity and stability, the study of cold-adapted enzymes can guide the fine-tuning of current industrial meso- and thermophilic enzymes for use at lower temperatures while clarifying major issues such as protein folding diseases. Several examples of how cold-adapted enzymes may be used to optimize existing industrial processes and develop new technologies are illustrated below.

20.3.1 Enzymes for detergents and personal care

While the specific detergent ingredients vary between geographic markets and specific uses, the cleaning methods are usually similar. Soil and stain removal from fabrics and hard surfaces (such as dishes) is facilitated by surfactants, alkaline builders, and the hydrolytic activity of a variety of enzymes, including proteases, amylases and lipases. Cellulases can also be used to clean fabrics indirectly by hydrolyzing glycosidic bonds, thus increasing softness and color brightness in cotton fabrics. A major benefit from developing detergents that efficiently hydrolyze soils and stains at low temperatures would be reduced energy consumption resulting in decreased associated costs and environmental impacts. Additionally, garment alterations that take place during warm- and hot-water wash cycles, such as fabric degradation, shrinkage and dye bleeding, will be reduced. Given the trend of decreasing wash temperatures, particularly in Europe and Japan, industrial enzyme companies have started developing detergent ingredients capable of working efficiently under low to medium-temperature conditions. Examples of products from Novozymes include: Kannase®, a detergent protease capable removing stains efficiently at wash temperatures of 10–20°C; Celluzyme®, a cellulase capable of efficient hydrolysis at temperatures down to 15°C; Savinase®, an alkaline protease capable of removing stains at moderate temperatures (45–55°C); and Duranyl®, able to remove starch-based stains at moderate temperatures. Genencor also offers Purafect® Prime and Properase®, two proteases effective under low-temperature wash conditions. Additionally, several patents have been filed for cold-adapted proteases derived from psychrophilic and psychrotolerant bacteria as well as Antarctic krill for use in the detergent industry (Hasan and Tamiya 1997, 1998; Takaiwa et al. 1997; Brenchley et al. 2001; Asenjo et al. 2006). One proteolytic extract in particular, both with and without surfactants and builders, exhibited higher stain removal effectiveness at temperatures between 0 and 37°C when compared with commercially-available enzymes (Brenchley et al. 2001). The use of cold-adapted enzymes may therefore be more economical than mesophilic homologs, as smaller amounts are required to achieve the same effect. Such characteristics of cold-adapted enzymes hold promise for decreasing not only energy requirements of washing but also the costs for manufacturing and use of liquid or granular detergents, stain removers, household cleaners, and industrial cleansing applications.

20.3.2 Food, pharmaceutical and cosmetic industries

Cold-adapted enzymes are particularly attractive for the processing of foods due to their high catalytic activity at temperatures that minimize spoilage and alterations in taste and nutritional values. Their inherent low structural stability also facilitates inactivation once a desired product is attained. Cold-adapted lipases and proteases can lower production costs by serving as rennet substitutes and

accelerating the maturation of slow-ripening cheeses that require specific low-temperature and low-moisture conditions. Cold-adapted proteases can also be used for tenderization and taste improvement of refrigerated meat products (He et al. 2004), and removal of undesirable tissues from seafood, such as the de-scaling and removal of fish skin and extraction of carotenoproteins from shellfish (Bjarnasen and Benediktsson 2001; Shahidi and Kamil 2001). Pectin-depolymerizing enzymes, such as polygalacturonases and pectate lyases, in addition to the debranching activities of various hemicellulases, can be used for the low-temperature degradation of pectin compounds in the fruit and vegetable processing industries (Nakagawa et al. 2004).

Cold-adapted β-galactosidases operating at neutral pH can improve the digestibility of dairy products for lactose-intolerant consumers and enhance sweetness at temperatures which minimize contamination. The activity of cold-adapted β-galactosidases operating at acidic pH can also reduce the pollution impact and increase the technical usefulness of whey (a product of the cheese industry) by producing glucose- and galactose-rich syrups that can be used as sweeteners in a variety of food products and substrates that are easily fermented by alcohol-producing microorganisms (Gerday et al. 2005). Cold-adapted β-galactosidases have also been shown to possess transglycosylation activities where lactose hydrolysis takes place with simultaneous transfer of the monosaccharides to higher oligosaccharides to form tri- and tetrasaccharides (Karasova-Lipovova et al. 2003; Benesova et al. 2005). Production of such galacto-oligosaccharides (either directly in milk or from whey as an additive in dairy products) can be used as additives in probiotic food items to enhance the growth of bifidobacteria in the large intestine, or as low-calorie sweeteners due to their resistance to metabolism in the small intestine.

In baking, enzymes such as xylanases, proteases, amylases, lipases and glucose oxidases, can modify the hemicellulose, gluten, starch and free sulfhydryl groups, respectively, during dough preparation and processing which generally take place at temperatures below 35°C. The combined actions of these enzymes can result in improved elasticity and machinability of the dough, resulting in a larger volume and improved crumb structure. A cold-adapted family 8 xylanase was recently shown to be more efficient in baking and yielded a larger loaf volume when compared with a widely-used commercial mesophilic enzyme preparation, indicating another benefit cold-adapted enzymes hold for the baking industry (Dutron et al. 2005; Collins et al. 2006).

Cold-adapted enzymes that exhibit high catalytic activities at low and ambient temperatures can also be exploited for the pharmaceutical industry. The increasing demand for enantiomerically-pure drugs and pharmaceutical intermediates has led to a rapid expansion of the use of biocatalysis in organic synthesis (Stinson 1998). Because low water conditions favor the synthesis and transesterification reactions of hydrolases, and enhance the solubility of many substrates involved in biocatalytic transformations, enzymes used for many organic synthesis applications must be able to operate efficiently in aqueous/organic and nonaqueous solvents (Schoemaker et al. 2003). As these conditions tend to impair enzyme activity by increasing the activation energy of reactions while limiting the conformational mobility needed for catalysis

(Bordusa 2002), the inherent flexibility of cold-adapted enzymes may be of particular significance when used in solvents that render meso- and thermophilic enzymes inactive (Owusu-Apenten 1999). Indeed, a heat-labile lipase from *Candida antarctica* has already found a broad range of applications, including modification of polysaccharides, desymetrization of complex drug intermediates and resolution of alcohols and amines (Suen et al. 2004). Enzymes produced by organisms found in cold and hypersaline habitats, such as the brine pockets of sea ice, may be particularly adept at catalyzing organic synthesis reactions in aqueous/organic and nonaqueous media, as salt-tolerant enzymes are well-adapted to low water activity conditions (Sellek and Chaudhuri 1999; van den Burg 2003).

In the cosmetic industry, cold-adapted enzymes can enhance the yield of biotransformations involving volatile substrates, such as flavor and fragrance compounds subject to evaporation at high temperatures (Trytek and Fiedurek 2005). Proteolytic enzymes active at ambient temperatures may also find use in topical gel-based treatments for skin scarring, infections and wound-healing (Asenjo et al. 2006).

20.3.3 Biofuels

As the human population continues to grow and exert unprecedented demands on the fossil fuel industry, there is increasing worldwide interest in developing alternative sources of energy. Biofuels, such as ethanol made from the fermentation of carbohydrates produced in plants, represent a renewable energy source that can provide a myriad of other benefits, including increased energy security, a reduction in greenhouse gas emissions, economic benefits for rural communities, and mitigating problems associated with disposal of agro-industrial residues (Wyman 2003). Despite these advantages, to compete with the existing fossil fuel industry and become commercially viable, the current cost of traditional biomass processing for ethanol must be reduced. In the conventional ethanol production process, a starchy feedstock such as corn is first milled and then slurried with water containing a heat-stable α-amylase mixture. This slurry is then cooked at high temperatures (105–150°C) to gelatinize and liquefy the starch in a step called liquefaction. The resulting mash is then cooled to ~60°C and a glucoamylase is added to convert the liquefied starch to fermentable sugars in a process called saccharification. In the final step of the process, yeast is added to the mash and incubated at temperatures below 35°C for 48–55 h to ferment the sugars to ethanol.

Although the conventional ethanol production process is highly optimized, it is not energetically or economically efficient as it requires high heat levels and specialized equipment, thus limiting the production capacity of biorefineries. In fact, some studies suggest the energy demand of the conventional cooking process is equivalent to 10–20% of the fuel value of ethanol produced (Robertson et al. 2006). To address this problem, industrial enzyme companies have been working with partners to develop a low-energy ethanol production process involving raw

starch hydrolysis, also known as cold hydrolysis. This process essentially eliminates the energy- and equipment-intensive liquefaction cooking step. Genencor has developed the STARGEN™ line of fungal enzyme products that contain α-amylases and glucoamylases capable of hydrolyzing uncooked starch in a simultaneous saccharification and fermentation process (SSF) that takes place at 32°C (Shetty et al. 2005). The combined activities of these enzymes catalyze the continuous release of fermentable glucose from granular starch which is directly utilized by the yeast, resulting in a streamlined ethanol production process.

Another industrial enzyme company, Novozymes, has partnered with Broin Companies in the development of Broin's BPX™ technology, a cold hydrolysis process also reliant upon fungal enzymes that efficiently converts starch to fermentable sugars without the traditional cooking process. In addition to significant energy savings, incorporation of the raw starch hydrolysis process into ethanol production offers numerous benefits, including higher ethanol yields, fewer unwanted side products, reductions in plant waste and emissions, and savings on capital expenses, by reducing the necessary equipment. Furthermore, SSF processes result in increased enzyme efficiencies by continuous removal of glucose and maltose during the process of fermentation that otherwise would result in end-product inhibition. At present, high enzyme concentrations are needed to counteract the decreased activity and specificity associated with the cold hydrolysis step, resulting in high enzyme costs. Due to their high activities and specificities at the low temperatures compatible with fermentation (28–35°C; Lin and Tanaka 2006), cold-adapted α-amylases and glucoamylases may afford a reduction in biocatalyst loading and prove to be an economically attractive alternative to the current use of mesophilic fungal enzymes.

Furthermore, while nearly all fuel ethanol is currently produced by fermentation of starchy crop-based sugars, industrial enzyme companies are pursuing methods for inexpensive ethanol production from low-cost lignocellulosic biomass, including agricultural waste, forestry waste, energy crops, and municipal solid waste. The complexity of lignocellulose matter requires the degradative action of many enzymes, and researchers are beginning to file patents for enzymes capable of hydrolyzing lignocellulose at low temperatures compatible with fermentation (e.g., Felby et al. 2006). Cold-adapted glycosyl hydrolases such as cellulases, xylanases and glucosidases may enable cost-effective lignocellulose biomass conversion, thus facilitating the development of an economically-viable and renewable source of fuel to meet the world's increasing energy demands.

20.3.4 Molecular biology

Cold-adapted enzymes can provide a number of benefits for the sequential processes used in molecular biology. A commercial cold-adapted alkaline phosphatase derived from an Antarctic bacterial strain (Rina et al. 2000; commercialized by New England Biolabs), has already found a variety of applications.

The activity of this enzyme catalyzes the removal of 5′ phosphoryl groups from nucleic acids, thus preparing templates for 5′ end labeling and furthermore preventing fragments from self-ligating. The benefit of this enzyme compared to mesophilic phosphatases lies in its heat-lability. Before proceeding to subsequent labeling or ligation steps, the phosphatase activity is easily removed by heating at 65°C for 10 min, thus bypassing the chemical purification steps needed to inactivate most mesophilic phosphatases. Cold-adapted DNA ligases could offer a significant advantage over mesophilic enzymes because ligation yields increase at low temperatures (Georlette et al. 2000). The mesophilic ligases used currently require long incubation times at low temperatures, thus increasing the risk of contamination and residual nuclease activity interference. Most enzymes presently used in molecular biology applications consist of mesophilic enzymes that work optimally with *E. coli* expression systems. However, the interest in developing low-temperature expression systems using psychrophilic hosts (see Chap. 21) will likely create the need for enzymes capable of catalyzing additional reactions at low temperatures (Allen et al. 2001).

20.3.5 Enzyme nanobiotechnology

In the field of materials science, chemical synthesis of mineral/organic composites and silica-based materials, such as nanostructured resins, molecular sieves and electronic materials, have traditionally required caustic chemicals and extremes of temperature, pressure, and pH. In nature, however, large quantities of silica are synthesized biologically from molecular precursors at low temperatures, pressures and neutral pH into complex nanostructures, such as the skeletons of diatoms, radiolarians and sponges, often with a precision that exceeds current engineering capabilities (Kisailus et al. 2005). A study of the mechanisms enabling the biological synthesis of silica spicules in the marine sponge, *Tethya aurantia*, isolated from temperate coastal waters, revealed that protein filaments consisting of catalytically active, structure-directing enzymes (called silicateins) were responsible for the polymerization of silica (Shimizu et al. 1998). Subsequent studies indicated the intact protein filaments and their native subunits were able to directly catalyze the hydrolysis and structurally direct the in vitro polycondensation of silicon and organosilicon to yield silica and silicones under low-temperature conditions (Cha et al. 1999). Since this discovery, numerous enzymatic and enzyme-inspired methods for the synthesis of nanostructured oxides at low temperatures and mild chemical conditions have been illustrated (Table 20.2). Due to the mild synthetic conditions as well as the high selectivity and precision when compared with traditional chemical techniques, these novel methods hold promise for enabling the synthesis of a new generation of polymers containing functional organic molecules. For example, such methods may facilitate the incorporation of enzymes into nanostructured materials, leading to numerous developments, including the design of sensors with environmental and biomedical applications and the synthesis of functional materials capable

Table 20.2 Enzymatic synthesis of nanostructured materials under low temperature and mild chemical conditions

Protein	Reaction catalyzed	Conventional synthetic path	Applications	Reference
Silicatein	Polymerization of silica and silicone at neutral pH and 20°C	Acid or base catalyst	Bio- and chemical sensors, functional nanomaterials, molecular sieves	Cha et al. (1999)
Silicatein	Synthesis of titanium dioxide at 16–20°C and near-neutral pH	Temperatures >500°C	Metal-oxide semiconductor	Sumerel et al. (2003)
Silicatein	Synthesis of spinel gallium oxide at room temperature	Temperatures >400°C	Gas-sensing semiconductor	Kisailus et al. (2005)
Silicatein	Synthesis of bimetallic perovskite-like material at 16°C	Temperatures >340°C, or an acid-promoted reaction	Ferroelectric, piezoelectric transducers, solid oxide fuel cells	Brutchey et al. (2006)
Immobilized silicatein	Fabrication of gold nanoparticles onto biofunctionalized nanowires	Unprecedented	Multidimensional devices of metal and metal oxides	Tahir et al. (2006)
Biomimetic approach (silicatein)	Bio-inspired synthesis of spinel gallium oxide	Unprecedented	Semiconductors	Kisailus et al. (2006)
Fungal protein (*Fusarium oxysporum*)	Synthesis of zirconia nanoparticles at 27°C	Extreme temperature and pH	Structural material: abrasive, coating for cutting tools, high temperature engine components	Bansal et al. (2004)
Fungal protein (*Fusarium oxysporum*)	Synthesis of bi-metallic alloy nanoparticles	Harsh reducing agents	Biomedical	Senapati et al. (2005)

of self-repair. Further investigations will likely reveal additional cold-adapted enzymes capable of synthesizing nanostructured materials at low temperatures and mild conditions, resulting in inexpensive, environmentally-friendly alternatives to traditional synthesis techniques.

20.4 Conclusions

The increasing trend towards sustainable and environmentally-benign industrial practices has spurred the research and development of clean and cost-effective technologies. In the same way that enzymatic catalysts derived from meso- and thermophilic organisms have replaced many harsh conventional processes to great economic benefit, cold-adapted enzymes can be exploited to further decrease industrial energy expenditures and costs. Cold-adapted enzymes are beneficial for their enhanced selectivity and high catalytic activity at low and moderate temperatures, in addition to their structural lability that can be exploited in multi-step processes requiring rapid and mild inactivation treatments. Furthermore, the inherent conformational plasticity of cold-adapted enzymes may be particularly suited to organic synthesis applications under the low water conditions used during the production of many fine chemicals and pharmaceutical intermediates. Despite their many benefits, cold-adapted enzymes remain under-utilized in industry. Recent technological advances, such as directly mining environmental metagenomic DNA coupled with genetic engineering techniques, promise to reveal and enhance the applicability of cold-adapted enzymes for a variety of industrial processes.

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Chapter 21
Heterologous Protein Expression in Psychrophilic Hosts

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21.1 Introduction

The vast number of candidate proteins generated from genome projects are creating enormous opportunities for biologists. However, efficient expression of genes in homologous/heterologous expression systems and rapid purification steps are actually major bottlenecks. In fact, although many recombinant proteins have been successfully produced from common prokaryotic (*Escherichia coli*) and eukaryotic (yeast and CHO cells) hosts, these conventional systems have often proved to be unproductive due to the peculiar properties of the protein to be produced. Indeed, beside the obvious impossibility of achieving a large scale production of thermally labile proteins at the normal *E. coli* growth temperature, degradation of the product by the host proteases and the incorrect folding of the nascent polypeptides, resulting in the proteins aggregation and accumulation as insoluble inclusion bodies, are sometimes observed (Speed et al. 1996).

To overcome the above mentioned limits of *E. coli* as host for the recombinant protein production, a rational experimental approach has consisted in lowering the

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cultivation temperature (Baneyx 1999), since this change has a pleiotropic consequence on the folding processes. There are many examples in literature describing the effectiveness of enhancing the solubility of a number of difficult proteins by this approach (Vasina and Baneyx 1997). Inclusion bodies formation is a process mainly driven by hydrophobic interactions which are directly dependent on temperature (Kiefhaber et al. 1991). Another interesting aspect linked to the growth of *E. coli* at suboptimal temperature is the partial elimination of heat-shock proteases, whose production is generally induced under over-expression conditions (Chesshyre and Hipkiss 1989), and the observed higher intracellular concentration of host-encoded chaperones at temperatures around 30°C (Mogk et al. 2002; Ferrer et al. 2003).

There are two main systems which optimize the recombinant protein production in *E. coli* at reduced temperatures. The first system takes advantage of the host response to temperature downshift, which induces the transcription and translation of the cold-shock genes while ceasing the expression of virtually all the other genes (Qing et al. 2004). The other one is based on the co-expression of the target protein with chaperones from a psychrophilic bacterium. The two chaperones (Cpn60 and Cpn10 from *Oleispira antarctica* RB8T) allow *E. coli* to grow at a reasonable rate at 4°C (Ferrer et al. 2004).

There is, however, a major drawback in the cultivation of *E. coli* at reduced temperatures: biomass production decreases with temperature, reducing the global process productivity, i.e. the total biomass produced divided by the process time. Therefore, the use of psychrophilic bacteria as alternative expression hosts is the compelling choice towards the exploitation of industrial processes at temperatures as low as 0°C.

To develop such a technology in cold-adapted bacteria, the availability of several genetic tools and structural/functional information are required, such as molecular signals for the autonomous DNA replication (origin of replication), marker genes enabling efficient selection schemes for the isolation of transformed psychrophilic cells, and gene-expression regulating sequences (promoters). These genetic tools should be derived from psychrophilic bacteria or should at least be able to work in the psychrophilic cellular context. In literature, there are only two reported examples of heterologous protein production in psychrophiles by using mesophile-derived replicons with a broad host-range profile.

In 1999, Remaut and coworkers reported the expression of the moderately thermolabile, eukaryotic luciferase (from *Photinus pyralis*) by cloning its coding gene into a pJB3-derived replicon (Blatny et al. 1997) under control of *E. coli*-derived transcriptional and translational signals, in an Antarctic strain growing at 15°C. The second example is the production of a β-galactosidase and the green fluorescent protein in the piezo-psychrophile *Photobacterim profundum* SS9 (Lauro et al. 2005). A broad-host range gene-expression vector was constructed by cloning the *E. coli* P_{BAD}/*araC* system from pJN105 (Newman and Fuqua 1999) into an RSF1010 (IncQ) derivative. The resulting plasmid was effective in the regulated production of the β-galactosidase in the cold-adapted bacterium, although the production yields resulted to be about one order of magnitude lower than what was obtained by the same system in *E. coli* (Lauro et al. 2005).

The isolation and molecular characterization of a true psychrophilic plasmid from the Antarctic Gram-negative bacterium *Pseudoalteromonas haloplanktis*

TAC125 (Tutino et al. 2001) have been instrumental in setting up several *E.coli*-psychrophile shuttle vectors for the efficient recombinant protein production. In the next sections, we will describe our "cold" gene-expression technology and some examples of its application to the production and secretion of "difficult" proteins.

21.2 The genetic system

In this section, we describe the bacterial host and all the genetic tools developed to achieve recombinant protein production. Features of psychrophilic gene-expression vectors set up are described in detail.

21.2.1 The psychrophilic host: Pseudoalteromonas haloplanktis *TAC125*

P. haloplanktis TAC125 is a Gram-negative bacterium isolated from an Antarctic coastal seawater sample collected in the vicinity of the French Antarctic station Dumont d'Urville, Terre Adélie. It can be classified as a eurypsychrophile (i.e. a bacterium growing in a wide range of low temperatures; Atlas and Bartha 1993) and was the first Antarctic Gram-negative bacterium of which the genome was fully determined and carefully annotated (Medigue et al. 2005). Genomic and metabolic features of this Gammaproteobacterium, accounting for its remarkable versatility and fast growth compared with other bacteria from aqueous environments, were discovered by combining genome sequencing and further in silico and in vivo analyses.

Amongst these relevant traits, it is worth mentioning that the bacterium seems to cope with the increased solubility of oxygen at low temperature by multiplying dioxygen scavenging while deleting whole pathways producing reactive oxygen species (ROS). In the latter direction is the unusual (if compared to other related bacteria, such as the vibrio or *Shewanella*) abolition of the ubiquitous molybdopterin-dependent metabolism and the deletion of all genes coding for enzymes using the molybdopterin cofactor. In general, *P. haloplanktis* TAC125 is remarkably well protected against ROS under cold conditions, a feature that could be very useful for the expression of foreign proteins at low temperatures.

Moreover, as seen in silico with its proteome composition (Medigue et al. 2005), it provides a way to resist the aging features involving asparagine cyclisation and deamidation. Indeed, *P. haloplanktis* TAC125 proteome reveals a concerted amino acid usage bias specific to psychrophiles: for instance, this proteome is enriched in asparagine residues compared to those from organisms growing at higher temperatures, making it an organism of choice for foreign protein production when deamidation ought to be put to a minimum (Weintraub and Manson 2004).

Being a eurypsychrophile, the duplication of *P. haloplanktis* TAC125 has been observed in the range 4–30°C, with an apparent optimal growth temperature at 20°C, where the observed duplication time in rich medium is 31 min (Tutino

et al. 1999). However, the bacterium still duplicates at fast speed even at lower temperatures (at 4°C, one cell division is completed in about 100 min; unpublished results from this laboratory) and, when provided with sufficient nutrients and aeration, it grows to very high density (up to $A_{600} = 20$) under laboratory settings, even at 0°C. This growth performance makes it one of the faster growing psychrophiles so far characterized.

Fast growth rates, combined with the ability of *P. haloplanktis* TAC125 to reach very high cell densities even under laboratory growth conditions and to be easily transformed by intergeneric conjugation (Duilio et al. 2004a), made this bacterium an attractive host for the development of an efficient gene-expression system at low temperatures.

Moreover, the knowledge of *P. haloplanktis* TAC125 genome has made feasible the development of an efficient genetic scheme for the construction of knock-out mutants in specific psychrophilic genes (unpublished results from this laboratory). Setting up genomic mutants is the obligatory step towards either the study of the role of a given gene, if any, or the construction of genetically engineered bacterial strains, possibly displaying improved features as host for recombinant protein production and secretion.

21.2.2 The psychrophilic gene-expression vector

A few other reported examples of recombinant protein production in psychrophiles made use of molecular signals (such as the origin of replication and the transcriptional promoter) derived from mesophiles. A different philosophy inspired the construction of our gene-expression systems, which derived from the proper assembly of true psychrophilic molecular signals into a modified *E. coli* cloning vector. Figure 21.1

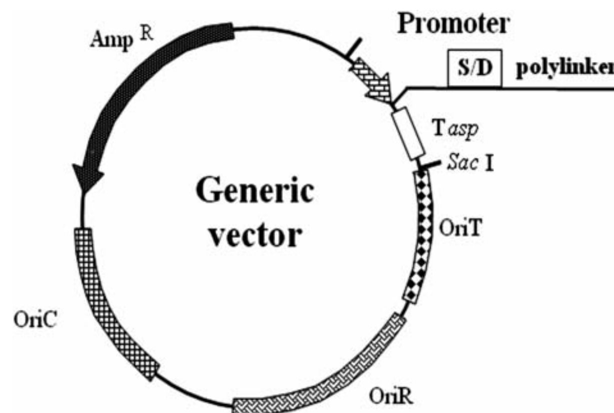

Fig. 21.1 Psychrophilic gene-expression vector

shows the scheme of a generic psychrophilic gene-expression vector set up to produce recombinant proteins in *P. haloplanktis* TAC125. The mesophilic signals consist of the pUC18-derived origin of replication (OriC) and a selection marker gene (a β-lactamase encoding gene), allowing the plasmid to replicate either in *E. coli* or in the psychrophilic host, i.e. a shuttle vector. Another crucial mesophilic signal is represented by the OriT sequence, the conjugational DNA transfer origin from the broad host range plasmid pJB3 (Blatny et al. 1997). OriT is recognized by some proteins (encoded by *mob* gene cluster) involved in the plasmid DNA mobilization from the donors to the psychrophilic recipient cells during conjugation (Duilio et al. 2004a).

In the following sections, we will describe in more detail some structural and functional results concerning the psychrophilic origin of replication (OriR) isolated from the *P. haloplanktis* TAC125 pMtBL plasmid and the cold-adapted promoters used for the recombinant protein production.

21.2.2.1 Psychrophilic origin of replication

We reported the isolation and molecular characterization of the first plasmids isolated from Antarctic marine bacteria, pTAUp from the strain *Psychrobacter sp*. TAD1 (Tutino et al. 2000) and pMtBL from *P. haloplanktis* TAC125 (Tutino et al. 2001).

We focused our attention on the small multicopy plasmid pMtBL, since this episome turned out to be an interesting extra-chromosomal element displaying unique molecular features. First of all, its nucleotide sequence (4081 bp) is still not related to any entry stored in the databanks and it has been found to be transcriptionally silent. Because of being cryptic, the inheritance of pMtBL totally relied on the efficiency of its replication functions. This function can be linked to a region of about 850 bp by an in vivo assay. In fact, the pMtBL autonomous replication sequence (ARS) (OriR) was defined as the smallest *Alu*I portion of the psychrophilic plasmid able to actively promote the duplication of an *E. coli* vector (unable to replicate by itself) in the cold-adapted host *P. haloplanktis* TAC125. Interestingly, no incompatibility but a simple competition was observed between the endogenous wild type plasmid and OriR-containing shuttle vectors actually carrying the selection resistance gene (amp^R). It might be that other functions, possibly related to a specific plasmid partitioning mechanism, are responsible of the stable pMtBL inheritance at a low copy number in *P. haloplanktis* TAC125 transconjugants.

pMtBL-derived shuttle vectors were used to successfully transform several psychrophilic bacteria, belonging either to *Pseudoalteromonas* and *Psychrobacter genera*, or unclassified marine bacteria (Tutino et al. 2001) and the piezopsychrophile *Photobacterium profundum* SS9 (F. Lauro, personal communication). These data suggest that pMtBL ARS can be used as a broad-host-range origin of replication and functionally support some structural observations on the OriR sequence, which highlighted its similarity to the OriV origin of replication from the broad host range RK2 plasmid (Fang and Helinski 1991).

21.2.2.2 Psychrophilic transcription initiation signals

A necessary background to investigate the adaptation of gene-expression mechanisms is represented by the study of RNA polymerase and its cognate molecular signals. This knowledge is of utmost importance to control and modulate the recombinant protein production in any gene-expression system, and, therefore, also in a cold-adapted one.

Concerning the psychrophilic transcription initiation signals, despite the steady increase in the number of sequenced cold-adapted genes, there is only one example of systematic analysis of the sequence of promoter regions in psychrophilic bacteria. The structural/functional characterization of *P. haloplanktis* TAC125 promoters (Duilio et al. 2004b) was carried out by random cloning of genomic DNA fragments and identification of promoter sequences by evaluating their capability to express a promoter-less reporter gene. A promoter-trap library was constructed by cloning *P. haloplanktis* TAC125 genomic DNA fragments into a shuttle psychrophilic vector upstream of the promoter-less β-galactosidase gene from *P. haloplanktis* TAE79 (*Ph*TAE79*lacZ*) (Hoyoux et al. 2001). The recombinant vectors were mobilized into *P. haloplanktis* TAC125 cells by intergeneric conjugation (Duilio et al 2004a), and several dark blue colonies, possibly containing strong promoters, were selected from X-Gal containing agar plates (Duilio et al. 2004b).

The determination of the putative transcription start site was obtained by primer extension analysis and a promoter consensus sequence for *P. haloplanktis* TAC125 was proposed on the basis of a sequence comparison between the various active promoters (Fig. 21.2).

The identified consensus sequences resulted to be very similar to those of the *E. coli* σ^{70}-dependent promoter. Indeed, as far as this small promoter sample is concerned, the cold-adapted promoters seem to diverge from the mesophilic ones mainly in the 2nd and 5th positions of -35 box, where the occurrence of T and C have more frequently been observed (Lisser and Margalit 1993).

Data collected in this study highlight the strong similarity between the defined psychrophilic transcriptional signals with the *E. coli* counterparts. Furthermore, this resemblance is not limited to the −35/−10 sequences but it is also supported by their functionality in the mesophilic context, since the *E. coli* transcriptional machinery initiates the m-RNA synthesis exactly from the transcriptional start point used by *P. haloplanktis* TAC125. This functional characterization supports the observed high sequence similarity between the mesophilic and the psychrophilic RNA-polymerases (Medigue et al. 2005).

The implementation of the above described psychrophilic promoters in the pMtBL-derived shuttle vectors resulted in the set up of cold-adapted gene-expression systems, characterized by the constitutive production of the recombinant protein.

However, efficient production can sometimes be achieved only by fine tuning the recombinant gene expression. This goal can be reached by using regulated promoters and efficient induction strategies. Indeed, physical separation between bacterial growth phase and expression of the desired proteins can either improve

A

```
                    -35                              -10          +1

P2-1    AGAATGAACTATTACCCGATGCAGTGAG-TCTGTATATAGAG
P2-2    GTCATTGGTATAAAACCCC--TAGCATGTTAAAATGCACGCTAT
P3      AGGCTTGATCTGTTTTCGG---ATCTCCCTATAATGCGACCCCA
P4      CAAGTCGGTAAAACCGAGTAAACTATGCGTATTATATTATGC
P6      GCCGTGAGTTGTTTGTAAT----TGCTGATATGCCGTTTATGACTTA
P14     TTATTTCAATGAGTTGCATAA---CAGCCTAAAATTAAGT
P15     TCTCTAGTTTGCTAAGCACC--AAATGAGTTACATTGTAACTT
P17     GTGGTAGCTTTTGCCCAT---CAACATGGTATTAAAGTGTTT
P25     CTTTTACAATGGCAAAGTCA-----CCGGTAATACTGTG
```

B

```
Consensus
              -35                          -10                       +1
E.coli        TTGACA -- (16-18 bp) -- TATAAT -- (5-8 bp) --
PhTAC125      TRGRTW -- (14-19 bp) -- TATRAY -- (4-12 bp)--
```

Fig. 21.2 Promoter consensus sequence for *P. haloplanktis* TAC125 Alignment of promoter regions from *Ph*TAC125 (**A**) and identification of a cold-adapted consensus sequence (**B**). The determined transcriptional start sites are *boldface* and *underlined. Dashes* indicate gaps introduced to maximize the alignment. Nucleotides highlighted in *black* are those considered for the definition of the consensus sequence shown in panel B. It consists of nucleotides that are present in any given position in more than 51% of the sequences. In *brackets* the average distances (in base pairs) between the promoter consensus sequences are reported. R: A or G; Y: T or C; W: A or T. *E.coli* promoter consensus sequence was derived from Lisser and Margalit (1993)

the productivity of the entire system or it can also play an important role in the production of toxic proteins for the host cells.

Recently, using a differential proteomic approach, we isolated and characterized a two-component system. This regulatory system is responsible for the transcriptional regulation of the gene coding for an outer membrane porin, and it is strongly induced by the presence of L-malate in the medium (Papa et al. 2006). The regulative region of the porine gene was used for the construction of an inducible cold expression vector, where the recombinant protein expression results to be under L-malate control. This inducible system was effective in the production of both psychrophilic and mesophilic proteins in *P. haloplanktis* TAC125 (Papa et al. 2007).

21.2.3 Molecular signals for protein addressing

Although the production of recombinant protein in the host cytoplasm is the preferred strategy in many processes due to higher production yields, this approach cannot be pursued when the wanted product requires the correct formation of

disulphide bonds to attain its catalytic competent conformation. Indeed, as for all Gram-negative bacteria, *P. haloplanktis* TAC125 cytoplasm is a reducing environment (due to the presence of reducing enzymes, such as thioredoxin and glutaredoxin) and the formation of disulphide bridges is confined in the periplasmic space, where the enzymes belonging to the Dsb family are located (Rietsch and Beckwith 1998). *P. haloplanktis* TAC125 genome analysis lead us to confirm the presence of at least one clear homologue for any *E. coli dsb* gene (from *dsbA* to *dsbG*) characterized so far. However, the psychrophilic bacterium notably diverges from the mesophilic counterpart since it possesses two adjacent chromosomal genes coding for two DsbA-like proteins (Madonna et al. 2006). Their transcriptional regulation mechanisms were investigated in detail. It turned out that both proteins are produced in vivo but at different expression rates during growth, suggesting their likely involvement in different but complementary cellular processes (Madonna et al. 2006).

Protein translocation from the cytoplasm to the periplasmic space can be achieved by three different routes: the Sec pathway (Matlack et al. 1998) which is a post-translational export; the Srp pathway (Luirink and Sinning 2004), which is a co-translational export and shares some components with the previous one; and the TAT pathway (Lee et al. 2006), which differs significantly from other pathways since it is able to translocate fully folded passengers. From the genome analysis, we know that *P. haloplanktis* TAC125 contains all the above mentioned export machinery (Medigue et al. 2005). Therefore, we set up a family of psychrophilic gene expression systems for the recombinant protein production in the periplasmic space. Gene fragments encoding two signal peptides from psychrophilic secreted proteins have been cloned under the control of constitutive promoters with different transcription efficiencies. Several chimerical proteins were generated, where the signal peptide was fused to the N-terminus of several mesophilic disulfide bond containing proteins, and their efficiency as periplasm addressing tag was tested. An example of successful use of these vectors for the soluble production of human nerve growth factor (hNGF) as reported in the last section of this chapter.

Furthermore, the final aim in recombinant protein production is to simultaneously reach a high specific recombinant protein production rate and a high product quality. One strategy to avoid quality problems and improve protein production process is to target the protein to the outer compartment of the host cell. This strategy avoids inclusion body formation and the majority of proteolytic proteins in the cytoplasm and achieves a primary purification reducing the costs of downstream process. In this context, the setting up of a gene expression system for the production and secretion of recombinant proteins in psychrophilic bacteria could conbine the effects of low temperatures on the recombinant product solubility with the advantages linked to extra-cellular protein targeting. Our research starting point was the study of the secretion of a psychrophilic protein: α-amylase from *P. haloplanktis* TAB23 (Feller et al. 1992). This exo-protein is produced and secreted as a larger precursor, with a long C-terminal propeptide that constitutes a structurally independent domain that neither exhibits any foldase function nor affects the amylase catalytic activity (Feller et al. 1998). Indeed, when the psychrophilic enzyme is produced by

recombinant cold-adapted bacteria (the source strain, *P. haloplanktis* TAC125 and *Psychrobacter sp*. TAD1) the propeptide is not mandatory for the α-amylase secretion (Tutino et al. 2002; Cusano et al. 2006a). Starting from the latter observation we set up a cold-adapted secretion system which makes use of the mature α-amylase as a carrier. This system was quite efficient, since several heterologous proteins were produced and secreted (Cusano et al. 2006b). A key aspect towards the optimisation of this secretion system is the identification of the specific α-amylase secretion pathway. Indeed, this information is instrumental in the construction of engineered cell hosts with an improved secretory potential. An in vivo complementation experiment was set up to identify the genes responsible for the α-amylase secretion in the cold-adapted bacteria chosen as host for the heterologous protein production (*P. haloplanktis* TAC125). By applying this strategy, a 37.5-Kb-long genomic fragment was selected, whose nucleotide sequence was determined and analyzed in silico. Surprisingly, the selected DNA portion does not code for any of the already characterized secretion pathways (Lee and Schneewind 2001). These results are strongly suggestive of a novel secretion machinery occurring in the Antarctic *P. haloplanktis* TAC125 strain. An extensive mutagenesis analysis to identify the psychrophilic functions specifically involved in the extra-cellular targeting is in progress.

21.3 Examples of heterologous protein expression in psychrophilic bacteria

21.3.1 **P. haloplanktis** *TAE79 β-galactosidase and* **Saccharomyces cerevisiae** *α-glucosidase production*

Two “difficult” proteins were produced to test performances of the cold expression system inducible by L-malate (Papa et al. 2007). These proteins (the psychrophilic β-galactosidase from *P. haloplanktis* TAE79 and the *S. cerevisiae* α-glucosidase) were chosen because they can hardly be expressed in the recombinant form in mesophilic hosts even at sub-optimal temperature conditions.When the β-galactosidase was produced in *E. coli* cells at 18°C, 20 mg of catalytically active enzyme was produced per liter of culture. Analogously, recombinant yeast α-glucosidase produced in *E. coli* aggregates in an insoluble form, the active soluble amount of protein being less than 1% of the total production (Le Thanh and Hoffmann 2005).

Both recombinant psychrophilic β-galactosidase and yeast α-glucosidase were produced in *P. haloplanktis* TAC125 (Fig. 21.3) as soluble and catalytically active enzymes. Structural and kinetic analysis of the recombinant proteins showed that both enzymes were nearly identical to their native counterparts. The absence of aggregated protein material might be due to the expression temperature that lowers hydrophobic interactions. However, since the optimal expression temperature determined for *P. haloplanktis* TAC125 is only marginally lower than that used for

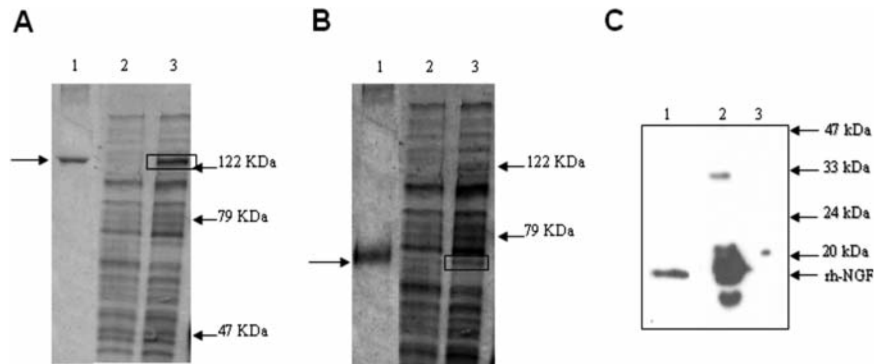

Fig. 21.3 Examples of recombinant protein production in *P. haloplanktis* TAC125 **A** Recombinant production of the thermally labile β-galactosidase from *Ph*TAE79 in *Ph*TAC125 cells. 7.5% SDS PAGE gel electrophoresis of protein extracts from *Ph*TAC125 cells harboring P(PSHAb0363) and grown in minimal medium in the absence (lane 2) and in the presence (lane 3) of L-malate, in comparison with β-galactosidase from *Ph*TAE79 used as control (lane 1). The recombinant protein is indicated by an *open box*. **B** Recombinant production of the mesophilic α-glucosidase from *Saccharomyces cerevisiae* in *Ph*TAC125 cells. 7.5% SDS PAGE gel electrophoresis of protein extract from *Ph*TAC125 cells harboring pUCRPGLUCP1 grown in minimal medium in the absence (lane 2) and in the presence (lane 3) of L-malate, in comparison with commercial α-glucosidase from yeast used as control (lane 1). The recombinant protein is indicated by an *open box*. **C** rh-NGF production and cellular localisation in recombinant *P. haloplanktis* TAC125. Western blotting analysis of periplasmic (lane 2) and cytoplasmic (lane 3) fractions of 4°C grown *P. haloplanktis* TAC125-pPM13*psDngf* recombinant cells. Polyclonal anti-h-NGF antibodies were used for immunodetection. As positive control, 50ng of rm-NGF proteins was loaded in lane 1

protein production in *E. coli* (15°C compared to 18°C), other factors must have an effect in preventing aggregation.

Experimental conditions for optimal protein production in the cold-inducible expression system were also defined. Low concentrations of L-malate and long induction times are effective for maximal protein production. Under optimal expression conditions, recombinant β-galactosidase is produced with high yields (620–720 mg l^{-1}), indicating that the inducible system can be very effective in the expression of psychrophilic proteins that are usually poorly produced in mesophilic hosts. A significantly lower production yield is observed for yeast α-glucosidase possibly due to the different codon usage between the eukaryotic and bacterial organisms. Nevertheless, the cold expression system yielded a satisfactory amount of this protein in a soluble and active form.

21.3.2 *hβ-NGF Production*

Another example of "difficult" proteins is the mature form of human nerve growth factor (hNGF), a neurotrophin which found promising applications as a therapy agent in several neurological disorders such as Alzheimer's disease (Lad et al. 2003).

hNGF is translated as prepro-protein, where the presequence mediates translocation into the endoplasmic reticulum, while the prosequence seems to facilitate the folding of the mature part, at least from the *E. coli* inclusion bodies (Rattenholl et al. 2001), and it is removed by a cellular specific protease to give the mature hNGF form. Therapeutical applications require the expression and purification of a large amount of functional protein. However, the recombinant production of this protein exhibits several problems in the conventional host *E. coli*, due to its tendency to form insoluble aggregates either when produced as prepro-protein or as mature form (Dicou et al. 1989; Rattenholl et al. 2001).

Vigentini et al. (2006) reported the expression of the mature form of human NGF gene (hNGF) in *P. haloplanktis* TAC125 and investigated the production and the cellular localization of the recombinant protein. The protein produced at 4°C was soluble and efficiently translocated in the host periplasmic space (Fig. 21.3). A gel exclusion chromatography also indicated that the protein was largely in a dimeric form, the quaternary structure required for its biologic activity (Harmer et al. 2003). Vigentini and coworkers investigated the influence of growth conditions and cultivation operational strategies on final biomass concentration and on rhNGF production to optimize the use of *P. haloplanktis* TAC125 as a novel host for recombinant protein production.

21.3.3 Secretion of several heterologous proteins

Cusano et al. (2006b) described the setting up and use of a "cold" gene-expression system implemented for the secretion of recombinant proteins in *P. haloplanktis* TAC125. As previously mentioned, such a system combines the advantages of extra-cellular protein secretion with the positive low temperature effect on the recombinant product solubility. The novel system makes use of the psychrophilic α-amylase from *P. haloplanktis* TAB23 (Feller et al. 1992) as a secretion carrier. This exo-protein is synthesised as a preproenzyme, made of: (1) a Sec-dependent signal peptide; (2) the mature enzyme; (3) a flexible spacer; and (4) a structurally independent C-terminal propeptide. The C-terminal propeptide is removed by the action of a host-secreted protease which recognizes and cleaves the -Ala-Ser-(↓)Ser-Thr- sequence contained in the flexible spacer. This event occurs when the precursor reaches the extra-cellular medium (Feller et al. 1998). It was demonstrated that the C-terminal propeptide is not mandatory for the *P. haloplanktis* TAB23 α-amylase recombinant secretion either in the source strain or in *P. haloplanktis* TAC125 (Tutino et al. 2002). Starting from the latter observation, the secretion of chimeric proteins obtained by the replacement of α-amylase C-terminal propeptide with a passenger protein was studied (Cusano et al. 2006b). The novel genetic system allows the easy in-frame cloning of any gene downstream of the mature psychrophilic α-amylase encoding region (Fig. 21.4). Three chimeric proteins, obtained by fusing intra-cellular proteins to the psychrophilic exoenzyme, were produced in *P. haloplanktis* TAC125 and their secretion was analyzed. The results demonstrated that the cold-adapted secretion

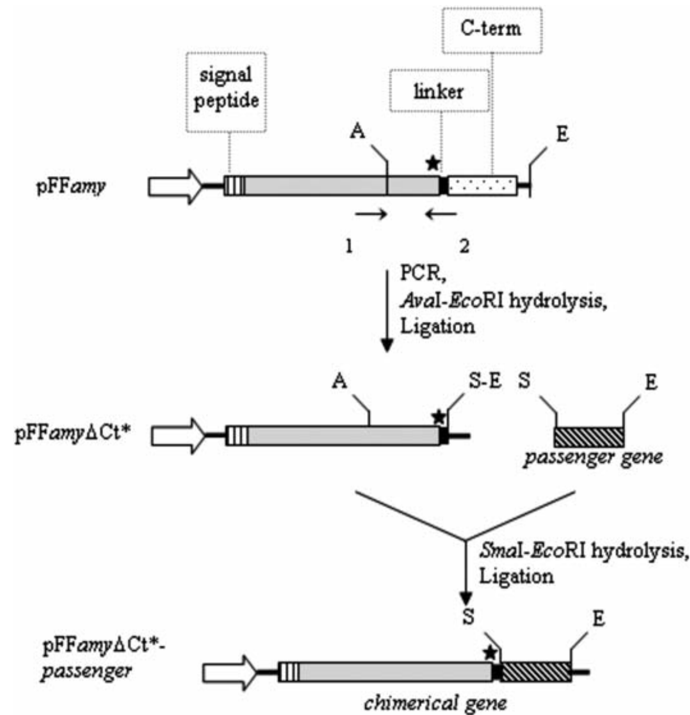

Fig. 21.4 Cold gene-expression system implemented for the secretion of recombinant proteins in *P. haloplanktis* TAC125 *White arrow*, *P. haloplanktis* TAC125 *aspC* promoter; signal peptide, sequence encoding *P. haloplanktis* TAB23 α-amylase signal peptide; C-term, α-amylase C-terminal propeptide encoding sequence; linker, α-amylase linker encoding sequence; A, *AvaI;* E, *EcoRI*; S, *SmaI* restriction endonuclease sites; *black arrows*, PCR primers. The *black star* indicates the presence of a sequence encoding the amino acid motif -Ala-Ser-Ser-Thr-, recognized and cleaved by a *P. haloplanktis* TAC125 secreted protease

system is efficient since all tested chimeras were translocated with a secretion yield always above 80%. Furthermore, reported activity data indicated that the system also allows the correct disulphide bond formation of chimera components (Cusano et al. 2006b).

21.4 Conclusions

In this chapter, we briefly summarized the recent achievements in recombinant protein production in psychrophilic bacteria. Over the last decade, we significantly enhanced the number of set ups of reliable genetic systems for the recombinant gene expression in an Antarctic marine Gram-negative bacterium. Our results clearly demonstrated that the production of recombinant proteins in

cold-adapted bacteria is not only a mature and reliable technology, but it is also a successful strategy to overcome the product solubility problems sometimes occurring in conventional systems such as in *E. coli*. In this context, *P. haloplanktis* TAC125 and the gene-expression systems set up have a valuable biotechnological potential as non-conventional systems for the production of "difficult" proteins. The next challenge towards their industrial application is to develop an efficient fermentation scheme to up-scale the recombinant protein production in automatic bioreactors.

However, there are other foreseeable applications of the cold-adapted gene-expression system, one of which is its use as a host for the screening of metagenomics libraries from psychrophilic environments (see Chap. 18). Indeed, it is widely accepted that up to 99.8% of microbes present in many environments are not readily cultivable (Streit and Schmitz 2004). Metagenomics try to overcome this bottleneck by cloning and expressing environmental-derived large DNA fragments in bacterial hosts (routinely, in *E. coli*). This strategy makes the discovery of novel biocatalysts easily achievable, best suited for specific industrial applications (Lorenz and Eck 2005). The prerequisite is that the observed metagenes are expressed in the heterologous host and the resulting products are stable. In this context, metagenomes from cold environments may encode thermal-labile enzymes, whose expression rate would be greatly enhanced by using a psychrophilic bacterial host system.

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Chapter 22
Cold-Adapted Fungi as a Source for Valuable Metabolites

Jens C. Frisvad

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22.1 Introduction

Filamentous fungi, bacteria, especially actinomycetes, plants, algae and many other kind or organisms can produce a vast array of different extrolites. Extrolites is an ecological term for outwards directed metabolites that potentially can play a role in the interaction between organisms (Frisvad et al. 2004, 2007). The collective metabolic term for these compounds is the exometabolome. Many extrolites are secreted, but some stay in the cell wall to protect the fungal, bacterial or plant spores or seeds. Plants, including those from Arctic and alpine areas, protect themselves from herbivores by producing an array of secondary metabolites (Palo 1984; Bryant et al. 1991). In the sea surrounding Antarctica there are many chemical interactions between mollusks and amphipods, algae, urchins and anemones, and between sponges and their predators (Amsler et al. 2001), and in Arctic ecosystems there are indications that secondary metabolites also play a role in the chemical interactions between species (Chapin III et al. 1992). However, these potential interactions have not been studied in any detail in microbiological systems.

Secondary metabolites are an important part of the extrolites that most filamentous fungi produce in large amounts and in an impressive diversity (Turner 1971; Turner and Aldridge 1983). These secondary metabolites are produced consistently and in species-specific profiles by filamentous fungi (Larsen et al. 2005). Bioactive secondary metabolites of potential use as drugs have been screened for in different habitats since the discovery of penicillin, and new, very important

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drugs have been discovered and developed for pharmaceutical use. The fungi producing these drug candidates have been isolated from soil, from plants such as endophytes or pathogens, from animals or from other sources (Schulz et al. 2002). Many of the fungal-derived drugs in use today have been isolated from fungi from temperate or tropical regions, but drugs derived from fungi isolated in cold areas are quite rare. One cold habitat that has been explored, and from which a large number of microbial bioactive secondary metabolites have been derived, is the sea (Rinehart 1992; Flam 1994; Bernan et al. 1997; Pietra 1997; Biabini and Laatch 1998; Faulkner 1999; Gudjarnnson 1999; Munro et al. 1999; Höller et al. 2000; Jensen and Fenical 2000; Verbist et al. 2000; Hentschel 2002; Bhadury et al. 2006; Ebel 2006; Konig et al. 2006; Newman and Hill 2006; Paul et al. 2006; Blunt et al. 2007). There are, however, only a few studies on the extrolite potential of fungi from Arctic, Antarctic and alpine areas.

22.2 Extrolites from cold-adapted fungi

Some protein and peptide extrolites from fungi have special properties and include hydrophobins (Linder et al., 2005), adhesins (Verstrepen and Klis, 2006), polypeptides (Newstead and Huner 2005) and extracellular enzymes. Enzymes from bacteria are treated elsewhere in this book (see Chapters 13 and 20); filamentous fungi also produce an array of different extracellular enzymes. Cold-adapted fungi have the ability to produce many extracellular enzymes and will use those for their nutrient uptake (Feller and Gerday 1997. 2003; Fenice et al. 1997; Gerday et al. 2000; Russell 2000; Bölter et al. 2002; Burg 2003; Gomes and Steiner 2004; Margesin et al. 2005). For example, insect exoskeletons can be broken down by chitinolytic enzymes from *Verticillium lacanii* working at low temperatures in Antarctica (Fenice et al. 1998). However, insect pathogenic fungi have rarely been reported from Antarctica (Bridge and Worland 2004). Some fungi produce keratinases to break down animal skin in Antarctica (Mercantini et al. 1993; Marshall 1998) including *Geomyces pannorum*. Ectomycorrhizal basidiomycetes such as *Hebeloma* also produce a series of enzymes working at low temperatures (Tibbett et al. 1988a, 1988b, 1989). All these enzymes may have low temperature optima and are of course of great interest for the biotechnological industry (Margesin and Schinner 1994; Hoshino et al. 1996; Bradner et al. 1999a, 1999b; Cavicchioli et al. 2002). Interestingly, some of the enzymes from cold-adapted fungi have mesophilic properties (Cairns et al. 1995). *Myrosclerotinia borealis* produces several polygalacturonases but also bioactive polypeptides in the sclerotia (Takasawa et al. 1997; Newstead and Huner 2005). Some of these enzymes or other proteins and smaller peptides may have growth inhibitory effects on other organisms, and some have antigenic properties.

Mycosporin-derived molecules may also be of biotechnological interest (Volkmann et al. 2003) because of their UV-absorbing properties. Lipids from psychrophilic and psychrotolerant fungi may have a desirable high relative amount of polyunsaturated

triglycerides and fatty acids (Kerekes and Nagy 1980; Hammonds and Smith 1986; Istokovics et al. 1998; Weete and Gandhi 1999; Weinstein et al. 2000), and sterols and carotenoids may also be exploited biotechnologically (Dexter and Cooke 1984). However, the most promising extrolite candidates for biotechnological exploration, apart from extracellular enzymes, are the secondary metabolites.

22.3 Secondary metabolites from cold-adapted fungi

Filamentous fungi can produce a large number of secondary metabolites. Fungi can be screened for these secondary metabolites by phenotypic methods such a HPLC-DAD-MS (Larsen et al. 2005). However, fungi from polar and alpine areas have not extensively been screened for bioactive secondary metabolites. Three groups of fungi, *Penicillium* species from refrigerated foods, psychrotolerant to mesophilic soil-borne fungi, and psychrotolerant fungi, have been examined for secondary metabolites and these are mentioned below.

Food-borne penicillia are common in refrigerated foods (Frisvad and Samson 2004), but many of these have also been found in Arctic glacier ice (Gunde-Cimerman et al. 2003) and in Antarctic bird nest and moss samples (McRae et al. 1999). There have been no or very few differences between the profiles of secondary metabolites of food-borne penicillia (Frisvad et al. 2004) or penicillia from cold ecosystems (Sonjak et al. 2006). For example, more than 100 isolates of *Penicillium crustosum* from glacial ice from Svalbard and further isolates from many different sources all produced terrestric acid, roquefortine C, penitrem A, and viridicatins, all representing a biosynthetic family of several members (Sonjak et al. 2005; 2007a). In addition, some of the Arctic strains produced andrastin A, but it is not yet known whether all *P. crustosum* isolates have the gene cluster necessary to produce andrastin A. It is also possible that andrastin A can only be produced by Arctic strains of *P. crustosum*, in which case it could be possible to find new secondary metabolites in already well-characterized strains from other species from cold habitats. However, the experience until now has been that secondary metabolite profiles are consistent in a species (Larsen et al. 2005). Therefore, new species may be more interesting candidates for finding new drug leads.

Some new species of *Penicillium* from Antarctica and Svalbard have been examined in our laboratory. *Penicillium antarcticum* (McRae et al. 1999) had already been recovered from Danish salami, a beach in Denmark, a water-bottling plant in the Atlantic, a beach in Australia and a saltern in Slovenia, so this species was not specific for Antarctica, but rather a marine salt-tolerant species of *Penicillium*. Strains of this halotolerant and psychrotolerant to mesophilic species produced patulin, penitrem A, asperentins and several other as yet unknown secondary metabolites (Frisvad, unpublished), irrespective of origin. In that case, known secondary metabolites in conjunction with interesting potential new drug candidates were found. Likewise, the new species *P. svalbardense* produced known and unknown secondary metabolites (Sonjak et al. 2007b). Like *P. antarcticum*,

P. svalbardense is also a psychrotolerant to mesophilic species, but it has until now only been found in glacial ice from Svalbard.

A series of psychrotolerant to mesophilic species have been found in soil or glacial ice samples from cold ecosystems, such as ice (Ma et al. 1999a, 1999b), alpine soil from Wyoming, Colorado and Switzerland, and tundra soil from Greenland. These include *Penicillium soppii* and *P. lanosum*, species that are mostly found in permanently cold soils (Frisvad et al. 2006). These two species are prolific producers of a series of interesting bioactive secondary metabolites. Isolates in both species can produce griseofulvin and cycloaspeptide A, and each species produces a series of other secondary metabolites in species-specific combinations (Frisvad et al. 2006). *P. antarcticum, P. svalbardense, P. soppii* and *P. lanosum* all grow and sporulate well at 25°C.

The most interesting discoveries were a series of new species of *Penicillium* that hardly grow at 25°C, if at all, and are close to being psychrophilic. The first of these have been described as *P. jamesonlandense* (Frisvad et al. 2006). This species produces the interesting cyclic peptides cycloaspeptide A and D, in addition to many other bioactive metabolites. New secondary metabolites were also discovered in the new species *P. ribium*, *P. rivulorum*, and *P. algidum*. For example, *P. ribium* produced the interesting cyclic nitropeptide psychrophilin A, in addition to cycloaspeptide A and D (Dalsgaard et al. 2004a; Frisvad et al. 2006), and *P. rivulorum* produced psychrophilin B and C and communesin G and H (Dalsgaard et al. 2004b, 2005a). *P. algidum* produced psychrophilin D and cycloaspeptide A and D (Dalsgaard et al. 2005b). It is interesting to note that these two series of cyclic peptides are only found in fungi from cold ecosystems. Some of these cyclic peptides are insecticidal and antimalarial (Dalsgaard et al. 2005b; Lewer et al. 2006), but there are probably other biological activities of those cyclic peptides.

22.4 Conclusions

As mentioned above, marine microorganisms appear to be very promising producers of new lead drug compounds, but fungi from other cold ecosystems appear to be equally valuable. New species from Arctic and alpine soils are maybe the most chemically inventive of all these, and the discovery of the nine new cyclic peptides, cycloaspeptide A to E and psychrophilin A to D, shows that maybe some secondary metabolites are entirely unique to cold ecosystems. Screening for new drug candidates should certainly be intensified, and new psychrotrophic species seem especially to be good candidates, rather than screening several strains of the same species.

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Chapter 23
Natural and Stimulated Biodegradation of Petroleum in Cold Marine Environments

Odd Gunnar Brakstad

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23.1 Introduction

The cold regions of the world represent some of the largest biotopes on the Earth, including the Arctic, Antarctic, and the permanently cold parts of the oceans. The oil industry is looking for new exploration and production activities both in the polar and the deepwater regions, increasing the risk for oil discharges in these vulnerable environments. Oil exploration and production activities are already going on in the North American and European Arctic, and in several countries

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technologies are being developed for offshore oil production at water depths of more than 3,000 m.

Biodegradation is an essential weathering process and important for understanding the fates and risks associated with oil discharges to marine environments. Hydrocarbon biodegradation has been the topic of several reviews (e.g., Atlas 1981, 1984; Leahy and Colwell 1990; Prince 1993; Swannell et al. 1996; Harayama et al. 1999, 2004; NRC 2003; Van Hamme et al. 2003; Venosa and Zhu 2003). Biodegradation of hydrocarbons in cold environments have been reviewed only by a few authors (e.g., Margesin and Schinner 1999, 2001). This review will focus on the interactions between discharged oil and microorganisms in cold marine environments, including the use of bioremediation actions.

23.2 Oil discharges in cold seawater

Hydrocarbons are released to marine recipients from a variety of natural and anthropogenic sources. Natural seeps provide a continuous source of crude oil and are widely distributed in the oceans. Several biogenically produced hydrocarbons from marine phytoplankton and prokaryotes also have chemical structures equal to petroleum hydrocarbons. Marine microorganisms are therefore constantly exposed to hydrocarbons, and hydrocarbonoclastic prokaryotes are present in all marine systems investigated so far.

In Arctic and Antarctic regions oil pollutions may be transported to ice-free shorelines or to the ice margins by prevailing seawater currents. In the latter case, the oil may be infested in the ice. Investigations of the fate of stranded or ice-infested oils are therefore important as part of the risk assessment and for the development of operational cleanup strategies, including bioremediation.

23.2.1 Petroleum characteristics and weathering

Petroleum is one of the most complex substances known, containing thousands of different compounds, many of which have never been characterized. It contains both water-soluble and oleophilic compounds, of which the hydrocarbons are best characterized. Petroleum hydrocarbons are basically separated into linear or cyclic alkanes, aromatic hydrocarbons, asphalthenes and resins, and the distributions of these compound groups vary considerably within petroleum oils (often categorized as paraffinic, asphalthenic, waxy and naphthenic oils). Although petroleum is associated with hydrocarbons, many oils also contain a variety of poorly characterized compounds, referred to as "unresolved complex mixtures" (UCM), which are recalcitrant to biodegradation (Gough and Rowland 1990; Watson et al. 2002).

23.2.2 Surface oil spills

Surface oil spills will result in a several weathering processes changing the physical–chemical characteristics of the oil. These processes are of importance for hydrocarbon degradation, and influenced by seawater temperature. Oil weathering processes include surface spreading of the oil, evaporation of volatile compounds, water-in-oil emulsions, oil-in-water dispersions, dissolution of small and charged (polar) compounds, and photo-oxidation (Brandvik 1997). A surface oil slick may spread by current and wind, and most of the surface generated by a thin oil film (sheen) with thickness <1 µm. Immediate surface evaporation processes result in losses of small C_5–C_{10} alkanes and monoaromatic compounds like BTEX (benzenes, toluenes, ethylbenzenes, xylenes), and many compounds in the sheen may rapidly biodegrade (Brakstad et al. 2004).

In seawater, the microbes degrade soluble oil compounds and act on the oil-water interfaces of small oil droplets, and laboratory studies have shown that biodegradation of oleophilic hydrocarbons at low temperatures is considerable (Brakstad and Bonaunet 2006). Hydrocarbon evaporation is reduced in cold seawater, or if the oil reaches ice-covered waters, since the oil surface is partly replaced by ice floes. This results in temporarily higher concentrations of volatile toxic compounds (e.g., BTEX) in the seawater. At high concentrations, these compounds may result in prolonged microbial lag-phases and delayed onset of biodegradation due to their acute toxicity (Atlas and Bartha 1972; Hokstad et al. 1999). For wax-rich oils with high pour points evaporation, dilution and dispersion may be reduced in cold sea water, since precipitated wax may build a matrix which limits the internal mixing of the oil and act as a diffusion barrier between the oil and the water.

The behaviour of oils in freezing environments includes spreading in ice, snow, under ice, and in water with ice present. Important parameters for spreading in ice are oil gravity and viscosity (Chen et al. 1974), but the oil–ice interfacial tension is also important (Kawamura et al. 1986). Oils were spread in the ice even below −15°C (Chen et al. 1974). Water-soluble hydrocarbons released from the ice may be transported through the brine channels (Faksness and Brandvik 2005), thereby coming in contact with sea-ice microbes in a fluid medium. The oil escapes the ice in the spring as the ice deteriorates by two general processes; (1) vertical rise of the oil through the brine channels in the ice, and (2) ablation of the ice surface down to the oil lens in the ice (Fingas and Hollebone 2003).

23.2.3 Sediment processes

Very few oils are dense enough to sink in seawater, but in spills close to the coast oil may mix with debris, sediment or organic matter to form tar balls which subsequently sink (Baker 1983). Adhesion to heavier particles most often takes place when the oil reaches the shoreline and strands on the beach. These layers of oil can

be covered by subsequent sediment deposition or alternatively uncovered during periods of erosion. In sub- or intertidal sediments, aerobic or anaerobic biodegradation become important processes for the removal of the oil. Biological processes like bioturbation are important for venting and oxygenation of the sediments, increasing the biodegradation.

23.2.4 Deepwater oil spills

A deep-water oil spill will most probably be caused by a subsea blowout. A rising hydrocarbon plume of dispersed oil droplets and gas bubbles will entrain dense water and, eventually, the suspension will not be buoyant (Johansen et al. 2003). After deepwater discharges, high pressures only have limited influence on dissolution rates and dispersion (Sawamura et al. 2001), and biodegradation becomes increasingly important for depletion of toxic volatile compounds. The majority of the oil in most deep-water releases will rise to the surface within several hours. The surface slick formed once the oil reaches the surface will be thinner than that seen during a shallow-water release or a surface release. This is due in part to the fractionation of oil droplets that results in a staged arrival of the oil at the surface and in part to diffusion or dispersion of the oil as it rises (NRC 2003). Compounds in the oil films will subsequently be biodegraded after re-dispersion by wave actions or due to the low film thickness.

23.3 Hydrocarbon-degrading microorganisms

During the last decades, nearly 200 bacterial, cyanobacterial, fungal, algal or diatom genera have been shown to degrade hydrocarbons (Prince 2005). Archaea have so far not been shown to oxidize hydrocarbons other than methane and therefore seem to play an insignificant role during oil biodegradation (Röling et al. 2004).

23.3.1 Hydrocarbon-degraders in cold marine environments

23.3.1.1 Arctic and Antarctic seawater, sediments and sea ice

Microbial communities in polar ocean waters or sea ice are relatively well described, based on 16S rDNA phylogenetic studies. Studies from Arctic or Antarctic oceans or sea ice by culture- or culture-independent techniques have shown abundance bacteria affiliated to the classes Alphaproteobacteria and Gammaproteobacteria, and to the phylum Cytophaga–Flavobacter–Bacteroidetes (CFB) (Bowman et al. 1997; Brown and Bowman 2001; Bano and Hollibaugh 2001; Junge et al. 2002; Brinkmeyer et al. 2003; Yakimov et al. 2003; Gerdes et al.

2005). Comparison of bacterial and archaeal phylotypes from Arctic and Antarctic seawater or ice suggested that most of the sequences are common between these environments (Brinkmeyer et al. 2003; Bano et al. 2004).

Several of the bacterial genera described in polar seawater and marine ice include members with the abilities to degrade petroleum hydrocarbons. Culture studies of psychrophilic or psychrotolerant hydrocarbon-degrading marine bacteria have indicated high phylogenetic diversity, including members of the genera *Sphingomonas*, *Marinobacter*, *Marinomonas*, *Halomonas*, *Psychrobacter*, *Psychromonas*, *Colwellia*, *Oleispira*, *Acinetobacter*, *Shewanella*, *Pseudoalteromonas*, *Pseudomonas, Cytophaga*, *Agreia*, *Arthrobacter* and *Rhodococcus* (Michaud et al. 2004; Yakimov et al. 2004; Deppe at al. 2005; Gerdes et al. 2005; Powell et al. 2005a; Brakstad and Bonaunet 2006). When marine Antarctic or Arctic inocula were used for oil biodegradation studies, typical shifts in bacterial communities were detected by changes in banding patterns in denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA PCR-amplified gene fragments, and the abundance of Gammaproteobacteria increased, as determined by fluorescence in-situ hybridization or by sequencing of the 16S rRNA genes (Yakimov et al. 2004; Gerdes et al. 2005).

Phylogenetic analyses of microbial communities in pristine marine sediments from Arctic or Antarctic environments revealed predominance of Gamma- and Deltaproteobacteria, members of the Cytophaga–Flavobacterium cluster, Planctomycetes and archaea (Ravenschlag et al. 2001; Bowman and McCuaig 2003; Bowman et al. 2003). Closer examination of the members of the Deltaproteobacteria revealed sequence alignments to members of sulphate-reducing bacteria of the *Desulfosarcina–Desulfococcus* group (Ravenschlag et al. 2000), and MPN-propagations of sulphate-reducing bacteria from Arctic sediments showed predominance of psychrophilic communities (Knoblauch et al. 1999). Controlled oil-contamination of pristine Antarctic marine sediments resulted in immediate increase in hydrocarbon-degrading bacteria by several orders of magnitude (Delille and Delille 2000; Powell et al. 2005b). Cloning and sequence analyses of bacterial communities from oil-polluted Arctic sediments showed that the oiled beaches resulted in increased abundance of Gammaproteobacteria, when compared to an unoiled reference beach (Grossmann et al. 2000).

23.3.1.2 Deep water

Only a few studies have described hydrocarbon-degrading microbes in deep water. Schwarz et al (1975) reported that microorganisms collected from sediments at a depth of 4,940 m in the Atlantic were capable of utilizing hydrocarbons (hexadecane) under both ambient and in situ pressures. Several organic-solvent-tolerant bacterial strains isolated from deep seabed environments (1,100–2,000 m depth) were capable of degrading n-alkanes (n-C_7–n-C_{16}) of crude oil, polyaromatic hydrocarbons, or cholesterol (Moriya and Horikoshi 1993). Investigations of natural oil-seep sediments at moderate or deep-sea depths (300–1,500 m) on the northwest shelf of Australia showed that 74% of isolated strains displayed preferential growth on hydrocarbon-selective media (Johnson and Hill 2003). Further studies from

cold hydrocarbon seeps in deep-sea sediments (Japan trench, depth 5,300 m) showed dominance of Deltaproteobacteria and Epsilonproteobacteria. The Deltaproteobacteria were mostly affiliated to the sulphate-reducing bacteria of the genus *Desulfosarcina* (Inagaki et al. 2002).

23.3.2 Hydrocarbon metabolism at low temperatures

23.3.2.1 Aerobic degradation

Aerobic degradation of aliphatic hydrocarbons (*n*-alkanes) involves a terminal or subterminal oxidation of the alkane. In most bacteria, the terminal oxidation process includes α- and ω-hydroxylation of the alkane to alcohols catalyzed by alkane monooxygenase (Harayama et al. 1999). Alkane metabolism is associated with the function of *alk* gene products. The *alkBFGHJKL* operon of *Pseudomonas putida* encodes the enzymes necessary for converting alkanes into acetyl-coenzyme A, while *alkST* encodes a rubredoxin reductase and a positive regulator for the *alkBFGHJKL* operon (Van Hamme et al. 2003).

For aromatic hydrocarbons, the ring structure is attacked by hydroxylation with generation of catechols, followed by transformation of the hydroxides to carboxylic and/or aldehyde groups and ring cleavage. The initial bacterial processes are catalyzed by dioxygenases (Cerniglia 1992). PAH-metabolism has been characterized in *P. putida*, notably using studies of naphthalene-catabolic plasmids. In this system, three operons are involved, one (*nahAaAbAcAdBFCED*) encodes the pathway for the conversion of naphthalene to salicylate, the second (*nahGTHINLOMKJ*) encoding conversion of salicylate to acetaldehyde and pyruvate, while the third operon (*nahR*) is a regulator for both the other operons (Van Hamme et al. 2003).

Investigations of hydrocarbon-catabolism in cold environments have primarily been performed in contaminated Arctic or Antarctic soils. Two strains of *Pseudomonas* sp. isolated from Arctic soils degraded C_5–C_{12} *n*-alkanes, toluene and naphthalene at both 5 and 25°C and possessed both the *alk* and *nah* catabolic pathways (Whyte at al. 1997). The psychrotrophic *Rhodococcus* sp. strain Q15 mineralized the alkanes dodecane (*n*-C_{12}) and hexadecane (*n*-C_{16}) at 0 and 5°C, and utilized a broad degree of C_{10}–C_{21} alkanes, branched alkanes and a substituted cycloalkane present in diesel fuel at 5°C (Whyte at al. 1998). This strain contains at least four alkane monooxygenase gene homologues (*albB1*, *alkB2*, *alkB3* and *alkB4*). The *alkB1* and *alkB2* homologues are part of an *alkB* gene cluster encoding rubredoxines, a transcriptional regulatory protein and a rubredoxine reductase. It is assumed that the explanation for the presence of the four monooxygenases in one strain is that each alkane monooxygenase is specific for a certain range of alkanes (Whyte et al. 2002b).

Genes encoding alkane monooxygenase (*alkB* and *alkM* genes) were examined in hydrocarbon-polluted and pristine soils from the Canadian Arctic and the Antarctic by culture-independent (PCR hybridization) and culture-dependent (colony hybridization) methods. Culture-independent methods showed that *alkB*

genes were prevalent, while *alkM* genes appeared only rarely, colony-hybridization results showed that *alkB* genes were more abundant in cold-adapted cultures than in mesophilic cultures from the same origin, while *alkM* genes were less abundant (Whyte et al. 2002a). The presence of a number of hydrocarbon degradation genes were examined in Antarctic petroleum-contaminated soils by PCR and hybridization method, including genes for alkane monooxygenase (*alkB* and *alkM*), naphthalene dioxygenase (*ndoB*), toluene dioxygenase (*todC1*), catechol-2,3-dioxygenase (*xylE* and *cat23*) and biphenyl dioxygenase (*bphA*), with PCR-primers and probes derived from hydrocarbon degradation genes of *P. putida*, *Rhodococcus* sp., *Acinetobacter* sp., or *P. pseudoalcaligenes*. All genes, except *alkM*, were detected in contaminated and control soils, and at higher frequencies than in polluted soils from Brazil (Luz et al. 2004).

23.3.2.2 Anaerobic degradation

Anaerobic degradation of alkenes or aromatic hydrocarbons has been reported in marine environments by sulphate- or nitrate-reducing bacteria (e.g., Aeckersberg et al. 1991, 1998; Rockne et al. 2000; Kniemeyer et al. 2003). In situ anaerobic degradation of petroleum alkanes has also been reported in marine sediments under natural conditions (Massias et al. 2003). It is also obvious that anaerobic degradation of petroleum hydrocarbons contributes to degradation in marine low-temperature anoxic environments, and low-temperature degradation of PAH-compounds have been reported with enrichment cultures from Arctic soils under anoxic and nitrate-reducing conditions at 7°C (Eriksson et al. 2003).

In recent years, the mechanisms behind anaerobic degradation of alkanes and aromatic hydrocarbons have been better understood. Anaerobic oxidation of *n*-alkanes and aromatic hydrocarbons has been demonstrated with sulphate or nitrate as electron acceptors, forming alkyl- or benzyl-substituted succinates via radicals with fumarate as co-substrate (Kropp et al. 2000; Krieger et al. 2001; Rabus et al. 2001; Spormann and Widdel 2000). Further metabolism of *n*-alkanes includes the conversion of succinyl-CoA to propionyl-CoA (Wilkes et al. 2002), while aromatic hydrocarbon conversion includes the intermediates acetyl-CoA and benzoyl-CoA (Leuthner and Heider 2000).

23.3.2.3 Microbial metabolism in sea ice

Although petroleum stimulates bacterial growth in sea ice, hydrocarbon biodegradation seems to be very slow. The ability to grow at subzero temperatures has been demonstrated by the bacterium "*Psychromonas ingrahamii*", which has been cultured at temperatures as low as −12°C (Breezee et al. 2004). Respiration in the ice has been associated with particles or surfaces in the brine-inclusion networks of the ice (Junge et al. 2003, 2004), caused by salting-out processes during ice development. Laboratory experiments showed that leucin was incorporated into proteins in a

psychrophilic bacterium (*Colwellia psychroerythraea*) down to temperatures of −20°C (Junge et al. 2006).

Microbes living in marine ice may adapt to their environments in several ways. Psychrophilic bacteria may produce extracellular polymeric substances (exopolysaccharides), which are associated with halotolerance and may have a cryoprotective role in the sea ice brine channels, as well as binding essential cationic trace metals (Nichols et al. 2005). Enzymes in bacteria isolated from sea ice may be cold-active, with catalytic activities well below the freezing point of seawater (Groudieva et al. 2004). Microbes exposed to large drops in temperature may produce cold-shock and cold-acclimation proteins (Berger et al. 1996). Cold-shock proteins are supposed to be involved in protein translation regulation, while cold-acclimation proteins show high catalytic activity at a low temperature and rapid inactivation at a moderate temperature (Fukunaga et al. 1999). Psychrophilic and halophilic bacteria are also capable of adjusting the lipid composition of their membranes in such a way that the proton permeability at the respective growth temperature remains constant by altering fatty acid composition and protein content of the membrane (Chintalapati et al 2004). Protein flexibility is also important for adaptions to low temperatures to avoid reduced stability (Fields 2001). In a study of the psychrophilic bacterium *Colwellia psychroerythraea* 34H, comparative genomic analysis suggested that the psychrophilic life is most likely conferred not by a unique set of genes, but by a collections of synergistic changes in overall genome content and amino acid composition (Methe et al. 2005).

23.4 Modelling of hydrocarbon degradation

23.4.1 Biodegradation rates

Due to the complexity of petroleum oil we cannot predict just one degradation rate for petroleum, since each of the thousands of compounds has individual rate constants. In a numerical fate and effect model for oil spills, biodegradation rates included rate coefficients for groups of hydrocarbons with similar characteristics, such as comparable boiling points (Reed et al. 2000). In addition, many hydrocarbons distribute between the water- and oil-phases after discharge to seawater, resulting in separate degradation rates for the water- and oil-phases. This was shown for some PAH compounds by comparing their biotransformation rates in water-soluble fractions and when immobilised in thin oil films (Brakstad and Faksness 2000; Brakstad and Bonaunet 2006).

Biodegradation rates are determined by nonlinear regression analyses, using first- or second-order approaches. In environments with low and relative constant biomass, like seawater, rate coefficients are often determined by first-order rate kinetics (Battersby 1990), which also is convenient for use in numerical fate models for oil discharges.

23.4.2 Temperature

Most marine biodegradation experiments have been performed at temperatures higher than those expected in polar or deep-water environments. One simplistic way of transferring data from warm to cold water environments is the Arrhenius plot (Arrhenius 1889). Ideally the plot should be linear, but experimental data have shown that Arrhenius plots may fit by more than one linear segment. For instance, when psychrotrophic toluene-degrading strains of *P. putida* isolated from a toluene-polluted were grown in media supplemented with benzoate or toluene the Arrhenius plots were best fitted by two linear segments at a temperature range from 30 to 4°C (Chablain et al. 1997). The linearity of the Arrhenius plot thus reflects microbial activities when the temperature range is not too wide (Nedwell 1999).

Temperature-dependent biodegradation has been suggested to follow a Q_{10} value. This value is the ratio of the first-order rate constant at a specific temperature to the rate constant at a temperature 10°C lower (Kurola 2006). The Q_{10}-values depend on the activation energy (E_a) of the specific chemical reaction, which is directly related to the activation enthalpy (ΔH^*); the lower the activation enthalpy, the lower the Q_{10}-value. The Q_{10}-values were determined in a seawater-based hydrocarbon biodegradation study with a heavy fuel oil (Bunker C), and with winter or summer water samples from the North Sea as inocula. When incubation temperatures of 4 to 18°C were used, Q_{10}-values of 2.4 and 2.1 were determined for waters in winter and summer, respectively, when biodegradation was measured as biological oxygen demand (Minas and Gunkel 1995).

23.5 Oil bioremediation in cold marine environments

Bioremediation is the stimulation of pollutant biodegradation by accelerated biodegradation, either by the indigenous microbes (biostimulation) or by introducing microbial cultures with high degradation potentials for the present contaminants (bioaugmentation). Since hydrocarbon-degraders are indigenous in seawater and shoreline sediments most marine bioremediation actions have focused on developing good biostimulation strategies, typically by applying dispersants, rate-limiting nutrients, or by the combination of these and other processes to accelerate the activities of natural degradation processes. Bioremediation offers cost-effective procedures to reduce the environmental impacts of marine oil spills (Prince 1993; Swannell et al. 1996; Prince and Clark 2004; Prince 2005; Prince and Atlas 2005). Prerequisites for accelerated biodegradation are hydrocarbon bioavailability (i.e. microbial access to the contaminants), enough oxygen for biodegradation (theoretical oxygen demand of 3.5 mg O_2 mg^{-1} hydrocarbon) and no limitations of essential nutrients.

23.5.1 Biostimulation

23.5.1.1 Shoreline sediments

Most biostimulation actions have focused on stranded oil, with application of fertilizers to increase the natural degradation by the indigenous microbial flora. Biostimulation treatment is often combined with mechanical treatment to improve oxygen and nutrient stimulations.

In marine environments, some growth- and biomass-stimulating factors are essential for oil biodegradation, especially nitrogen and phosphorous, and the addition of these nutrients is common practice in bioremediation. A balanced nutrient availability is important for biodegradation and the composition of hydrocarbon-degrading communities, since nutrient amendments in some instances have inhibited microbial activities (Braddock et al. 1997). It is therefore important to avoid excess of nutrients which can cause detrimental effects such as eutrophication. During biostimulation actions, molar carbon/nitrogen/phosphorous ratios of 100/10/1 have often been used (e.g., Bouchez et al. 1995; Obbard et al. 2004). However, results from laboratory studies have also shown that microbial sub-populations may require more than one N/P ratio for optimal degradation of different hydrocarbons (Smith et al. 1998). Nutrient products are available as briquettes, granules or liquid fertilizers. Liquid inorganic fertilizers have proven effective but require frequent application, and therefore oleophilic slow-release nutrient formulations have been developed which promote hydrocarbon degraders at the oil–water interface.

For improved results, bioremediation actions may be combined with other cleanup procedures. Surf washing and the use of surfactants may increase the surface area of the oil and hence increase oil degradation. Ex situ technologies like land farming (spreading the polluted sediments over a larger area for better oxygenation), composting and biopiling may be used for treating oily waste during spill treatment (Lynch and Moffat 2005).

Several field biostimulation trials have been conducted on Arctic beaches on Svalbard, either in Ny Ålesund (78°55′N, 11°56′E) or in the Van Mijen fjord close to the small mining community of Svea (77°56′N, 16°43′E). Experiments performed by SINTEF and the oil company ELF in the 1980s in Ny Ålesund with the slow-release oleophilic fertilizer Inipol EA22 indicated that application of the fertilizer to oil in beach sediments resulted in increased biodegradation in coarse sediments, but not for oil in finer sediments (Sveum and Ladousse 1989). During a full-scale trial during the ITOSS program in 1997 several remediation actions were tested on intermediate fuel oil (IF-30) artificially stranded on mixed (sand and pebble) intertidal shorelines. The remediation methods included sediment relocation (surf washing), mixing (tilling) and bioremediation (Guenette et al. 2003; Sergy et al. 2003). Fertilizers used included both soluble (prilled ammonium nitrate and superphosphate ($Ca(H_2PO_4)_2$)) and commercial slow-release (Inipol SP1) chemicals, applied to the top of the sediments during the first 2 months of the experiment. The introduction of the fertilizers resulted in elevated levels of bioavailable nitrogen and

phosphorous in the oiled sediments. The biodegradation rates were approximately doubled over a period of 1 year in the oiled sediments that received fertilizers when compared to non-treated oiled sediments, and no acute toxicity was associated with the bioremediation treatment (Prince et al. 2003). Mixing/tilling also seemed to result in increased microbial activities for limited periods by increasing the sediment permeability (Owens et al. 2003).

Biostimulation field experiments have also been conducted in Antarctic environments. An Arabian crude oil (topped at 150°C) was added to several 1-m^2 enclosures in intertidal sandy beaches on the main island of the Kerguelen Archipelago (49°19′S, 69°42.5′E). Different fertilizers were added to the top of the oil, including the slow-release Inipol EAP 22 and various experimental mixtures consisting of dry fish compost, with or without supplements of urea, phosphate and charged or neutral surfactants (Pelletier et al. 2004). During a 300-day experiment the oil was eventually depleted in both untreated and treated sediments in this cold environment (seawater temperatures 3–4°C), but the various fertilizers accelerated the biodegradation rates. It was also observed that a fertilizer with a neutral surfactant reduced the toxicity of the oil during the last 3 months of the experiment.

Bioremediation was used as an oil beach cleaning technology on a full-scale oil spill in Arctic environments during the Exxon Valdez accident in March 1989. This spill in the Prince William Sound of Alaska resulted in the release of 41 million litres of Alaskan North Slope crude oil. Bioremediation was used extensively, employing the fertilizers Inipol EAP 22 and Customblen (slow-release granulated fertilizer). Approximately 50,000 kg nitrogen and 5,000 kg phosphorous were applied to the shorelines over the summers of 1989–1992 (Bragg et al. 1994). For a low-energy beach containing both surface and subsurface oil and treated with both fertilizers, it was estimated that the fertilizers enhanced oil biodegradation by 5.5 times over non-treated controls (Bragg et al. 1994).

23.5.1.2 Seawater

Most remediation actions in seawater have focused more on mechanical removal, e.g., by use of oil booms and skimmers, than on accelerated biodegradation. As an alternative to mechanical treatments, chemical oil dispersants have been used. Dispersants are used primarily to remove much of the oil from the water surface in order to reduce the impacts on seabird and mammal populations close to an oil spill, but have also been suggested for improving hydrocarbon biodegradation in the seawater column. Dispersants are mixtures of surface-active agents and will reduce the surface tension of the oil, resulting in the formation of small oil droplets which will rapidly dilute. The dispersants reduce the average oil-droplet size, resulting in higher surface/volume ratios, and thereby increasing the bioavailability of the dispersed oil. However, this may also increase the concentrations of toxic compounds in the water column (Yamada et al. 2003).

Biodegradation studies with chemical dispersal of oils have shown conflicting results. In a cold water mesocosm experiment (−1.8 to 5.5°C) in 3.5-m^3 tanks, dispersed light crude oils (Forties and Western Sweet Blend) were treated with the dispersant Corexit 9527 or different surfactant mixtures, and this chemical dispersal resulted in higher biodegradation rates than in untreated oil during a 63-day experiment if water temperatures were >0°C (Siron et al. 1995). In microcosm studies with seawater temperatures of 8°C, the dispersant Corexit 9500 was added to Alaskan North Slope crude oil. The dispersants resulted in rapid colonization of oil droplets by bacteria, and heterotrophic and oil-degrading microbes proliferated in the microcosms (Davies et al. 2001; MacNaughton et al. 2003). However, the amendments of dispersants resulted in only slow or negligible biodegradation of the oil when compared to naturally dispersed oil (Davis et al. 2001; Lindstrom and Braddock 2002; MacNaughton et al. 2003). It was suggested that carbon mineralization at least initially was the result of dispersant mineralization (Lindstrom and Braddock 2002). However, if the more dispersible and biodegradable Forties oil was used at a seawater temperature of 15°C, the biodegradation effect of dispersant was better (Swannell and Daniel 1999; MacNaughton et al. 2003). Studies of the correlation between the droplet area of dispersed oil and oil degradation indicated that both dispersed area and dispersant chemistry controlled the degradation and depended on the surfactant blend hydrophile–lipophile balance and treatment levels (Varadaraj et al. 1995).

Fertilizers have stimulated biodegradation of crude oils in cold seawater under controlled experimental conditions. The slow-release fertilizer Inipol EAP 22 was added to Antarctic seawater contaminated with "Arabian light" crude oil in a mesocosm study. The addition of the fertilizer enhanced both the concentrations of heterotrophic and oil-degrading bacteria and increased the rate of biodegradation during a 40-day experiment both in ice-covered and ice-free seawater (Delille et al. 1998).

23.5.1.3 Sea ice

Only a few studies have investigated the possibilities for accelerated biodegradation of petroleum in sea ice. In this environment, the microbes are primarily active in the brine channels where fluidity is maintained at temperatures well below freezing point. During freezing periods, the brine channels, which may constitute up to 30% of the ice volume, may reach salinities >80 psu, while ice melting results in ice surface salinities well below that of seawater in the upper parts of the ice (Krembs et al. 2001).

A study on bioremediation of diesel fuel and "Arabian light" crude oil in land-fast Antarctic ice showed that the oils induced a negative effect on ice-microalgae growth, reducing the phytoplankton bloom, but addition of the fertilizer Inipol EAP 22 stimulated the microalgae development (Fiala and Delille 1999). During the winter of 2004, a field bioremediation experiment was set up by the Alfred Wegener Institute in Van Mijenfjord on Svalbard, in which fertilizers were applied to oil-polluted Arctic fjord ice. However, no data from this experiment have yet been published.

23.5.2 Bioaugmentation

Bioaugmentation has been proposed as a bioremediation method for soil and sediments, often as a supplement to biostimulation treatments. Introduction of exogenic hydrocarbonoclastic bacteria for detoxification of hydrocarbon-polluted cold environments have been reported to have variable success. In a study of diesel oil-contaminated Alpine soil, a psychrophilic diesel oil-degrading inoculum was added to the contaminated soil, but biostimulation with fertilizers proved more efficient than the bioaugmentation for improved biodegradation activity (Margesin and Schinner 1997). In microcosm experiments performed in Antarctic gas-oil polluted soil (Jubany Station, King George Island, South Shetland Islands), inoculation of the psychrotolerant strain B-2-2 resulted in 75% hydrocarbon removal after initial 35% hydrocarbon removal by biostimulation methods when compared to abiotic controls (Ruberto et al. 2003).

Several bioaugmentation studies from marine environments have been reported, although none of these are from cold waters. A laboratory biodegradation and toxicity study of 12 commercially available bioaugmentation products applied to weathered oil (Alaska North Slope) in seawater at 20°C showed that 3 of the products enhanced biodegradation better than nutrient-amended controls, but only 1 product resulted in reduced toxicity (Aldrett et al. 1997). In a marine sediment microcosm study, the aromatics-degrading bacterial strain *Cycloclasticus* sp. E2 was identified as playing an important role during degradation of naphthalene in combination with biostimulation treatment (Miyasaka et al. 2006). Interestingly, bacteria of this genus were also abundant during bioremediation treatment of Arctic oiled beaches on Svalbard (Grossman et al. 2000).

23.6 Conclusions

The cold environments of the Earth are increasingly exposed to petroleum exploration, production and transport, and these activities increase the risk of accidental oil discharges. Since hydrocarbon-degrading microorganisms are present in most of these environments they may play a crucial role during decontamination of toxic hydrocarbons in the seawater column and in sediments. Microbial activities have also been measured in marine ice at temperatures lower than −10°C, indicating that slow hydrocarbon biodegradation also may appear in oil-contaminated ice, primarily of water-soluble compounds present in the brine channels of the ice. As our knowledge of microbial ecology, metabolic processes, and interactions with their environment in the cold environments are constantly increasing we will be able to design improved regimes for bioremediation in these vulnerable environments.

Bioremediation is still a “promising” technology for treatment of polluted cold marine environments. It is cheap, labour-effective and generates no harmful

by-products. To decrease the time frame for the restitution of polluted environments combinations of bioremediation techniques with different chemical and mechanical treatments are required.

Recent advances within genomics and proteomics are also important to address the molecular mechanisms of psychrophilic biodegradation. These methods may be used for optimizing bioremediation methods, and to improve the monitoring of the treatment efficiencies. Examples are microarrays and real-time PCR methods, which may be used for measurements of the expression of hydrocarbon-catabolic genes.

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Chapter 24
Microbial Adaptation to Boreal Saturated Subsurface: Implications in Bioremediation of Polychlorophenols

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24.1 Introduction

Saturated subsurface environments pose challenges to the intrinsic microbiology. Prevailing environmental conditions (temperature, pH, bioavailability of substrates and nutrients) affect microbial biodegradation activity, which is often favored by certain redox conditions. Microbial adaptation in each redox environment proceeds by selection and enrichment of indigenous bacteria, evolution of novel catabolic pathways and horizontal gene transfer (Wilson et al. 1985; van der Meer et al. 1998; Tiirola et al. 2002b). Formation of biofilms enables microbial retainment, co-operation among microorganisms and enhanced gene transfer among organisms (Singh et al. 2006).

Chlorophenols are toxic and persistent pollutants mainly originating from anthropogenic sources. Polychlorophenols, i.e. tri-, tetra-, and/or pentachlorophenol

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(TCP, TeCP and PCP, respectively), have been applied in wood preservatives. Although the use of chlorophenols as wide-spectrum biocides in the lumber industry is currently banned in many countries, these chemicals remain typical and recalcitrant groundwater contaminants in low-temperature environments in countries where mechanical wood processing has represented an important branch of industry (Valo et al. 1984; Goerlitz et al. 1985; Mueller et al. 1989). In Europe, typical chlorophenol formulations were produced by nucleophilic substitution-based synthesis processes followed by dissolution as respective sodium salts. In North America, on the other hand, the chlorinated phenol formulations were dissolved in organic solvents such as kerosene. These two product alternatives result in completely different contaminant transport and remediation scenarios.

The fate of a given contaminant is affected by factors such as density, toxicity, volatility and water solubility of the chemical and characteristics of the receiving environment. Degradation and biotransformation of chlorophenols by bacteria and fungi has been reported under aerobic and anaerobic conditions (for reviews, see Häggblom 1992; Mohn and Tiedje 1992; Häggblom and Valo 1995; McAllister et al. 1996; Puhakka and Melin 1996, 1998; Häggblom et al. 2000). However, polychlorinated phenols are not degraded in a reasonable time in many soils, sediments and groundwaters (e.g., Mueller et al. 1991; Suominen et al. 2001). Several chlorophenol-degrading microorganisms have been isolated and characterized physiologically, and their biodegradative enzymes and metabolic pathways have been described. These studies combined with results from on-site and in situ bioremediation processes have outlined the frontiers of bacterial adaptation into chlorophenols in cold groundwater. In this review, special emphasis has been given to the bacterial communities and their interactions in oligotrophic and microaerophilic conditions.

24.2 Physicochemical properties of chlorophenols

Polychlorophenols, especially pentachlorophenol, have been widely used throughout the world in wood preservative and pesticide formulations. Furthermore, chlorophenols are intermediates of herbicide synthesis processes as well as potential metabolites in the biodegradation of phenoxy acid herbicides (Tuxen et al. 2006). In addition to industrial production and usage, chlorophenols are produced from naturally occurring phenols as a result of chlorine bleaching of wood pulp in the paper industry and through the chlorination of domestic water supplies and swimming pools (reviewed by Jensen 1996). The degree of protonation of chlorophenols governs their chemical behavior and fate in the environment (WHO 1989). The aqueous solubility of chlorophenols increases with pH (Zullei 1981) and decreases with the number of chlorine substitutents (Wightman and Fein 1999). Protonated chlorophenols readily adsorb onto organic matter (Lafrance et al. 1994), solids (Stapleton et al. 1994) and bacteria (Daughney and Pein 1998).

24.3 Polychlorophenol degradation is controlled by redox conditions

The activity of microorganisms is often limited to certain redox environments suitable for their specific physiological properties. Consumption of most energy-yielding electron acceptors leads to zones dominated by redox conditions typically proceeding from O_2 Mn(IV), Fe(III) and SO_4^{2-} reduction, and methanogenesis, followed by succession in the respective microbiota. If dissolved oxygen is depleted, biodegradation can continue only in three cases: (1) if oxygen is transported from outside the plume to support aerobic biodegradation; (2) if evolution brings up new microbial strains which are capable to degrade polychlorophenols in intermediate redox conditions; or (3) if the redox potential is low enough to enable reductive dehalogenation under methanogenic conditions. Monochlorophenols can be degraded under a wide range of redox conditions (Table 24.1) (for reviews, see Häggblom 1992; Mohn and Tiedje 1992; Häggblom and Valo 1995; McAllister et al. 1996; Puhakka and Melin 1996, 1998; Häggblom et al. 2000). However, the biodegradation of polychlorophenols does not occur or is lacking under nitrate, iron or sulfate reducing conditions (Table 24.1), which limits in situ bioremediation of groundwater. Reductive dehalogenation requires methanogenic conditions, which in turn are possible in an excess of electron donors (Mohn et al. 1999), which is unlikely to occur in oligotrophic aquifers. Furthermore, anaerobic degradation of chlorophenols is often partial and does not always lead to mineralization (Magar et al. 1999; Beaudet et al. 1998; Gardin et al. 2001). Methanogenesis is also known to be inhibited by relatively low PCP concentrations (0.2–3.0 mg l^{-1}) (Duff et al. 1995). Further, Chang et al. (2004) showed that reductive dechlorination of monochlorophenols competes in hydrogenotrophic microbial consortia with nitrate and sulfate reduction. Anaerobic processes are typically slower and yield smaller amounts of biomass than aerobic processes, since anaerobic microbes gain less energy in their catabolism than aerobic ones. In sequential anaerobic/aerobic bioreactors, accumulating monochlorophenols can be further degraded in the aerobic processes (Magar et al. 1999; Ehlers and Rose 2006), but this may be challenging during in situ bioremediation.

24.4 The Kärkölä case: long-term monitoring of the chlorophenol-contaminated groundwater and on site bioremediation

Aerobic biodegradation of chlorophenols, natural organic matter (NOM) and decaying microbial biomass can lead the environment to anoxic conditions, which leads to elevated soluble concentrations of NH_4^+ and Fe^{2+}, explained by O_2-limitation of iron oxidation and nitrification under microaerobic conditions (Langwaldt et al. 2005). This phenomenon has been seen in one of the most studied and described cases of

Table 24.1 Biotransformation and mineralization of chlorophenols under different redox conditions

Prevalent redox condition	MCP	DCP	TCP	TeCP	PCP	Comment	Reference
Aerobic	+	+	+	+	+	Mineralization	for a review, see Puhakka et al. (1995)
Nitrate-reducing	+	NS	NS	NS	NS	Dechlorination	Bae et al. (2002)
	NS	-	NS	NS	-	No transformation observed	Puhakka et al. (1992)
	+	NS	NS	NS	NS		Melin et al. (1993)
	+	+	NS	NS	NS	Dechlorination followed by phenol degradation	Sanford and Tiedje (1997) for a review, see Häggblom et al. (2000)
Iron(III)-reducing	+	NS	NS	NS	NS	Biodegradation	Kazumi et al. (1995)
	+	+	NS	NS	NS	Dechlorination	He and Sanford 2003)
Sulfidogenic	+	NS	NS	NS	NS	Mineralization	Häggblom and Young (1995)
	+	+	+	NS	NS	Reductive dechlorination	Ehlers and Rose (2006)
Methanogenic	+	+	+	+	+	Mineralization	Kennes et al. (1996)
	-	+	+	+	+	Dechlorination, accumulation of 4-MCP	Magar et al. (1999)
Dehalorespiration	+	+	NS	NS	NS	Reductive dechlorination only of ortho-chlorophenols	Sun et al. (2000)

MCP Monochlorophenols, *DCP* Dichlorophenols, *TCP* Trichlorophenols, *TeCP* Tetrachlorophenols, *PCP* Pentachlorophenol, *NS* not studied

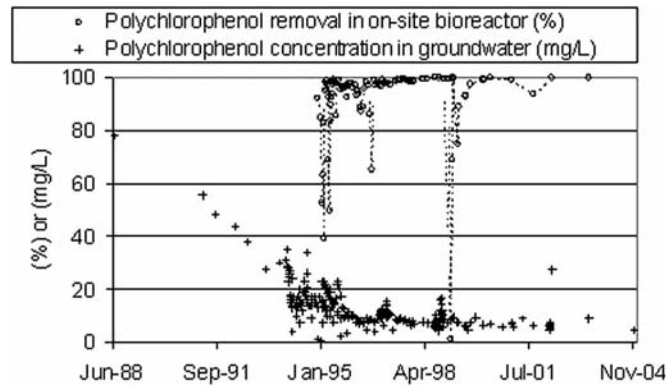

Fig. 24.1 Summed concentration of 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) in a contaminated groundwater at Kärkölä, Finland, and removal of chlorophenols in an on-site bioreactor. Failures in oxygen supplies in 1996 and 1999 resulted temporary losses of chlorophenol removal

polychlorophenol-contaminated groundwater, which is situated in Kärkölä, Finland (Langwaldt et al. 2005). A sawmill upstream of the contaminated aquifer used the wood preservative Ky-5 from the 1930s until 1984 (Nystén 1994). In 1988, groundwater samples showed chlorophenol concentrations as high as 189 mg l^{-1} (Herkamaa 1988) consisting of 2,4,6-TCP, 2,3,4,6-TeCP and PCP. Sediment studies of the recipient Lake Valkjärvi indicated that groundwater contamination occurred from the late 1960s to the early 1970s (Lampi et al. 1992).

Limitations of the on-site bioremediation of chlorophenols were originally thought to be the low groundwater temperature, which causes slow degradation rates (Melin et al. 1998a), and too low contaminant concentration to allow sufficient biomass buildup in suspended systems (Langwaldt and Puhakka 2000). A high-rate on-site bioremediation process based on fluidized-bed biofilm technique was successfully developed and applied at Kärkölä and operated at 5–8°C for 10 years between 1994 and 2004 (Järvinen and Puhakka 1994; Järvinen et al. 1994; Melin et al. 1998a,b; Puhakka et al. 1995, 2000; Järvinen 2001). The full-scale process treated contaminated groundwater with an average efficiency of over 99% (Puhakka et al. 2000; Järvinen 2001). The operation demonstrated the feasibility of on-site bioremediation of chlorophenols in ambient groundwater temperatures. The groundwater was aerated, supplemented with $(NH_4)_2HPO_4$ and fed to the bioreactor. Since 1996, the concentrations of polychlorophenols in the groundwater (Fig. 24.1) pumped to the bioreactor decreased only moderately (Järvinen 2001).

24.5 In situ bioremediation of groundwater polychlorophenols

In situ remediation of contaminated groundwater is considered more efficient and economical than pump-and-treat systems (e.g., Edel and Voigt 2001). Experiences of pilot- and full-scale in situ bioremediation of groundwater contaminated with

polychlorophenols are limited to PCP (Burnett and Man 2000), mixtures of creosote oils with PCP and TeCP (Piotrowski et al. 1994; EPA 1998; Borazjani et al. 1999), chlorophenols in chlorobenzene mixed plume (Zettler 1996; Junge and Schäfer 1997), and a mixture of chlorophenols in the case of Kärkölä. In all these case studies, comparison of the chlorophenol concentrations upstream and downstream of contamination source demonstrated efficient chlorophenol removal at low temperatures. In most cases, the aerobic biodegradation of chlorophenols competed with ferrous iron oxidation for supplied oxygen (Zettler 1996; Junge and Schäfer 1997; Langwaldt and Puhakka 1999; Burnett and Man 2000). Consumption of supplied oxygen by ammonium oxidation was also reported (Zettler 1996; Junge and Schäfer 1997). In the case of Kärkölä, biodegradation was limited only by availability of oxygen, as demonstrated in pilot-scale study (Langwaldt and Puhakka 2003).

24.6 Distribution and origin of polychlorophenol degraders

24.6.1 Diversity of culturable polychlorophenol degraders

Many of the well-known PCP and polychlorophenol-degrading bacteria belong, in the current taxonomical classification, to the genera *Sphingobium* or *Novosphingobium* (Karlson et al. 1995; Nohynek et al. 1996; Cassidy et al. 1999; Tiirola et al. 2005). Besides sphingomonads, other well-known aerobic polychlorophenol degraders have been classified to *Ralstonia* (Louie et al. 2002), *Pseudomonas* (Shah and Thakur 2003; Kao et al. 2005), *Burkholderia* (Tomasi et al. 1995), *Streptomyces* (Golovleva et al. 1992), *Arthrobacter* (Wieser et al. 1997), *Mycobacterium* (Häggblom et al. 1994), and *Rhodococcus* (Briglia et al. 1996). The phylogenetic analysis of groundwater bacteria isolated from a chlorophenol-contaminated aquifer (Männistö et al. 1999) showed that the aerobic polychlorophenol-degrading capacity is widely distributed among cold-adapted bacteria, as it was detected with various groundwater isolates assigned to *Nocardioides*, *Pseudomonas*, *Ralstonia*, *Flavobacterium* and *Caulobacter,* as well as in the phylogenetically diverse group of sphingomonads (Fig. 24.2).

Cultivation-independent studies have also revealed the principal role of sphingomonads as aerobic chlorophenol degraders in different environments. A bacterium matching the dominant molecular fingerprint and 16S rRNA gene sequence in an on-site bioremediation process of the chlorophenol-contaminated Kärkölä aquifer was sphingomonad, and was characterized as *Novosphingobium lentum* MT1^T, a polychlorophenol degrader at 8°C (Tiirola et al. 2002a, 2005). Stable isotope probing (SIP) (Radajewski et al. 2000) and denaturing gradient gel analysis (DGGE) of the RNA pool has been used for the cultivation-independent analysis of indigeneous chlorophenol degraders of grassland soil (Mahmood et al. 2005). The majority of sequenced DGGE bands that appeared or increased in relative intensity were most closely related to *Pseudomonas*, *Burkholderia* and *Sphingomonas* (Mahmood et al. 2005).

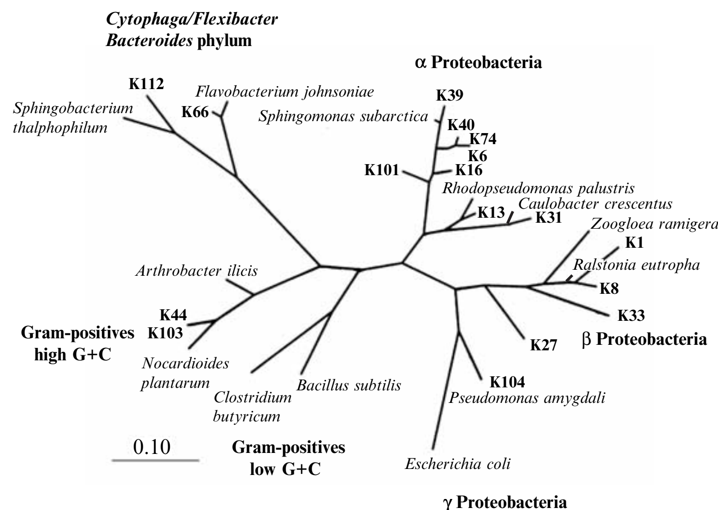

Fig. 24.2 Phylogenetic analysis of polychlorophenol degrading Kärkölä strains shows that chlorophenol degradation ability was widespread among sphingomonads but also present in four other lineages of bacteria. The strains isolated from contaminated groundwater in Kärkölä are indicated by "K". The tree was based on maximum-likelihood analysis of partial 16S rRNA sequences (adopted from Männistö et al. 1999 with permission from Springer-Verlag)

Analysis of the anaerobic polychlorophenol dehalogenating cultures has brought up two genera, *Desulfomonile* (Mohn and Kennedy 1992) and *Desulfitobacterium* (Dennie et al. 1998). Isolation of anaerobic dehalogenating bacteria has proven to be challenging, since those bacteria are often interdependent on their syntrophic partners (Becker et al. 2005). Another challenge is to provide the chlorinated substrate at levels that are nontoxic yet adequate to sustain growth and meeting the specific nutritional requirements of the dehalorespiring organism (Holliger and Schumacher 1994). Furthermore, many fungus species can degrade chlorophenols; e.g., white rot fungus *Phanerochaete chrysosporium* has been shown to metabolize PCP to pentachloroanisole (Lamar and Dietrich 1990) or 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (Mileski et al. 1988). However, what has been disregarded is that the degradation of xenobiotics by white rot fungi may in fact be due to a symbiotic relation between bacteria and the fungus partner (Laine et al. 2003).

Several soil studies have revealed that bioaugmentation of various chlorophenol degraders can enhance the degradation of chlorophenols (reviewed by Häggblom and Valo 1995). Exceptionally high chlorophenol concentrations, unfavorable redox conditions and mixtures of several toxic compounds may still form situations in which either indigenous or inoculated organisms are not able to adapt. Microbial evolution can be accelerated by genetic engineering. Dai and Copley (2004) successfully used genome shuffling to improve *Sphingobium chlorophenolicum* ATCC 39723 resistance to high levels of PCP. The same strain has recently been coverted to a hexachlorobenzene degrader by inserting the gene cassette encoding a

cytochrome P-450cam by homologous recombination (Yan et al. 2006). However, ethical problems may prevent the use and distribution of genetically modified organisms in practical bioremediation.

24.6.2 Natural evolution of polychlorophenol degradation

Contamination history is a principal factor determining the capacity for self-remediability towards xenobiotics. The acclimation of an aquifer to simulated consecutive accidental spills has been found to be directly proportional to the cumulative load of each single chlorophenol (Antizar-Ladislao et al. 2003). Polychlorophenol degradation ability is known to be also present in some pristine environments. Microorganisms of a forest soil that had never been exposed before to human-generated chloroorganic compounds were able to efficiently degrade up to 5,000 ppm of 2,4,6-TCP (Sanchez et al. 2004). In a microcosm experiment, 80-90% of added 5 ppm of 2,4-DCP and 2,4,6-TCP was removed in pristine sandy and clay forest soils at 20°C in 30 days (Jordan et al. 2002). In a comparative study (Baggi et al. 2004), golf course soil treated with 2,4,6-trichlorophenol derivative (Prochloraz) for several years showed better ability in removing TCP than pristine landfill soil. Indigenous bacterial community was capable of degrading PCP at an approximate rate of 1.2 mg kg^{-1} day^{-1} in pristine grassland soil (Mahmood et al. 2005). Several bacterial isolates from uncontaminated aquifer water showed the ability to degrade TeCP and PCP (Männistö et al. 2001a).

The principal question when predicting the capacity of intrinsic bioremediation of subsurface is how new dehalogenases arise or spread in nature. Besides selection of the most competitive organisms, a range of genetic mechanisms, such as gene transfer, mutational drift, and genetic recombination and transposition, can accelerate the evolution of catabolic pathways in bacteria. Many catabolic genes of xenobiotic compounds are located in transmissible plasmids. In view of chlorophenol-monooxygenases, location in a catabolic plasmid has been only shown in *Pseudomonas* sp. PCP103 (Thakur et al. 2002). Sphingomonads initiate the chlorophenol degradation by the enzyme PCP hydroxylase, PcpB (Xun and Orser 1991; Lange et al. 1996; Cai and Xun 2002; Tiirola et al. 2002b). It has been suggested that *pcpB*/PcpB should be considered a model system for the study of recent evolution of catabolic pathways among bacteria (Crawford et al. 2006). Despite efforts (Basta et al. 2005), no catabolic plasmids have been attributed to polychlorophenol degradation of sphingomonads. The *pcpB* gene sequences with 100% identity have been obtained from PCP contaminated sites in different continents, from North America (Beaulieu et al. 2000) and Europe (Tiirola et al. 2002b). Highly identical (98% identity) sequences of the *pcpB* gene of *Sphingomonas chlorophenolica* ATCC39723 have been obtained from two non-degrading *Pseudomonas* sp. and *Ralstonia* sp. strains isolated from PCP contaminated soil (Saboo and Gealt 1998). Hybridization with the *pcpB* gene probe revealed that the gene was absent in

non-degrading sphingomonads (Tiirola et al. 2002b). Comparative analysis of the *pcpB* gene sequences obtained from genetically diverse group of eight sphingomonad strains isolated from contaminated Kärkölä groundwater site showed *pcpB* gene similarity of 98.9–100% sequence identity, which suggested that a recent gene transfer had facilitated chlorophenol degradation among genetically different sphingomonads (Tiirola et al. 2002b). Furthermore, the *pcpB* gene was spontaneously deleted when sphingomonads were cultivated without polychlorophenols (Tiirola et al. 2002a). In conclusion, the adaptation of sphingomonads to chlorophenols seems to be based on their potential to mobilize and share the *pcpB* gene encoded pathway. Further sequencing of the flanking sites of the gene cluster should reveal whether the gene is associated with transposable elements or insertion sequences.

24.7 Microbial responses to low temperatures

A general rule of thumb in chemical reactions is that reaction rate decreases by a factor of two for a 10°C decrease in temperature. The effect of temperature on growth and aerobic biodegradation of polychlorophenols has been studied by using 12 chlorophenol-degrading bacterial isolates (Männistö and Puhakka 2002). All these bacteria had optimum growth temperature between 23 and 30°C, maximum between 33 and 39°C and theoretical minimum between −33 and −1°C, as obtained from Ratkowsky plots, and the ratio of growth rate at 20°C to growth rate at 10°C was between 1.7 and 3.4 (Männistö and Puhakka 2002) (Fig. 24.3). These results show that the chlorophenol-degrading bacteria in the groundwater were characteristically psychrotolerant, not psychrophilic. Psychrotolerance gives a specific advantage to microorganisms over psychrophiles under varying temperature conditions. Some of the Kärkölä study strains degraded TeCP faster at 8°C than at 23°C (Männistö et al. 2001b). If the enzyme pathway is working better in cold than in warm conditions, it is possible that the catabolic gene cluster was originally recruited by a real cold adapted organism.

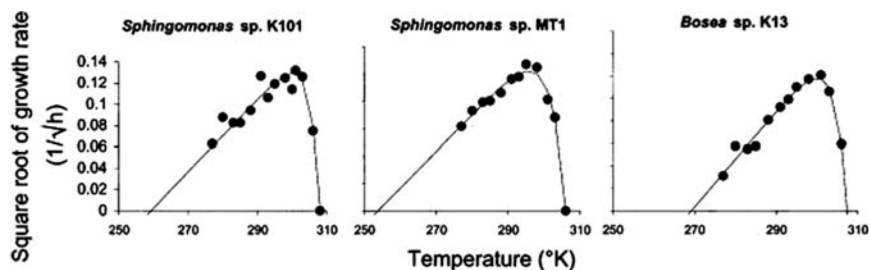

Fig. 24.3 Growth rates at different temperatures and fitted lines of Ratkowsky equation for the selected isolates from boreal groundwater (Männistö and Puhakka (2002) with permission from Blackwell)

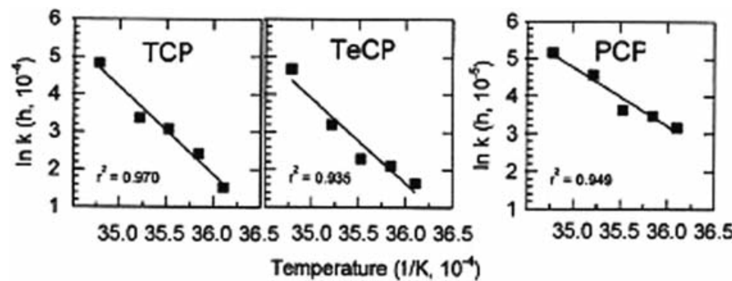

Fig. 24.4 Temperature effects on specific 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) biodegradation rates in fluidized-bed bioreactors employing celite R-633 as biomass carrier. Modified from Melin et al. (1998a) with permission from Elsevier

Melin et al. (1998a) demonstrated the strong temperature dependence of polychlorophenol biodegradation in fluidized-bed reactors treating contaminated groundwater (Fig. 24.4). In the studied temperature range (4–16.5°C), a temperature decrease of 10°C resulted in over seven times slower degradation rates. Arrhenius plots provided the activation energies for TCP and TeCP, 125–194 kJ mol^{-1}, and for PCP 59–130 kJ mol^{-1} (Melin et al. 1998a) (Fig. 24.4). This shows the strong temperature dependence of polychlorophenol biodegradation, since the activation energies for most microbial systems is about 33–50 kJ mol^{-1} (Tchobanoglous and Schroeder 1985). In fixed-bed reactors with bacteria from the Kärkölä aquifer, the highest measured biodegradation rates for total polychlorophenols were 0.3 and 2.7 mg l^{-1} h^{-1} at mean temperatures of 9°C and 23°C, respectively (Langwaldt et al. 1998, and unpublished data). Thus, a decrease by 10°C would cause over six times slower chlorophenol-biodegradation rates. Polychlorophenol degrading bacteria show a variety of low temperature induced modifications of fatty acid compositions to maintain membrane fluidity (Männistö et al. 2000; Männistö and Puhakka 2001). The unsaturation of fatty acids at low temperature to increase membrane fluidity (Männistö and Puhakka 2001) is opposite to the observed saturation of fatty acids by bacteria exposed to phenols (Heipieper et al. 1992). In laboratory-scale fluidized-bed bioreactors at 5–7°C, over 99.9% polychlorophenol biodegradation was achieved at a chlorphenol loading rate of up to 30.8 mg l^{-1} h^{-1} (Järvinen et al. 1994).

24.8 Microbial adaptation to oligotrophic conditions

Pristine groundwater is oligotrophic with dissolved oxygen concentration (DOC) usually below 2 mg l^{-1} (Thurman 1986), therefore the limited DOC content is the major growth-limiting factor for microorganisms. Although the oligotrophic conditions

of groundwater affect the generation time of microorganisms and their physiological state (Morita 1993), total cell counts are often from 10^3 to 10^7 cells ml^{-1} in groundwater (e.g., Männistö et al. 2001a; Langwaldt et al. 2005). In aquifer environments with low nutrient content, microorganisms prefer life at interfaces such as attached on solids. Biofilm formation allows improved substrate access. Microbial adaptation to changing conditions involves intercellular changes that result in improved substrate intake and utilization. Bacteria growing under oligotrophic conditions sustain a diversity of active catabolic enzymes by derepression, thus allowing efficient utilization of the various and alternating substrates at the microlevel (Egli 1995). Bacteria can increase the amount of transport enzymes or substrate-specific porins in the outer membrane, resulting in an observable increase of the apparent V_{max} (Kurlandzka et al. 1991; Death et al. 1993). Further, the transport system can be altered to a high affinity one, resulting in an observable decrease of the apparent K_m (Tros et al. 1996). A multiphase transport system with variable kinetics in relation to substrate concentration is another strategy to improve substrate uptake.

The NOM originates from the topsoil, buried sedimentary organic matter, and decaying biomass, but in groundwater NOM consists mainly of humic substances (Wallis et al. 1979). Koch et al. (2002) showed up to 27% biodegradation of humic substances by groundwater bacteria. Heterotrophic bacteria excrete extracellular NOM-degrading enzymes liberating monomers of NOM. Microbial hydrolysis and uptake of monomers are closely coupled processes (Langwaldt et al. 2005). Groundwater NOM can support the growth of xenobiotic-degrading bacteria and of biofilm-forming bacteria to form a habitat for contaminant-degrading bacteria. Microbial utilization of xenobiotics at initial concentration as low as 0.1 pg l^{-1} is likely due to their simultaneous biodegradation together with uncharacterized DOC (Subba-Rao et al. 1982). Kovárová-Kovar and Egli (1998) used the Monod model to calculate the effect of uncharacterized DOC, initial concentration 0.01–1000 mg l^{-1}, on xenobiotic biodegradation assuming simultaneous utilization of both substrates. An equal amount of uncharacterized DOC to pollutant greatly reduces lag phase and enhances biodegradation rate. In the simulation, the xenobiotic biodegradation rate became independent of the DOC concentration, when it was one order of magnitude larger than the xenobotic concentration, which was set to 1 mg l^{-1} (Kovárová-Kovar and Egli 1998).

24.9 Biodegradation of polychlorophenols under microaerophilic conditions

In groundwater, available dissolved oxygen (DO) may be consumed by oxidation of dissolved (ammonium, ferrous iron, manganese) and solid (iron oxides) reduced species, NOM and the xenobiotics. Aerobic heterotrophic bacteria compete for DO with nitrifying and iron-oxidizing bacteria. The competition for DO by in situ biodegradation of NOM and oxidation of ferrous iron may limit in situ bioremediation of xenobiotics. For instance, at Kärkölä in the mid-1990s, aerobic conditions

prevailed in the pristine groundwater outside the plume, but the plume was anoxic and contained elevated concentrations of ammonium and ferrous iron of up to 1.5 and 27.7 mg l^{-1}, respectively (Langwaldt et al. 2005). In the same aquifer, aerobic in situ biodegradation of NOM was also indicated by lower concentrations of DOC outside than inside the plume (Langwaldt et al. 2005).

Männistö et al. (2001b) studied the biodegradation of TCP by an isolate MT1, which is dominant in the fluidized-bed bioreactor (Tiirola et al. 2002a), and two groundwater isolates. In these experiments performed at 8°C, the oxygen content in the headspace was 2–21%, equal to 1–11.5 mg DO l^{-1} at total saturation. The effect of oxygen partial pressure was negligible compared to the differences of obtained oxygen consumption rates for TCP biodegradation (0.01–0.22 μmol DO l^{-1} min^{-1}) by three different isolates (Männistö et al. 2001b). On rich media, these strains were microaerophilic (Männistö and Puhakka 2002). Biodegradation of PCP was also shown for oxygen concentrations in the headspace from 0.02 to 99.8% (Valo et al. 1985) and from 2 to 21% (Hurst et al. 1997). Valo et al. (1985) indicated biodegradation of PCP at 0.002% oxygen in the headspace. Thus intrinsic biodegradation of polychlorophenols is also likely to occur under microaerobic conditions.

In batch assays using an enrichment culture from the Kärkölä fluidized-bed bioreactor, oxygen consumption rates for 2,4,6-trichlorophenol-biodegradation were up to 0.07 μmol DO l^{-1} min^{-1} at microaerobic conditions (Langwaldt and Puhakka 2002). In a full-scale fluidized-bed bioreactor at Kärkölä, mean rate for TCP-biodegradation was 0.3 μmol DO l^{-1} min^{-1} (Langwaldt and Puhakka 2002). The highest determined oxygen consumption rates for TCP biodegradation in fixed-bed reactor experiments were 0.6 and 0.9 μmol DO l^{-1} min^{-1}, respectively, at about 8°C (Langwaldt et al. 1998; Langwaldt and Puhakka 1999). Biodegradation rates for TCP of up to 1.5 μmol DO l^{-1} min^{-1} were obtained with a culture enriched on pentachlorophenol (Rutgers et al. 1992). However, Järvinen and Puhakka (1994) achieved, in fluidized-bed bioreactor tests, polychlorophenol removal rates corresponding to 25 μmol l^{-1} min^{-1} DO-consumption rates. The independence of polychlorophenol-biodegradation rates from microaerobic and aerobic conditions promises successful process performance under changing dissolved oxygen concentrations as typical for full scale in situ bioremediation applications.

24.10 Competition for oxygen by iron and chlorophenol oxidation

In laboratory experiments on the competition for DO with groundwater-enriched cultures, DO was consumed at higher rates by TCP-oxidizing bacteria than by iron-oxidizing bacteria at both microaerobic, 1 mg DO l^{-1}, and aerobic, 11.5 mg DO l^{-1}, conditions (Langwaldt and Puhakka 2003). Thus, aerobic biodegradation of TCP was not limited by simultaneously occurring Fe(II)-oxidation (Langwaldt and Puhakka 2002, 2003). Further, the biological oxidation of ferrous iron by a ground-

water-enriched culture followed zero-order kinetics and was completely inhibited by PCP at 6.1 mg l^{-1} (Langwaldt and Puhakka 2003). Further, biodegradation of polychlorophenols consumed oxygen at higher rates than Fe(II)-oxidation in full-scale studies (Langwaldt and Puhakka 2002).

24.11 Competition for oxygen by polychlorophenol, iron and natural organic matter oxidation

The competition for oxygen by Fe(II)-oxidation and biodegradation of NOM and chlorophenols was monitored in a full-scale fluidized-bed bioreactor at Kärkölä (Langwaldt and Puhakka 2002). Concentrations of NOM in the contaminated groundwater were up to 31.8 mg DOC l^{-1}. In the bioreactor, DO was consumed for polychlorophenol biodegradation (52%), NOM biodegradation (36%) and Fe(II)-oxidation (12%) (Langwaldt and Puhakka 2002). The groundwater DOC was partially biodegraded under aerobic conditions (20–27%) (Langwaldt et al. 1998; Langwaldt and Puhakka 1999, 2002). Also, other groundwater studies have shown that the biodegradable fraction of NOM is 20–27% (Järvinen et al. 1994; Goel et al. 1995; Kroth et al. 2001). The oxidation of ferrous iron and NOM did not control the bioremediation of polychlorophenols (Langwaldt and Puhakka 1999, 2002). Similar results were obtained by Tuxen et al. (2006) showing that oxidation of organic matter accounted for most of the dissolved oxygen consumption. These experiments demonstrate that competing oxidation processes are factors to be considered in intrinsic xenobiotic biodegradation, but oxygen consumption by chlorophenol-degrading bacteria has potential to compete with other oxygen consuming reactions.

24.12 Conclusions

The ability to degrade polychlorophenols is a common trait in many phylogenetically different microbes even in pristine environments, such as pristine soils and groundwaters. Furthermore, in oligotrophic groundwater environments with limited carbon sources, long-term chlorophenol contamination leads to bacterial acclimation, implicated by bacterial selection and horizontal gene transfer. Horizontal gene transfer has been shown by the *pcpB* pathway of shingomonads; these bacteria are well-known for their ability to degrade various haloaromatics. Other microorganisms seem to utilize several other functionally similar chlorophenol monooxygenase pathways, some of which are known from other Proteobacteria. Low temperature on-site high-rate bioreactor treatment has demonstrated the long-term viability of bioremediation of polychlorophenols in boreal environments. Biodegradation of chlorophenols by indigenous subsurface bacteria has been shown to efficiently proceed even in low oxygen concentrations and at low temperatures which prevail in groundwaters. However, microbial adaptation to

anoxic conditions is limited in the case of polychlorophenol biodegradation. The biodegradation of DCP, TCP, TeCP and PCP is unstable or lacking under nitrate, iron or sulfate reducing conditions. In a study focusing in a long-term contaminated Kärkölä groundwater, biodegradation of polychlorophenols was oxygen-limited, showing potential for in situ remediation by supplementation of oxygen. Biodegradation of polychlorophenols outcompeted other oxygen consuming processes such as iron oxidation under aerobic and microaerobic conditions, and thus bioremediation of polychlorophenol-contaminated groundwater could be predicted to proceed concomitant with NOM and iron oxidation at ambient temperatures.

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Chapter 25
Biological Iron Oxidation and Sulfate Reduction in the Treatment of Acid Mine Drainage at Low Temperatures

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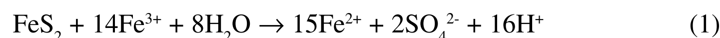
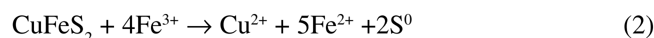
25.1 Introduction

Acid mine drainage (AMD) is the result of exposure of sulfidic seams to the oxidizing and leaching action of water—rain water, humidity, and groundwater—and is exacerbated by microorganisms that catalyze the solubilization of sulfide minerals by the regeneration of Fe^{3+} and oxidation of their dissolution products as the source of energy. Typical AMD has a low pH and a high sulfate and ferric iron concentration although other metals may also be present. The chemical composition of AMD varies with sulfide minerals associated with coal and metal mines. At low pH, ferric iron in AMD participates in the leaching action, as shown for pyrite (FeS_2) and chalcopyrite ($CuFeS_2$):



$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (1)$$



$$CuFeS_2 + 4Fe^{3+} \rightarrow Cu^{2+} + 5Fe^{2+} + 2S^0 \quad (2)$$

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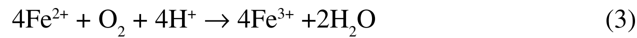
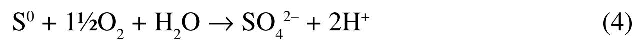
Ferrous iron thus formed is re-oxidized to ferric iron by acidophilic bacteria and archaea and sulfur (S^0) is oxidized to sulfate:

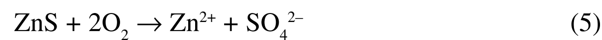


$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (3)$$



$$S^0 + 1½O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (4)$$

Oxidative dissolution of sulfide minerals can be initiated in the absence of ferric iron, shown as the net reaction for oxidative solubilization of sphalerite (ZnS):



$$ZnS + 2O_2 \rightarrow Zn^{2+} + SO_4^{2-} \quad (5)$$

The oxidation also involves acidophilic microorganisms as they oxidize the S-entity through intermediates (elemental S and sulfooxyanions) to sulfate in acid solutions. A number of inorganic sulfur compounds such as polysulfides, elemental S, thiosulfate, polythionates, sulfite, and sulfanes are produced in the intermediate steps of the oxidative dissolution, and they vary in dominant species and concentration with the sulfide minerals and the bioleaching microbial community. Sulfate production leads to sulfuric acid formation under acid leaching conditions, thus enhancing the proton attack of some mineral matrices – not only sulfides but also some accessory carbonates, oxides, and silicates.

Adverse environmental effects of AMD are not limited to the immediate source area but extend downstream, altering the chemical composition of water through introduction of sulfuric acid and dissolved metals. Sediments in altered habitats may become enriched with metals that are toxic to plants, fish, and sediment fauna. Associated with these changes is the precipitation of various Fe(III) hydroxysulfate and oxyhydroxide particles which can travel downstream in receiving water and thus carry sorbed metals to other locations. Contamination of potable water sources is also evident as AMD is pervasive in ambient surface environments as well as groundwater aquifers. The problem is of global proportions because it is invariably associated wherever mine sites have exposed sulfide mineral seams.

Microbiological oxidation of Fe^{2+} occurs over a wide range of temperatures; up to about 85°C, and numerous iron and sulfur oxidizing mesoacidophiles and thermoacidophiles have been described in the literature. In the low temperature range, iron oxidation by bacteria continues at temperatures down to 0°C. The response of their activity to low temperatures suggests that they are psychrotrophic organisms (also termed psychrotolerant or eurypsychrophilic microorganisms). By definition, these microorganisms grow over a wide range of temperatures extending into the mesophilic range. The psychrotolerant microorganisms could be better termed as having thermotolerant psychrophilic properties. While they have their optima at mesophilic temperature ranges, their growth and metabolic rate at low temperatures are sufficiently high to allow the organisms to thrive at low temperatures. In this paper, the term psychrotrophic is used to describe this situation.

Truly psychrophilic iron or sulfur oxidizing microorganisms have not been reported in the literature. Psychrophiles are also known as stenopsychrophilic

microorganisms and they grow at temperatures of up to about 25°C and with optimum at <15°C. The upper mesophilic temperature range of >25°C is prohibitive to growth of psychrophiles. Low temperature environments are found in high and low latitudes as well as high altitudes, and on a global scale they account for about 75% of the biosphere according to some estimates (Cavicchioli 2006).

The oxidation of sulfide minerals and the consequent formation of AMD have been documented in permafrost and boreal environments (Elberling 2004). The generation of AMD in low temperature environments has been reported, for example, for the Yukon Territory, Canada (Elberling 2005) and Greenland (Langdahl and Ingvorsen 1997). In a heap leaching test with of a copper oxide, the winter season had a minimum outdoor temperature of –54°C (McNaughton and Schlitt 2000). Despite problems with the flow systems for transferring liquors, the heap maintained its temperature over the test period due to the exothermic reactions involved in the oxidative dissolution of sulfides over time. In another example, the oxidation of sulfide in pyritic tailings in Baffin Island, Canada, continued at 0°C (Elberling 2001; Elberling et al. 2000). Laboratory experiments with tailings samples removed from the same area demonstrated oxygen consumption at subzero temperatures (Elberling 2005). Søndergaard et al. (2007) investigated runoff of acid drainage from pyrite-containing coal mine waste rocks in Svalbard and reported biogeochemical activity at the site, verified by determination of viable counts of iron-oxidizing acidophiles. In the winter, waste sulfidic rock piles can have temperatures above 0°C in these latitudes (78°N) because of the exothermic nature of pyrite oxidation, but the drainage will not flush until the spring thaw (Elberling et al. 2007).

The dissolution of sulfide minerals and oxidation of Fe^{2+} have been reported in samples from a sulfide ore deposit in northern Greenland (Langdahl and Ingvorsen 1997) and a test heap leaching operation in Canada (McNaughton and Schlitt 2000). Bioleaching of a multimetal sulfide ore sample in shake flasks and laboratory-scale column reactors has been reported at 4°C (Ahonen and Tuovinen 1991, 1992), as well as iron and sulfur oxidation in shake flask cultures (Ahonen and Tuovinen 1989, 1990). Mine waste burial in permafrost has been proposed as a means of reducing the formation and dissemination of AMD. Depending on the hydrogeology of the site, high salinity liquid water may still be present at −10°C (Elberling 2001, 2005; Meldrum et al. 2001; Kyhn and Elberling 2001) which may allow biological activity and the formation of AMD. The contribution of microorganisms to AMD formation in highly saline (>0.5 M Cl^-) environments has not been unequivocally elucidated.

25.2 Biological oxidation of iron and sulfur at low temperatures

Perhaps due to its ease of culture and lack of molecular ecological tools, acidophilic iron-oxidizing bacteria assigned to *Acidithiobacillus ferrooxidans* were originally concluded to dominate microbial communities in mine drainage that contained Fe^{2+}

and reduced S compounds. Molecular phylogenetic techniques have subsequently demonstrated that other acidophilic microorganisms are often numerically dominant, but this varies with pH, temperature and other site characteristics. At low temperatures, *At. ferrooxidans* is the only acidophilic, psychrotrophic Fe^{2+} oxidizer reported to date. The available 16S rRNA gene sequences from these studies (Dopson et al. 2007; Kupka et al. 2007) form a clade within *At. ferrooxidans* (Fig. 25.1).

Three clones closely aligning with *At. ferrooxidans* were identified from 5°C enrichment cultures derived from boreal, AMD-impacted environments (Kupka et al. 2007). Two of these clones, SS3 and SS5, were from a mine site in Norilsk, Siberia, and clone SK5 was from a tailings impoundment in Kristineberg, northern Sweden (Kupka et al. 2007). The representative of clone SS3 was subsequently isolated in pure culture and grew with tetrathionate and Fe^{2+} as substrate at 5°C (Fig. 25.2). The iron oxidation data suggested that the strain was psychrotrophic because the rates were highest at 30°C. Bioleaching experiments with a pyrite concentrate and strain SS3 at 5°C revealed that the iron in the solution was

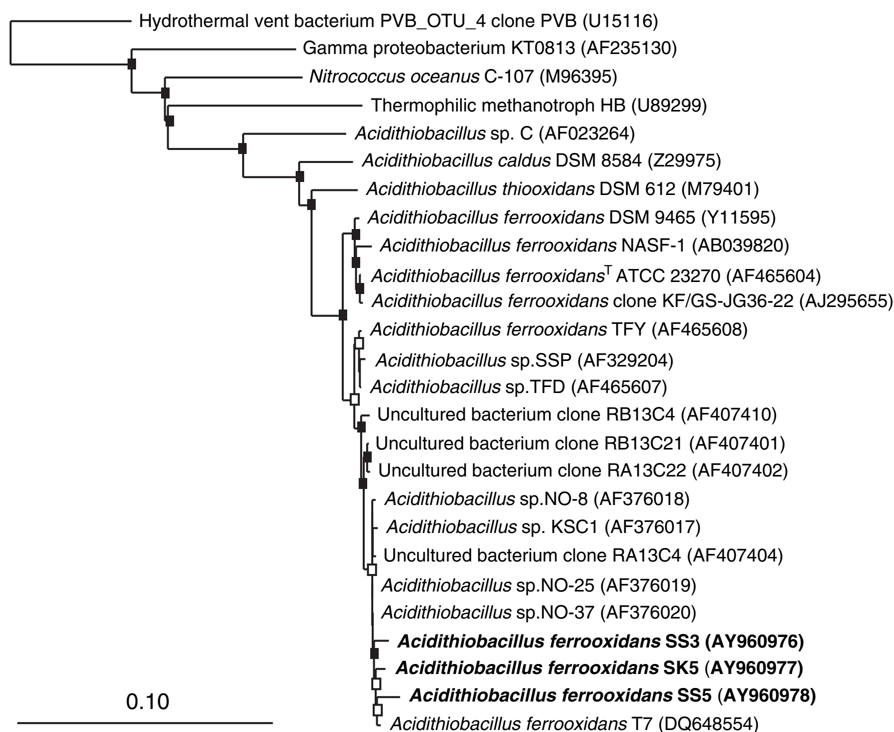


Fig. 25.1 Neighbor-joining tree of *At. ferrooxidans* 16S rRNA gene sequence from strains isolated from cold temperature environments (*in bold*) and sequences from the database (*accession numbers in parenthesis*). Phylogenetic analysis was carried out by the maximum likelihood, distance neighbor-joining, and DNA parsimony methods in ARB software and the nodes supported by all 3 trees (■) and 2 trees (□) have been marked. The *scale bar* corresponds to 10% sequence similarity

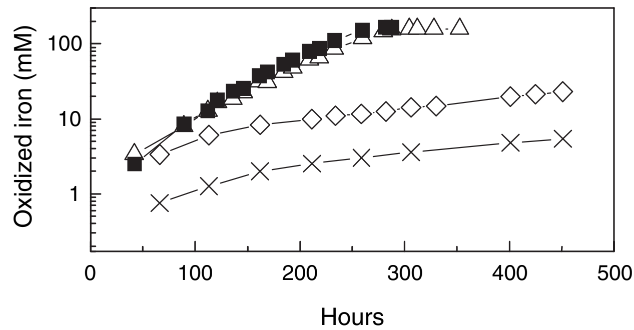


Fig. 25.2 Iron oxidation by the psychrotrophic culture *At. ferrooxidans* SS3 at pH 2.0 (Δ) and pH 2.8 (■), and a mesophilic reference culture *At. ferrooxidans* (◊, initial pH 2.0). Iron oxidation in a sterile control is also shown (×, initial pH 2.0). Modified from Kupka et al. (2007)

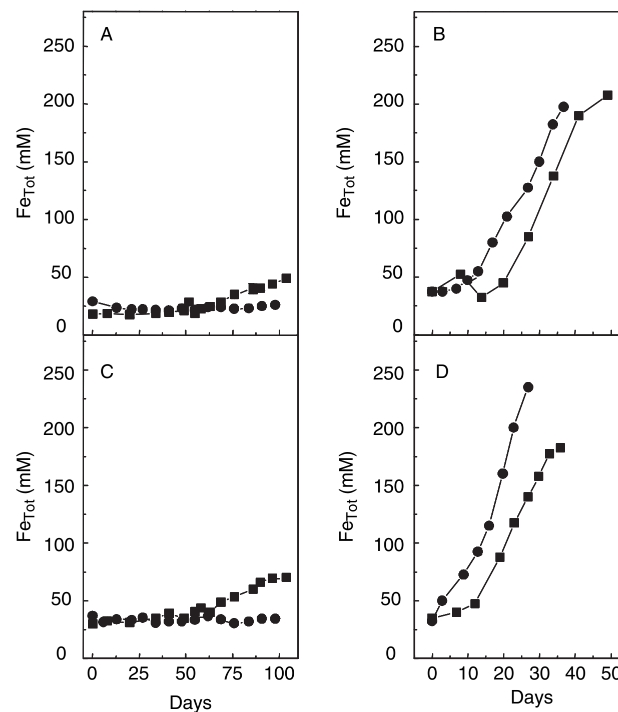


Fig. 25.3 Changes in the total soluble Fe concentration during the bioleaching of a pyrite (**A,B**) and pyrite/arsenopyrite (**C,D**) concentrate (all 4% wt/vol) by the psychrotrophic culture *At. ferrooxidans* SS3 (■) and a mesophilic reference culture *At. ferrooxidans* (●) at 4°C (**A,C**) and 30°C (**B,D**). Modified from Dopson et al. (2007)

completely oxidized (Dopson et al. 2007). These results suggested that pyrite oxidation under low temperature conditions may be limited by the rate of the Fe^{3+}- and proton mediated attack of the pyrite matrix rather than the biological oxidation and regeneration of Fe^{2+} (Fig. 25.3). However, a careful analysis of the kinetics and limiting factors of the bioleaching of sulfide ores and concentrates at low temperatures has yet to be reported.

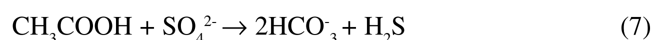
Ferroni et al. (1986) enriched iron-oxidizers from samples obtained from a closed uranium mine in the Elliot Lake area, Ontario, Canada. These cultures had generation times of 247 ± 46 h at 2°C and 103 ± 29 h at 6°C, compared to 12 h in the mesophilic range of 25–30°C. Other enrichment cultures and isolates from two closed uranium mine sites that grew at 4°C were identified as predominantly *At. ferrooxidans*, but all isolates grew fastest at temperatures over 21°C (Berthelot et al. 1993). Leduc et al. (2002) isolated psychrotrophic *At. ferrooxidans* at 4°C from Canadian sites using a direct plating technique based on agarose as the solidifying agent and Fe^{2+} as substrate. Numerous studies have addressed the effect of temperature on the kinetics of growth and substrate oxidation by iron oxidizing bacteria. Predictably, a decrease in the temperature lowers the biological activity (Kovalenko et al. 1981; Ahonen and Tuovinen 1989, 1990; Ahonen et al. 1990; Okereke and Stevens 1991). The microbiological oxidation of S compounds at low temperatures in boreal environments is very poorly characterized beyond the assertion that *At. ferrooxidans* is also able to oxidize various S compounds. In general, the minimum temperature supporting the biological oxidation of Fe^{2+} and S has not been well defined.

25.3 Biological reduction of sulfate at low temperatures

The microbiological reduction of sulfate has proven to be a powerful tool in mitigation of AMD due to alkalinity generation and precipitation of metal sulfides (Hao 2000; Huisman et al. 2006). Organic matter is needed as electron donor for the reduction, as shown for the lactate-dependent sulfate reduction (sulfidogenic lactate oxidation) by sulfate-reducing bacteria (SRB):

$$2\,CH_3CHOHCOOH + SO_4^{2-} \rightarrow 2CH_3COOH + 2HCO_3^- + H_2S \qquad (6)$$

The anaerobic oxidation of organic compounds by SRB may be incomplete, leading to acetate formation in the preceding example. Another group of SRB can couple anaerobic acetate oxidation to sulfate reduction (i.e., sulfidogenic acetate oxidation):

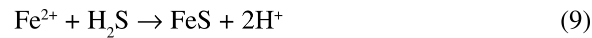


$$CH_3COOH + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \qquad (7)$$

Biogenic sulfide thus formed precipitates metals (M^{2+}):

$$H_2S + M^{2+} \rightarrow MS + 2H^+ \qquad (8)$$

Precipitation of Fe^{2+}, for example, is usually presented as:



$$Fe^{2+} + H_2S \rightarrow FeS + 2H^+ \qquad (9)$$

However, the reaction yields multiple Fe-sulfides such as mackinawite (FeS), greigite (Fe_3S_4), and pyrite, demonstrating that there is a series of reactions involved in forming solid solutions of Fe-sulfides. For SRB systems, the pathways and reactions for the formation of multiple Fe-sulfides are not well understood.

The biocarbonate alkalinity serves to neutralize the acidity of AMD:

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O \qquad (10)$$

Some attempts have been made to install constructed wetlands and permeable barriers in boreal AMD sites and biological activities in these systems are subject to seasonal temperature fluctuations. Early comprehensive reviews on sulfate-reducers (e.g., Postgate 1984) rarely raised the question of sulfate reduction at temperatures <4°C. Most of the sea floor sediment, where sulfate-reduction is traditionally considered to constitute a major process, is at the perennial temperature <4°C. More than 50% of the organic carbon in marine sediments has been shown to be mineralized by coupling with sulfate reduction (Jørgensen 1982; Canfield et al. 1993).

Marine environments have revealed a great diversity of psychrophilic sulfate-reducers, which are abundant in sediments because of the low temperature (<5°C) prevailing throughout the year. A psychrophilic sulfate reducer, *Desulforhopalus vacuolatus*, was initially enriched for with lactate–thiosulfate combination from estuarine sediments of a Danish fjord (Isaksen and Teske 1996). The isolate oxidized propionate and lactate incompletely to acetate and displayed optimum growth at 18–19°C. The isolate did not grow at >24°C but could reduce sulfate at temperatures as high as 38°C. Isaksen and Teske (1996) coined this bacterium as a moderate psychrophile to differentiate it from those psychrophilic bacteria that have their optimum growth temperature in the range of 5–15°C. However, the term moderately psychrophilic microorganisms has not found wide acceptance in the literature. Studies of marine sediments in Arctic Svalbard (Knoblauch et al. 1999a, 1999b) have yielded five novel psychrophilic isolates belonging to three new genera, *Desulfofrigus*, *Desulfofaba*, and *Desulfotalea* (Table 25.1). Figure 25.4 shows a phylogenetic tree with emphasis on psychrophilic SRB. In general, the information available on SRB in marine environments clearly shows that these populations are also diverse at low temperature ranges. In contrast to the permanently cold marine sediments, low-temperature terrestrial environments undergo seasonal temperature variations that influence microbial distribution and activity. Ambient temperature can vary considerably during daily or annual cycles in the terrestrial environments in northern climates or shallow water sediments. Rabus et al. (2002) suggested that psychrotolerance may be regarded as the phenotype that has adapted to survive under periodic changes from low to intermediate temperatures. Molecular evolutionary analysis to dissect such phenotypic adaptation has not been reported.

Table 25.1 Examples of psychrophilic and psychrotrophic sulfate reducing bacteria

Species	Temperature		Origin	Oxidation of acetate	Reference
	Range (C°)	Optimum (C°)			
Desulfofaba gelida	−1.8 to 10	7	Marine sediments off the coast of Svalbard	No	Knoblauch et al. (1999b)
Desulfofrigus fragile	−1.8 to 27	18	Marine sediments off the coast of Svalbard	No	Knoblauch et al. (1999b)
Desulfofrigus oceanence	−1.8 to 16	10	Marine sediments off the coast of Svalbard	Yes	Knoblauch et al. (1999b)
Desulforhopalus vacuolatus	0 to 24	18–19	Temperate estuary (Kysing Fjord, Denmark)	No	Isaksen and Teske (1996)
Desulfotalea arctica	−1.8 to 26	18	Marine sediments off the coast of Svalbard	No	Knoblauch et al. (1999b)
Desulfotalea psychrophila	−1.8 to 19	10	Marine sediments off the coast of Svalbard	No	Knoblauch et al. (1999b)
Desulfovibrio cuneatus	0 to 33	28	Oxic freshwater sediment	No	Sass et al. (1998)
Desulfovibrio ferrireducens	−2 to 30	23	Fjord sediments on the west coast of Svalbard	No	Vandieken et al. (2006)
Desulfovibrio frigidus	−2 to 25	20–23	Fjord sediments on the west coast of Svalbard	No	Vandieken et al. (2006)
Desulfovibrio litoralis	0–33	28	Oxic freshwater sediment	No	Sass et al. (1998)

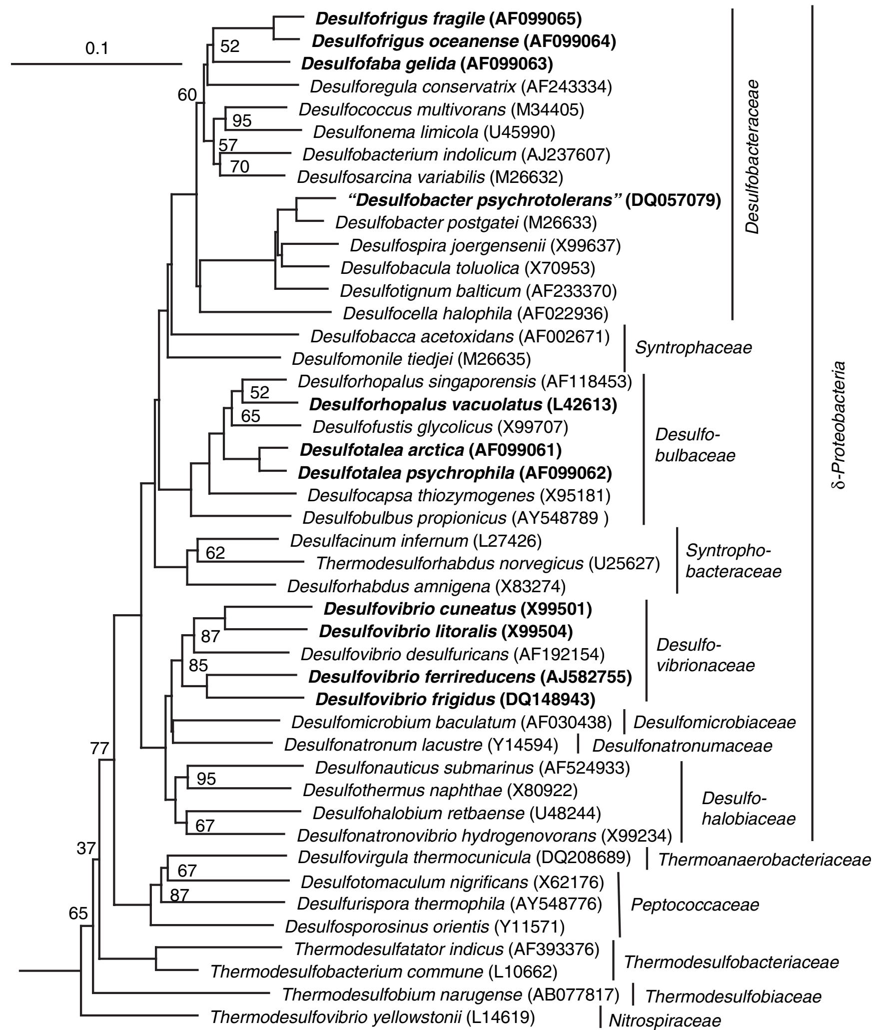


Fig. 25.4 Phylogenetic tree generated using distance matrix and neighbor-joining methods based on the 16S rRNA genes of sulfate-reducing bacteria. Psychrophilic and psychrotrophic species are shown with bold font. *Archaeoglobus fulgidus* (X05567) was used as an outgroup for rooting the tree. *Numbers* at nodes represent boostrap percentages based on 1,000 samplings. *Bar* 0.1 nucleotide changes per position

A study of SRB diversity in a northern mining location in the Kola Peninsula revealed that both mesophilic and psychrophilic incomplete-oxidizers were abundant in mine tailings and wetland sediments. *Desulfobulbus* spp. accounted for up to 15% of all cells (Karnachuk, unpublished results). The *Desulfotalea–Desulfofustis* group and *Desulfovibrio* spp. were the second and the third most

dominant groups, respectively. Mesophilic *Desulfobulbus* spp. were also important among cultivatable sulfate reducers isolated from the mining environments in another northern mining area in Norilsk, located above the Arctic Circle in the permafrost zone (Karnachuk et al. 2005). Acetate-oxidizing *Desulfosarcina–Desulfococcus* dominated in acetate and benzoate enrichments in the Norilsk samples. Direct analysis of a SRB community at Kola Peninsula sediments by fluorescent in situ hybridization (FISH) revealed very low numbers of complete oxidizers, including the *Desulfosarcina–Desulfococcus*, *Desulfobacterium* and the *Desulfobacter–Desulfobacula* groups.

Praharaj and Fortin (2004) reported high sulfate reduction rates of 100 to 1,000 nmol S cm^{-3} day^{-1} for samples of mine tailings in northern Ontario, Canada during the summer time at in situ temperature of 30°C. The general trend in the sulfate reduction rates was a decrease to less than 10 nmol S cm^{-3} day^{-1} during the spring time (at ambient temperature 12°C). The authors indicated that the temperature is one of the factors controlling the activity of SRB during the cold season.

Karnachuk et al. (2005) measured sulfate reduction rates, which ranged from 0.05 to 30 nmol S cm^{-3} day^{-1}, in mine tailings and tundra wetland receiving drainage water from tailings in the Norilsk mining area. The in situ sediment temperatures varied between 2 and 10°C. The measured rates were of the same order of magnitude as reported for the permanently cold marine Arctic and Antarctic sediments (Nedwell 1989; Isaksen and Jørgensen 1996; Lein et al. 1996; Knoblauch et al. 1999a). The rates were, in general, in the same range (Meier et al. 2004) or one order of magnitude lower (Herlihy and Mills 1985; Blodau et al. 1998; Edenborn and Brickett 2001) than those determined in wetland and lake sediments impacted by AMD in the temperate climate.

25.4 Passive treatment of AMD

25.4.1 Constructed wetlands

Passive treatment systems can be relatively low cost as compared to chemical treatment systems, which require continuous addition of a chemical downstream from the source. Constructed wetlands are commonly used treatment systems for AMD from metal and coal mines. A typical full-scale treatment involves the flow of AMD through a constructed wetland where iron oxidation takes place in the surface zone, resulting in the precipitation of Fe(III) hydroxysulfates. The underlying zone is anaerobic and contains a rich organic layer such as compost or animal manure. The organic matter is readily biodegradable and remains anaerobic because dissolved O_2 is depleted upon microbial decomposition of the substrate. The high sulfate concentration in AMD and the anaerobic organic layer promote SRB, and sulfate reduction leads to the formation of Fe- and other metal sulfides. Limestone is usually also present in the bottom layer to aid in the neutralization.

The oxic surface zones in constructed wetlands are populated by iron-oxidizing microorganisms. Their diversity has not been elucidated extensively in wetland environments. In a constructed wetland treating AMD (pH 3–4) from an abandoned coal mine, the *At. ferrooxidands/At. thiooxidans* group accounted for up to 70% of the microbial community in Fe(III)-rich precipitates formed in the oxic surface layer (Nicomrat et al. 2006a, 2006b). Neither archaea nor *L. ferrooxidans* type bacteria, tested with PCR- and FISH-based techniques, were significant in this constructed wetland environment. This finding is in contrast to microbial diversity in AMD from metal mines that have been shown to contain *L. ferrooxidans*, *Sulfobacillus* spp., *At. caldus*, and various thermophilic iron-oxidizing archaea. However, it is not understood how the AMD microbial communities from metal mines change when the effluent enters a constructed wetland system. Major differences are the higher acidity, elevated temperatures, and concentrations of various soluble metals in AMD from metal mines as compared to coal mines. Microbial communities in constructed AMD-treating wetland systems usually also contain heterotrophic iron-oxidizers. Organic matter enters through runoff from surrounding soil and wetland embankments. Constructed wetland systems often involve a series of treatment cells with chemical changes that improve the water quality downstream from the inlet. Mass balances for neutralization and precipitation of iron, other metals and sulfate precipitation are rarely available.

Iron precipitation in oxic surface layers in wetlands leads to schwertmannite (ideal formula $Fe_{16}O_{16}(SO_4)_2(OH)_{12}\cdot nH_2O$, $n = 10$–12) formation and it is noteworthy that sulfate is co-precipitated with Fe^{3+}. Jarosites (e.g., $KFe_3(SO_4)_2(OH)_6$) are also common, but their precipitation is usually controlled by the availability of K^+ and other monovalent ions. Goethite (α-FeOOH) is found in oxic zones in some AMD wetlands (Gagliano et al. 2004). Notably, its formation does not involve sulfate precipitation. Wetland treatment accumulates Fe(III) precipitates over time. If these precipitates are not removed periodically, wetland cells display laminar surface flow conditions with little or no contact of AMD with the underlying aerobic–anaerobic interface. Ideally, the anaerobic layer is intended to provide carbon and electron donors to SRB which will then reduce sulfate in AMD to sulfide. Fe-sulfides have been detected in anaerobic sediments (Gagliano et al. 2004), suggesting that active sulfate reduction occurs in bottom layers of AMD wetlands. In contrast, the role of the biological reduction of ferric iron in the biogeochemistry of AMD in constructed wetlands is not clear.

25.4.2 In situ remediation

In situ treatment processes can be carried out by stimulating SRB directly in the contaminated water body in water-filled underground workings and open pits. Thus, the mine serves as a sedimentation basin for sulfide sludge formed by precipitation of metals (Kuyucak et al. 1991; Kuyucak and St-Germain 1994; Christensen et al. 1996). In such cases, the associated sludge handling costs can be minimized or

eliminated in a low cost, low maintenance system (Christensen et al. 1996). The dosing of organic compounds as electron donors to a mine water system should be balanced with the concentration of metals and acidity to ensure proper metal removal and to avoid harmful or excessively high overloads of organic material or nutrients (Christensen et al. 1996). The in situ treatment of AMD may be particularly useful in small mine water systems, where the sulfate reduction process can be contained and properly controlled. A prerequisite for the in situ treatment within a flooded mine is that the mine is deep enough to prevent a complete turnover (mixing) of the water mass as otherwise the bottom sediments may be oxidized and a further leaching of metals may occur (Mustikkamäki 2000).

Castro et al. (1999) used varying amounts of waste from a potato-processing plant and composted manure to stimulate SRB in 5-l bottles containing open pit water (1,200 mg sulfate l^{-1}, 100 mg Fe l^{-1}, and 5 mg As l^{-1} at pH 6) at 15°C. In some instances, the numbers of sulfate reducers increased whilst the sulfate, Fe, and As concentrations decreased and the pH rose to near neutrality. Christensen et al. (1996) treated AMD in columns (total volume 31.4 l) with sand and gravel as the solid matrix and whey as the carbon and energy source for SRB at 15°C. According to Zadow (1986), whey contains on average 50 g lactose, 6 g protein, 6 g minerals, and 0.5 g fat per liter. After an initial lag phase, the pH increased, the redox potential decreased, dissolved Cu, Zn, Fe, and Al concentrations were reduced, and a black sludge was formed. Acetate accumulated suggesting incomplete oxidation of organic matter in the whey (Christensen et al. 1996).

In situ treatment of AMD has also been evaluated in a flooded open pit in a multiyear, commercial scale experiment in Finland (Mustikkamäki 2000). The pit was 46 m deep with a volume of 220,000 m^3 (Table 25.2). The temperature at the bottom of the pit is 4°C all year round. After the addition of 42 m^3 swine manure and 66 m^3 silage solution to the pit to promote sulfate reduction, the average Zn concentration in the water decreased from 3.5 mg l^{-1} to 0.8 mg l^{-1}, and the concentration of Zn was <0.1 mg l^{-1} below a water depth of 25 m, suggesting precipitation and stratification (Mustikkamäki 2000). Other in situ treatment trials in Finland have been conducted at the Kotalahti underground mine, the Hammaslahti open pits, and the Kangasjärvi open pit (Table 25.2) (Mustikkamäki 2000). Canty (1999) also reported high removal efficiencies for metals (Al, Cd, Co, and Zn) and increased pH in mine water flowing through organic substrate placed in mine shafts.

Infiltration beds are used for treating surface water contaminated with AMD in a manner analogous to the employment of reactive barriers in the restoration of groundwater aquifers (Vestola 2004). Infiltration beds are usually constructed in ditches so that the water flows through the bed material. The bed is rich in organic matter that supports the growth and activity of sulfate reducers. The organic layer (e.g., peat, compost, or saw dust) is covered with a geoliner that helps to maintain anaerobic conditions. The organic matter can be supplemented with inorganic nutrients and inoculated with sulfate reducers to enhance the efficiency of the infiltration bed. The material should also have a suitable hydraulic conductivity. An ideal material combination in infiltration beds should retain its treatment capacity for years

Table 25.2 Examples of processes for in situ treatment of acid mine drainage (AMD)

Application	Mine site	Additions	Treatment efficiency	Reference
In situ bioreactor	Ruostesuo open pit mine, Finland (mine water volume 220,000 m^3)	42 m^3 swine manure and 66 m^3 silage solution	Zn 3.5 → 0.8 mg l^{-1}	Mustikkamäki (2000)
In situ bioreactor	Kotalahti underground mine, Finland (mine water volume 3 million m^3)	600 m^3 mixture of swine manure and silage solution	Ni 1.5 → 0.5 mg l^{-1}	Mustikkamäki (2000)
In situ bioreactor	Hammaslahti open pit mine, Finland (mine water volume 3 million m^3)	200 m^3 swine manure	Zn 0.7 → 0.4 mg l^{-1}	Mustikkamäki (2000)
In situ bioreactor	Kangasjärvi open pit mine, Finland	Swine manure and bottom sediments from Kangasjärvi lake	Zn 50 → 40 mg l^{-1}	Mustikkamäki 2000)
Injection of substrates into boreholes	Burgas Copper Mine, Bulgaria (5–21°C)	Acetate-bearing waste product and ammonium phosphate	pH: 6.4–6.8 → 7.3–8.0 Eh: −33 to +23 mV → −120 to −41 mV Sulfate: 460–1045 → 185–325 mg l^{-1} U: 0.3–1.7 → 0.07–0.6 mg l^{-1} Zn: 0.07–1.54 → 0.01–0.21 mg l^{-1} Mo: 0.32–3.32 → 0.01–1.40 mg l^{-1} Mn: 0.1–2.44 → 0.01–0.64 mg l^{-1}	Groudev et al. (1998)
Infiltration bed	Pyhäsalmi mine, Finland	Limestone and peat	Sulfate: 72.0–93.7% Cu: 94.5–99.2% Zn: 76.1–97.4% Fe: 85.1–95.6% Mn: 76.7–95.6%	Riekkola-Vanhanen (1999)

(continued)

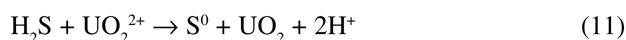
Table 25.2 (continued)

Application	Mine site	Additions	Treatment efficiency	Reference
Permeable reactive barrier (HRT[a] = 80–370 days)	Nickel Rim Mine, Ontario, Canada (2–16°C)	Gravel, limestone, municipal and leaf compost and wood chips	Sulfate 2.2–5.6 g $l^{-1}a^{-1}$ Fe 0.6–2.1 g $l^{-1}a^{-1}$	Herbert et al. (1998), Benner et al. (2002)
Permeable reactive barrier (HRT = 6 days)	Industrial site in British Columbia, Canada	Gravel, limestone, leaf compost	pH 6.4 → 6.9 Cu 3630 → 10.5 μg l^{-1} Cd 15.3 → 0.2 μg l^{-1} Co 5.3 → 1.1 μg l^{-1} Ni 131 → 33 μg l^{-1} Zn 2410 → 136 μg l^{-1}	Ludwig et al. (2002), Gibert et al. (2002)

[a]HRT, hydraulic retention time

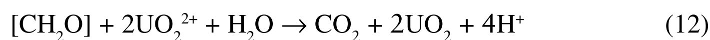
without clogging due to metal precipitates or excessive slime formation (Vestola 2004). Infiltration beds have been used to treat contaminated surface water flowing from an old waste dump at the Pyhäsalmi mine, Finland since 1994 (Table 25.2). The infiltration beds (18 m long, 3 m wide, and 1.5 m high) consisted of 12 tons of limestone and 45 m^3 of freshly excavated peat (Riekkola-Vanhanen 1999). The first section consisted of limestone, followed by three alternating sections of peat and limestone. During the summer, Fe, Zn, and Cu in the water precipitated in the infiltration beds as the corresponding sulfides, and concentrations in the water were reduced by 95–99% for Cu, 76–97% for Zn, 85–96% for Fe, 77–96% for Mn, and 72–94% for sulfate (Riekkola-Vanhanen 1999). The long-term capacity of the system is difficult to predict and the efficiency may be restored by periodic replacement of the organic layer.

A further in situ method for the treatment of AMD is the injection of a carbon and energy source below the surface to promote the action of SRB. Groudev et al. (1998) reported an increase in the numbers and activity of SRB in contaminated groundwater near the Burgas Copper Mines, Bulgaria, after injecting an acetate-bearing waste product and ammonium phosphate via boreholes. However, it was not clear how the increases in the SRB numbers reflected changes in the diversity and composition of the SRB population at the site. The temperature during the treatment varied in the range of 5–21°C. Biogenic sulfide precipitated metals as the corresponding sulfides and may also have acted as a reductant for U(VI) reduction:



$$H_2S + UO_2^{2+} \rightarrow S^0 + UO_2 + 2H^+ \quad (11)$$

The role of direct biological reduction of U(VI) as electron acceptor coupled with anaerobic oxidation of organic matter [CH_2O] is also plausible, represented in a simplified version as:



$$[CH_2O] + 2UO_2^{2+} + H_2O \rightarrow CO_2 + 2UO_2 + 4H^+ \quad (12)$$

The process improved at summer temperatures but even at 5–7°C the concentrations of metals were below the permissible discharge levels (Groudev et al. 1998).

Permeable reactive barriers can be used to treat AMD-contaminated groundwater in trenches or ditches, with compost as a carbon and energy source for supporting sulfate reduction, metal precipitation, and generation of alkalinity. A full-scale permeable reactive barrier was installed in 1995 at the Nickel Rim Mine near Sudbury, Ontario, Canada (Table 24.2). The reactive mixture in the barrier was composed of gravel, limestone, municipal and leaf compost, and wood chips (Herbert et al. 1998; Benner et al. 2002). The temperatures in the aquifer fluctuated from 2°C in the winter to 16°C in the summer. The fluctuations were dampened with depth although the temperature gradient was inverted semi-annually, with the highest temperatures at the surface during the summer and the highest temperatures at the base of the barrier during winter. The sulfate reduction rate in the summer was nearly twice as high as in winter. The initial annual

rates of sulfate and Fe removal within the barrier were 3.8–5.6 and 1.0–2.1 g l^{-1} (68–100 and 18–38 mmol l^{-1}), respectively, although the rates declined by 30–50% over the first 38 months. The extent of sulfate reduction and Fe precipitation within the barrier was both spatially and temporally variable. Spatial differences were primarily the result of different retention times due to hydraulic conductivity variations of the reactive material. Temporal variations were likely the result of a decline in organic C availability and reactivity over time and seasonal variations in the rate of sulfate reduction (Benner et al. 2002).

25.5 Active treatment of AMD

25.5.1 Application of Fe^{2+} oxidation

One bioremediation approach is the aeration of AMD to promote the oxidation of Fe^{2+} to Fe^{3+} by iron-oxidizers such as *At. ferrooxidans* and *Leptospirillum ferrooxidans*. The aeration is important because the O_2 demand for iron oxidation is usually substantially higher than the O_2 concentration dissolved in acid mine drainage (Hustwit et al. 1992). Fe^{3+} thus produced as well as other metals can subsequently be precipitated by increasing the pH (usually by the addition of lime), and then the precipitates are separated from the water. Other transition metals or arsenic may be co-precipitated in this process. A commercial scale aeration lagoon employing iron-oxidizing microorganisms for the oxidation step has been employed, for example, in Japan (Yabuuchi and Imanaga 1976). Murayama et al. (1986) reported on a biological treatment of acid mine drainage installed at the Matsuo mine (Iwate Prefecture), which involved bacterial oxidation of iron in a 580-m^3 oxidation basin with about 1-h hydraulic retention time. Another example of Fe^{2+} oxidation and precipitation based AMD treatment is the use of an immobilized biomass in a packed bed iron-oxidation bioreactor (Omura et al. 1991), although this system has not been used in full scale. The Fe^{2+} oxidation rates can be relatively high if not limited by oxygen availability. Iron oxidation in stirred tank bioreactors have also been evaluated for acid mine treatment (Kirby and Brady 1998) and may be a useful approach in bench-scale testing applications. Packed bed and fluidized bed bioreactors have been developed for various bioleaching applications (e.g., Kinnunen and Puhakka 2004; Ozkaya et al. 2007) where there is need to regenerate ferric iron at fast rates via the bacterial oxidation step. The bioprocess technology developed in that area may lend itself to the biotreatment of acid mine drainage.

A major kinetic consideration for the process is the temperature-dependent rate of Fe^{2+} oxidation. A type of bioreactor utilized to treat AMD is a rotating biological contactor (RBC) that uses immobilized iron-oxidizing microorganisms that come into contact with the AMD to oxidize the Fe^{2+} to Fe^{3+} that is subsequently precipitated. A pilot RBC was evaluated for the treatment of AMD from a U.S. coal mine (Olem and Unz 1977). The RBC was operated over an 11-month period,

during which the mean AMD water temperature was 11°C (range 9–12°C), the ambient air temperature was ≥4°C, and the AMD contained a mean Fe^{2+} concentration of 160 mg l^{-1} (range 15–310 mg l^{-1}). The RBC was colonized by the indigenous population of iron-oxidizing microorganisms from the AMD. The RBC treated 0.22 m^3 $day^{-1}{\cdot}m^{-2}$ AMD with 91% iron oxidation efficiency, thus demonstrating the potential use of RBC's as an integrated low temperature treatment process. Olem and Unz (1980) tested a prototype RBC treating several acid coal mine waters between 0–10°C. They reported that, although iron oxidation rates were slightly reduced, the oxidation occurred as low as at 0.4°C.

Nakamura et al. (1986) tested an experimental RBC using dilute mineral salts medium, which was supplemented with 500 mg Fe^{2+} l^{-1} and inoculated with iron oxidizing bacteria from an acid stream. The rate constant of iron oxidation was unaffected by the temperature in the range of 10–40 °C although the rate constant decreased at 5°C (Nakamura et al. 1986). Furthermore, the rate constant was similar in the range of pH 1.3–2.6 and 500–1,500 mg Fe^{2+} l^{-1}. The lack of an increase in the rate constant as a function of the temperature suggests a very low activation energy of iron oxidation. This may be a result of excessive encrustation of the RBC with Fe(III) precipitates, thereby acting as a diffusion barrier for the fluxes of reactants and products. The encrustation could have included jarosites because K^+ was included in the mineral salts medium.

25.5.2 Application of sulfate reduction

Numerous reactor designs for enhancing biological sulfate reduction and treatment of AMD have been reported in the literature (Hao 2000; Johnson 2000; Hulshoff Pol et al. 2001; Lens et al. 2002). In general, reactor types in low temperature applications are more limited as opposed to those in the temperate climate. The most common types are the anaerobic filter reactors (Table 25.3). Dvorak et al. (1992) reported the use of an underground pilot-scale upflow anaerobic filter reactor for the treatment of AMD at 10°C in an experimental mine set up (Table 25.3). The anaerobic filter reactor system consisted of three 200-l reactor barrels in a series, filled with spent mushroom compost. The average hydraulic retention time within the pore space of the compost was 5 days. The anaerobic filter reactors precipitated 85% and 99.9% of the initial 53 mg Fe l^{-1} and 7 mg Al l^{-1}, respectively, and 17% of the initial 1000 mg sulfate l^{-1}. This resulted in neutralization of the AMD from pH 3.2 to 6.4 and removal of Fe as sulfides, whereas Al was hydrolyzed to insoluble hydroxides (Dvorak et al. 1992).

Farmer et al. (1995) tested pilot scale upflow anaerobic filter reactors for acid mine water treatment. Two anaerobic filter reactors (volumes 230 l and 490 l) were packed with a mixture of composted dairy cow manure and paper products (96% by weight) and alfalfa hay (4%) and the hydraulic retention time was 50–100 h. The anaerobic filter reactors removed Zn, Mn, and sulfate from the acid mine water (Table 25.3), but the initially circumneutral pH of the influent did not substantially

Table 25.3 Examples of low-temperature bioreactor systems for the treatment of acid mine water or metallurgical wastewaters using SRB

Reactor and process type	Operation	Substrate(s)	pH		Metals[a]			Sulfate			Reference(s)
			Feed	Effluent	Feed (mg l^{-1})	Removal (g $m^{-3}d^{-1}$)	Removal (%)	Feed (mg l^{-1})	Removal (g $m^{-3}d^{-1}$)	Removal (%)	
Three sequential upflow AFRs[b] with limestone	V = 3×200 l HRT[c] = 120 h T = 10 °C	Mushroom compost	3.2	6.4	Al 7 Fe 53	1.4 9.0	100 85	1,000	34	17	Dvorak et al. (1992)
Upflow AFRs (pilot plant)	V = 490 l HRT = 50–100 h T = 12–16°C	Cow manure, paper products, alfalfa	6.7–7.2	6.7–7.0	Zn 49–57 Mn 1.8–2.4	11–27 0.3–1.0	96–99 71–91	390–440	70–170	70–79	Farmer et al. (1995)
Upflow AFRs (pilot plant)	V = 230 l HRT = 50–100 h T = 14–15°C	Cow manure, paper products, alfalfa	6.7–7.2	6.4–7.1	Zn 49–57 Mn 1.8–2.4	10–26 0.3–1.0	89–98 74–91	390–440	60–145	58–68	Farmer et al. (1995)
Upflow anaerobic mine reactor (no packing material)	V = 10.6 l HRT = 936 h T = 10°C	Acetate	NR[d]	NR	Al 270[e] Cu 350[e] Fe 580[e] Mn 220[e] Zn 640[e]	15[e] 21[e] 24[e] 2.3[e] 37[e]	92.5 99.4 67.8 16.9 96.6	NR	25	60	Govind et al. (1999)
Downflow AFR	V = 7.9 l HRT = 6.6 h T = 5 °C	Spent horse manure plus methanol	4.2	5.7–6.7	Fe 100	300–335[e]	83–92e	900	980–1,310[e]	30–40[e]	Tsukamoto et al. (2004)

(continued)

Table 25.3 (continued)

Downflow AFR	V = 7.91 HRT = 6.6 h T = 5°C	Spent horse manure plus ethanol	4.2	5.0–7.3	Fe 100	320–360[e]	88–99[e]	900	1,090–1,450[e]	33–44[e]	Tsukamoto et al. (2004)
Downflow AFR with limestone	V = 79.5 m^3 HRT = 1500 h T = 2–4°C	Cow manure, planter mix soil	3.8	NR	Mn, Fe, Ni, Cu, Zn, Cd 8.4 mM	0.11 mol $m^{-3}d^{-1}$	82	2,600	8	19	Wildeman et al. (1994, 1995)
FBR[f]	V = 0.625 l[g] HRT = 24 h T = 8°C	Ethanol	7.2	7.0	60	60	99.9	1,000	250	25	Sahinkaya et al. (2007a)

[a] Only metals with an initial concentration of 1 mg l^{-1} or above are given
[b] AFR, anaerobic filter reactor
[c] HRT, hydraulic retention time
[d] NR, not reported
[e] Data estimated from the published graphs
[f] FBR, fluidized bed reactor
[g] volume of the fluidized bed

change. The numbers of SRB in the packing material after 25 days of operation were $>10^6$ colony forming units g^{-1} substrate (Farmer et al. 1995), but it is not clear how representative these counts were for the total SRB community.

Tsukamoto et al. (2004) investigated the possibility of extending the lifetime of a downflow anaerobic filter reactor by adding alternative electron and carbon sources (methanol or ethanol) to decomposed horse, manure at low temperatures. The column was inoculated with a mixed culture which had previously been enriched for a year in a column with horse manure straw as substrate. The inoculum was not characterized in the study. The laboratory scale anaerobic filter reactors were initially operated at 15°C and stepwise cooled to 5°C. After the process was acclimated at 15°C, the performance of the bioreactor system was not adversely influenced by further reduction of the temperature to 6°C, suggesting that the bioreactor system had high capacity to operate at low temperatures. The rate limitation was not explored further in the study. At a hydraulic retention time of 6.6 h, the extent of Fe precipitation (initial concentration 100 mg l^{-1}) was 83–92% and 88–99% in anaerobic filter reactors receiving methanol and ethanol, respectively. The corresponding extent of sulfate reduction (initial concentration 900 mg l^{-1}) was 30–40% with methanol and 33–44% with ethanol (Table 25.3) (Tsukamoto et al. 2004).

Sulfidogenic acetate production and incomplete oxidation of substrates such as lactate generate alkalinity, which helps in the neutralization of acid mine waters. Most known psychrotrophic SRB are incomplete oxidizers (Table 25.1) and are thus unable to utilize acetate as sole energy and carbon source (Isaksen and Teske 1996; Sass et al. 1998; Knoblauch et al. 1999a; Vandieken et al. 2006). This situation creates a challenge in the treatment of AMD at low temperatures. Low temperatures also slow down microbial activity and the decay rate of cells, and reduce the solubility of metal sulfides and gases including H_2S (Hulshoff Pol et al. 2001; Benner et al. 2002).

Sahinkaya et al. (2007a) reported on treatment of AMD with a fluidized bed bioreactor operated with ethanol at 8°C (Table 25.3). Ethanol and sulfate removal rates increased with time and after one month of operation the rates reached 320 and 265 mg l^{-1} day^{-1}, respectively. Subsequent removal rates did not significantly change during 130 days of operation. Although iron (initial concentration of 60 mg l^{-1}) was virtually completely removed through precipitation, the efficiency of sulfate removal averaged only 35 ± 4% between days 30–130, and this was associated with acetate accumulation (Sahinkaya et al. 2007a). Several attempts were made to obtain acetate oxidation at 8°C. Switching the electron donor from ethanol to acetate, inoculation of two different low-temperature enrichments, and operating the fluidized bed reactor for up to 321 days did not result in the enrichment of acetate-oxidizing SRB (Sahinkaya et al. 2007b). Govind et al. (1999) studied the kinetics of acetate-limited sulfidogenic growth at 10, 25, 35, and 45°C in bench scale batch bioreactors. At 10°C, the value of the half-saturation coefficient for acetate (K_{Ac}) was much higher than those determined for higher temperatures (25–45°C), indicating a low affinity to acetate at low temperature. Since the study involved a mixed culture, the results may reflect the selection of a different sub-

population of sulfate reducers at 10°C as compared to the higher temperatures. In a genotypically and phenotypically homogenous culture, a change in the K_{AC} value as a function of the temperature would indicate a complex cascade of regulation and biochemical reactions that would culminate in a change in the affinity to a nutrient in the medium.

Groudeva et al. (1996) compared the efficiency of downflow anaerobic filter reactors operated at 10, 20, and 30°C to treat acidic (pH 2.1–2.7) mine water containing sulfate (1,760—3,740 mg l^{-1}), Fe (450—1,080 mg l^{-1}), Cu (20–70 mg l^{-1}), and As (10–20 mg l^{-1}). The removal peaked at 30°C, but the process was also efficient at 10°C. The temperature coefficient Q_{10} in the range from 10 to 30°C was approximately 1.7 (Groudeva et al. 1996), which is in the normal range of chemical and biochemical reactions. Wildeman et al. (1994, 1995) evaluated the relationship between temperature, sulfate reduction activity, oxidation-reduction potential (Eh), and contaminant removal in downflow anaerobic filter reactors containing cattle manure, planter mix soil, and limestone chips. An increase in the temperature from 2 to 24°C decreased the Eh from 320 to 50 mV, indicating enhanced sulfate reduction activity as the temperature increased. This kinetic response is consistent with the temperature effect that can be predicted for mesophiles from a typical Arrhenius plot.

25.6 Conclusions

The generation and need for abatement of AMD are globally relevant issues regardless of the climate. This review emphasizes that microbiological approaches to AMD abatement are feasible in boreal environments where the ambient temperature is traditionally not considered to be favorable for outdoor bioprocesses. The biooxidation of iron and bioleaching of sulfide minerals, the key mechanisms in AMD formation, involve microorganisms that seem to have acclimated to low temperature lifestyle and display psychrotrophic features of iron oxidation. Similarly, the biological reduction of sulfate is not suppressed in AMD-impacted, low-temperature environments. The biological oxidation of iron and reduction of sulfate are processes that can be designed for AMD abatement involving active or passive treatment in cold climate environments. One challenge in sulfate reduction is the availability of suitable organic matter as the electron donor for on-site treatment of AMD in boreal environments. Most cold geographic regions with past histories of metal or coal mining and AMD have seasonally warm summer temperatures. From an ecological perspective, psychrotrophic characteristics of iron oxidizers and sulfate reducers may help survival under ambient temperature fluctuations. AMD abatement via biological processes is an attractive alternative to chemical treatment but much of the previous on-site treatment has been phenomenological and is in need of optimization to maximize the benefit of the biological approach. The pollution potential and need of treatment of AMD may have served as an additional stimulus in the mining industry for developing heap bioleaching applications leading to the metal recovery and improvements in the waste

management. High-rate biological reduction of sulfate also has applications in the treatment of metallurgical effluents as it can be linked to resource recovery and containment through sulfide precipitation.

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