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).

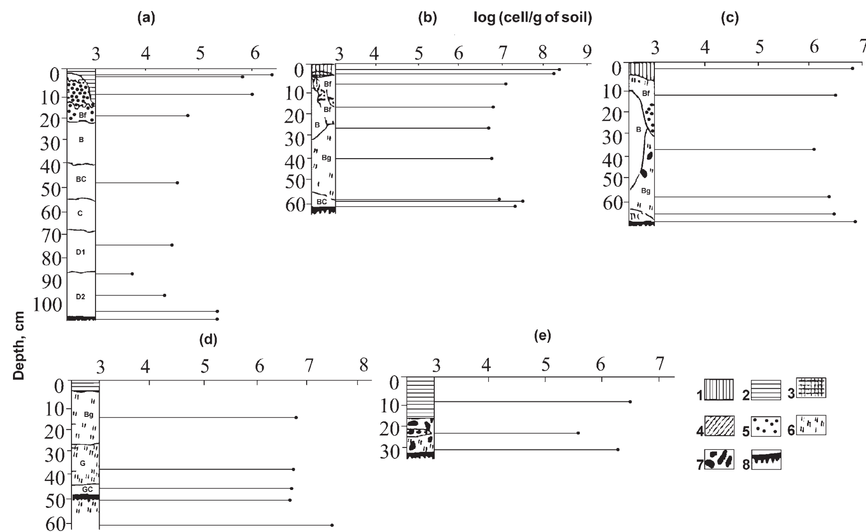

Fig. 6.1 Bimodal profile distribution of microorganisms in tundra and north taiga soil cover. *Key*: *1* forest litter; *2* peaty horizon; *3* mucky horizon; *4* soddy horizon; *5* iron-enriched horizon; *6* morphological features; *7* organic inclusions in mineral horizons; *8* permafrost table

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

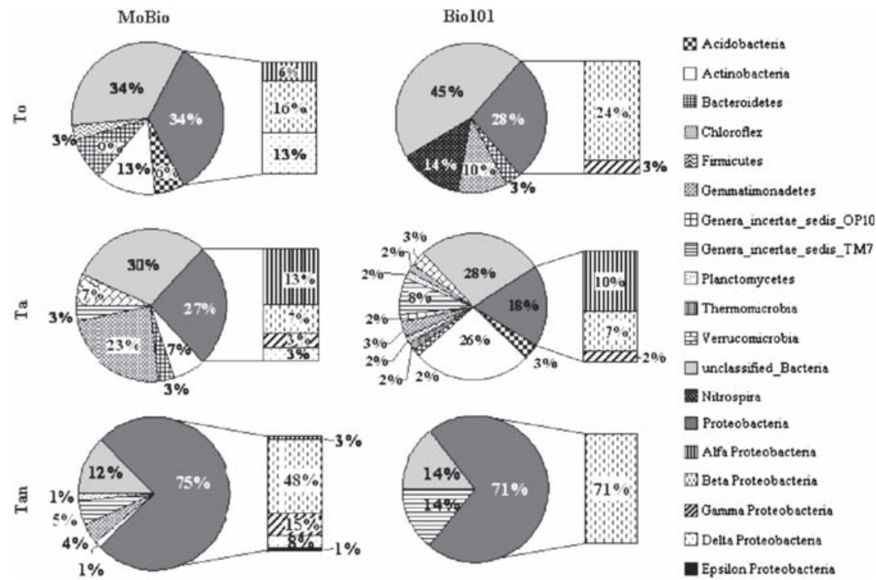

Fig. 6.2 Bacterial diversity in one sample of Arctic soil as obtained after aerobic (T_a) and anaerobic (T_{an}) enrichments in comparison to original community (T_0). The total community genomic DNA for each variant was isolated using MoBio and Bio101 kits. The proportion of different subdivisions of 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)

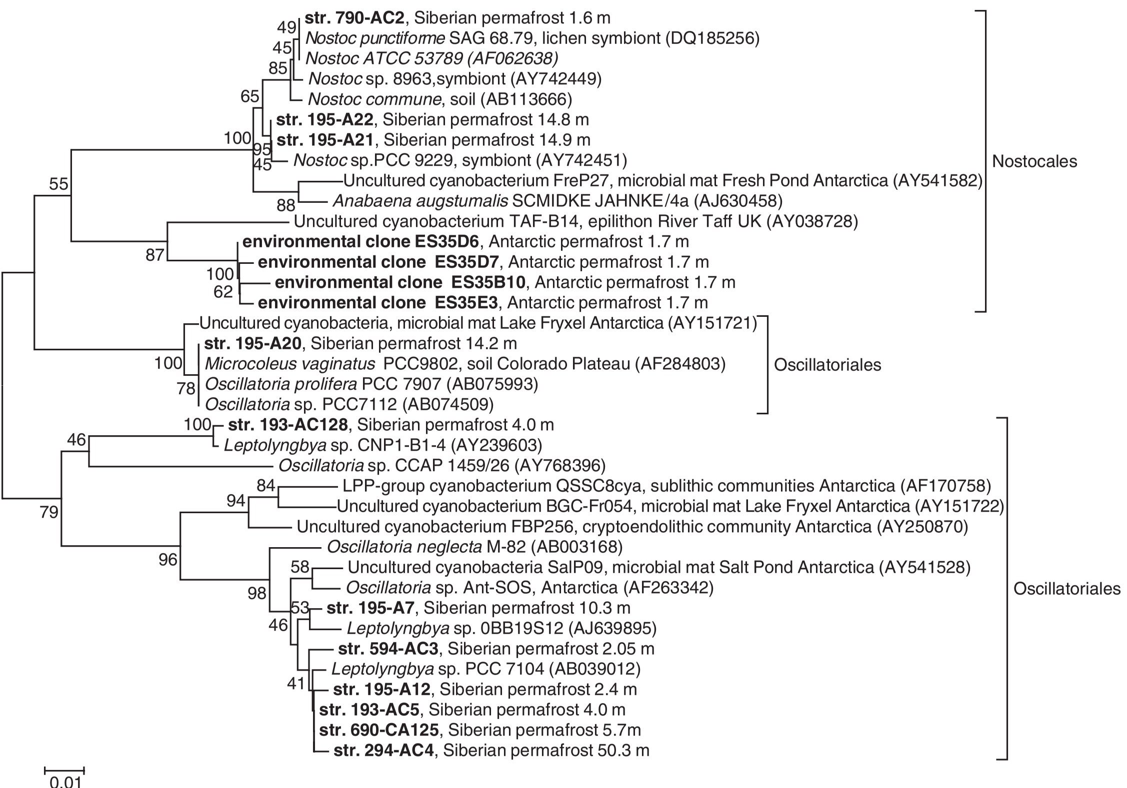

Fig. 6.3 Phylogenetic relationships of cyanobacterial isolates and environmental clones derived from Siberian and Antarctic permafrost. Tree was produced by the neighbor-joining method (Saitou and Nei 1987). Bootstrap values, expressed as percentages of 100 replications, higher than 40% are shown

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

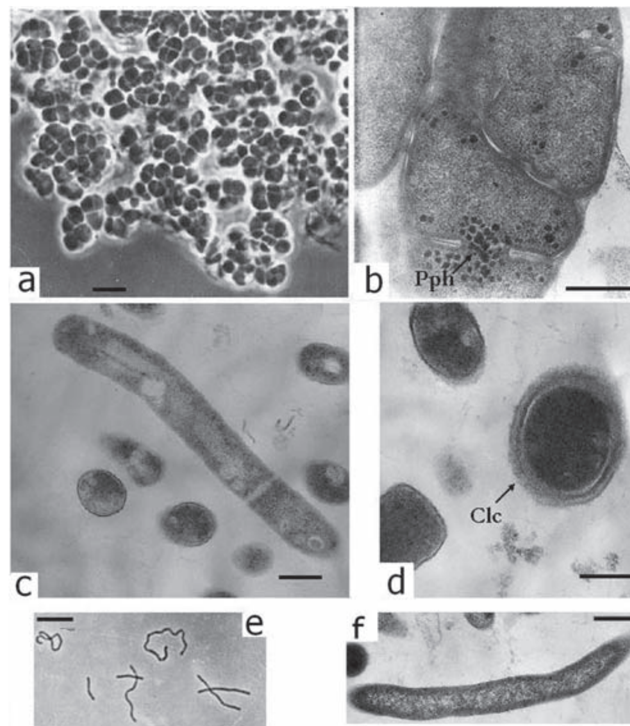

Fig. 6.4 Micrographs of methanogenic permafrost isolates. *Methanosarcina mazei* strain JL01: **a** phase contrast image, bar 10 mm; **b** ultrathin section, bar 0.5 mm. *Methanobacterium* sp. strain M2: **c** phase contrast image, bar 10 mm; **d** ultrathin section, bar 0.5 mm. *Methanobacterium* sp. 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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