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Research Paper

Bacterial Community in Ancient Siberian Permafrost as Characterized by Culture and Culture-Independent Methods

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ABSTRACT

The microbial composition of ancient permafrost sediments from the Kolyma lowland of Northeast Eurasia was examined through culture and culture-independent approaches. These sediments have been continuously frozen for 5,000 to 2–3 million years. A total of 265 Bacteria 16S rRNA gene sequences were amplified from the permafrost total-community genomic DNA and screened by amplified ribosomal 16S rRNA restriction analysis. Members of three major lineages were found: gamma-Proteobacteria (mostly Xanthomonadaceae), Actinobacteria, and Firmicutes. We also determined partial 16S rRNA gene sequences of 49 isolates from a collection of 462 aerobes isolated from these sediments. The bacteria included Actinomycetales (*Arthrobacter* and Microbacteriaceae); followed by the Firmicutes (*Exiguobacterium* and *Planomicrobium*); the Bacteroidetes (*Flavobacterium*); the gamma-Proteobacteria (*Psychrobacter*); and the alpha-Proteobacteria (*Sphingomonas*). Both culture and culture-independent approaches showed the presence of high and low G+C Gram-positive bacteria and gamma-Proteobacteria. Some of the 16S rRNA gene sequences of environmental clones matched those of *Arthrobacter* isolates. Two-thirds of the isolates grew at −2.5°C, indicating that they are psychroactive, and all are closely related to phylogenetic groups with strains from other cold environments, mostly commonly from Antarctica. The culturable and non-culturable microorganisms found in the terrestrial permafrost provide a prototype for possible life on the cryogenic planets of the Solar System. Key Words: Psychrotroph—Arctic—*Arthrobacter*—*Psychrobacter-Exiguobacterium*—16S rRNA phylogeny. Astrobiology 6, 400–414.

INTRODUCTION

LOW TEMPERATURE is a dominant feature of the planets beyond Earth, and even on Earth the oceans and soils from temperate regions to the poles experience temperatures of 4°C or lower. Hence, understanding microbial adaptations to cold is fundamental to understanding biological

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strategies for life on the other planets. Permafrost is a particularly relevant model for possible life on Mars because of similar temperature, moisture, and soil chemical and physical conditions.

Permafrost defines frozen soil, sediment, or bedrock that is frozen continuously for 2 years or more. It occupies over 20% of Earth's land surface (Gilichinsky and Wagener, 1995). Permafrost is a harsh, but stable, environment where subfreezing temperature (−12°C to −20°C in the Arctic), low water activity (a_w = 0.8–0.85), and inaccessibility of organic matter to the resident microorganisms remain constant for thousands to several million years (Gilichinsky, 2002). Microbial populations that inhabit this environment experience long-term exposure to low temperature, low water activity, low nutrient availability, and constant gamma radiation from soil minerals as well as cell aging processes. Nevertheless, these buried soils and sediments harbor a variety of microorganisms (Gilichinsky *et al.*, 1995; Vorobyova *et al.*, 1997) that have retained viability or might maintain low metabolic activity (Rivkina *et al.*, 2000; Bakermans *et al.*, 2003) over geological time periods and, upon thawing, renew or accelerate their physiological activity.

Despite the description of an increasing number of culturable isolates from Siberian permafrost (Soina *et al.*, 1991; Gilichinsky *et al.*, 1995; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000), relatively little information is available on the composition of the microbial community in this unique environment. This is primarily due to the reliance on standard culture-based methods for the recovery and identification of individual permafrost isolates, and the focus on specific physiological groups of microorganisms, rather than on gaining a more comprehensive view of the entire subsurface microbial community.

Permafrost habitats have remained isolated from the outside world for thousands to millions of years and provide a unique, natural culture collection for studying microorganisms that have been separated from the global gene pool of surface biota. 16S rRNA gene sequence analysis of the total-community genomic DNA has been used to characterize Archaea within the permafrost sediments (Tiedje *et al.*, 1998). Nevertheless, no comparative studies of the 16S rRNA gene diversity of culturable and non-culturable permafrost bacteria have been reported to date. Recently, we used improved isolation procedures for recovering aerobic heterotrophic bacteria from these ancient sediments, which include direct plating and permafrost soil enrichment with or without adding nutrient media and cryoprotectants, all carried out at 4°C to increase the diversity of genotypes recovered (Vishnivetskaya *et al.*, 2000). Herein, we reported the permafrost bacterial community of the Kolyma Region characterized by 16S rRNA gene sequences amplified from the total-community genomic DNA and from representative members of our culture collection obtained under conditions more similar to permafrost.

Subzero temperatures are a common phenomenon in space: most of the planets of the Solar System, their satellites, and comets and asteroids are of a cryogenic nature. The culturable and non-culturable microorganisms found in the terrestrial permafrost provide analogues that could be used in the search for possible ecosystems, potential inhabitants, or biosignatures, especially specific biomarkers, on extraterrestrial cryogenic bodies.

MATERIALS AND METHODS

Collection of the permafrost samples

The sampling sites were located in a tundra ecosystem in the Kolyma lowland of Northeast Eurasia, 152°–162°E, 68°–72°N, which is adjacent to the East Siberian Sea. The distances among boreholes ranged from 50 to 300 km. This area is characterized by Arctic climate with a mean annual air temperature of −13.4°C. The seasonal thawing of the soil surface usually starts in June and reaches its maximum, 0.3–0.5 m, in mid-September. Below this "active" layer the ground is permanently frozen and reaches a thickness of up to 800 m. The permafrost mean annual temperature is stable at −10°C. The permafrost samples were obtained by slow rotary drilling in the summers of 1991–1997 without the use of any solutions or drilling mud to minimize possible contamination. Evaluation of the sampling method as well as description of contamination controls were done by Khlebnikova *et al.* (1990) and confirmed by Juck *et al.* (2005) and Gilichinsky *et al.* (authors' unpublished data). These studies revealed a penetration level for contaminants of less than 1 cm from the core exterior. Contamination of the processed core interiors with the *Serratia marcescens* (Khlebnikova *et al.*, 1990) or with a

Pseudomonas strain expressing the green fluorescent protein (Juck *et al.*, 2005) was not detected by culture-dependent and culture-independent microbial analyses. In the field, the handling of permafrost cores was done in a sterile mobile hood with precautions to ensure aseptic manipulation, transportation, and storage of the samples (Khlebnikova *et al.*, 1990). The surface (1–1.5 cm) of extracted frozen core was trimmed away with a sterile knife to eliminate surface contamination from the drilling. The frozen core was then immediately divided into sections approximately 5 cm long, placed in presterilized aluminum tins, sealed, and placed in frozen storage. All samples remained frozen throughout this process and during transport. The cores were stored at −10°C to mimic the *in situ* conditions. In the laboratory, frozen samples were fractured in a class II positive-flow hood with a sterile knife, and only sections internal to the core were taken for microbiological analysis using sterile forceps (Shi *et al.*, 1997; Rivkina *et al.*, 1998).

Sediment characterization

The permafrost samples from depths of 1.2–68.6 m used in this study are listed in Table 1. The locations and characteristics of sampling sites and cores were also detailed previously (Rivkina *et al.*, 1998; Vishnivetskaya *et al.*, 2000). For DNA extractions, four permafrost samples were chosen (Table 1): Site A, sample 14, marine sediments from borehole 4/91, Point Chukochii, East Siberian Sea coast; Site B, sample 22, lake-swamp sediments from borehole 3/92, Kon'kovaya River; Site C, sample 20, lake-alluvium sediments from borehole 5/94, Chukochya River; and Site F, sample 45, buried soil from borehole 5/93, Khomus-Yuriakh River. Total bacterial cell counts were determined by counting cells stained with 5-(4,6-dichlorotriazin-2-yl)-aminofluorescein (DTAF) following the protocol of Bloem *et al.* (1995) as adapted to our facilities (Vishnivetskaya *et al.*, 2000).

Total DNA extraction from permafrost

The permafrost samples (5 g) were placed in sterile ceramic mortars after freezing in liquid nitrogen and ground until powder-like. The ground sample was mixed with 13.5 ml of sterile DNA extraction buffer [100 m*M* Tris-HCl, 100 m*M* sodium EDTA, 100 m*M* sodium phosphate (pH 8.0), 1.5 *M* NaCl, and 1% hexadecyltrimethylammonium bromide], containing 75 μg/ml proteinase K and 250 μg/ml achromopeptidase. Achromopeptidase was used to increase DNA yield from Gram-positive bacteria. Further steps for DNA extraction by high temperature/salt/sodium dodecyl sulfate-based lysis method were described previously (Zhou *et al.*, 1996). To increase yield of DNA, the DNA extraction from each sample was repeated three times. The crude DNA was separated by electrophoresis in 1% low melting point agarose and purified by filtration with Microcon YM-100 columns (Millipore Corp., Billerica, MA). The concentration of DNA was estimated at 260 nm. All reagents used for the DNA extraction and further analyses were molecular biology grade. Solutions were fresh made in DNase-free water and autoclaved.

Strain isolation from permafrost

We previously reported improved isolation procedures and the initial characterization of 238 strains from permafrost sediments (Vishnivetskaya *et al.*, 2000). Using different enrichment techniques, from four to over 50 bacterial strains were isolated from each sample. In spite of the fact that the permafrost strains were isolated without exposure to temperature above 4°C, all isolates were psychrotolerant and could grow at both 4°C and 24°C. We grouped the strains using phenotypic criteria (Balkwill *et al.*, 1989), which included: pigmentation; colony morphology; cell morphology; motility; catalase production; Gram reaction; rep-polymerase chain reaction (PCR) genomic fingerprinting; antibiotic resistance to ampicillin, chloramphenicol, streptomycin, erythromycin, and tetracycline; and growth at low temperatures. All tests were performed at 24°C except for the latter. Forty-nine strains representing the observed diversity were selected for partial 16S rRNA gene sequencing. In a number of cases, several strains from the same rep-PCR cluster were sequenced to assess their phylogenetic homogeneity or to compare more intimately similar strains isolated from different depths.

16S rRNA gene amplification from permafrost sediment-derived genomic DNA and from the bacterial isolates

Near-complete 16S rRNA genes were selectively amplified from purified total-community genomic DNA using moderately degenerate, Bacteria-specific primers targeted to *Escherichia coli*

TABLE 1. CHARACTERISTICS OF THE PERMAFROST SAMPLES

Site[a]	Borehole number	Sample number	Depth (m)	Age (YBP)[b]	Ice content (%)	pH	Organic C (%)	DNA yield (μg/g)	Bacteria cell counts			Distinct colony types
									Theoretical (cells/g)[c]	Total (cells/g)[d]	Culturable (CFU/g)[e]	
A	5/91	1359	1.2	10 K	47	7.2	3.2			7.8×10^7	1.8×10^3	6
	4/91	14	21.0	100–120	27	7.7	1.2	2.8	7×10^8	7.4×10^7	2.2×10^2	2[f]
		2671	31.4	K	27	7.4				7.3×10^7	8×10^2	2
B	1/97	392	0.15	Modern	24	5.6	4.1			2.5×10^8	3.2×10^5	11
		348	3.0	20–30 K	28	6.8–7.1	0.6–1.0			1.8×10^8	3.3×10^4	6
		130	4.5		40					1.2×10^8	7.6×10^4	4
		109	6.5		47					2.6×10^8	6.1×10^5	6
		293	8.0		31					2.8×10^8	6.7×10^5	4
		262	12.0		26					4.6×10^8	6.6×10^5	3
		273	12.5		22					2.4×10^8	3.1×10^4	7
		215	13.0		24					1.7×10^8	2.4×10^4	7
		235	13.5		27					3.3×10^8	3.7×10^4	5
	3/92	22	7.2	600 K	31	7.1	0.99	1.6	4×10^8	2.6×10^8	1.2×10^3	5[f]
C	5/94	190	5.5	200–600	17	7.3	0.9			8.1×10^7	4×10^2	4
		20	7.6–7.7	K	41	7.5	0.94	1.3	3.3×10^8	8.0×10^7	5.6×10^1	4[f]
	1/91	3361	55.8	2–3 M	33	7.0	3.2			2.4×10^7	8×10^2	3
	2/94	255	68.6		21	7.3	1.1			2.1×10^7	4×10^2	2
D	3/96	309	8.0	20–30 K	ND	ND	1.4			8.6×10^7	2.6×10^3	8
E	17/91	0198	7.5		22	7.8	1.2			5×10^7	1.3×10^3	1
F	6KhYu	007	8.0		ND	ND	ND			ND	8.0×10^5	5[f]
	5/93	45	40.0	1.5–2 M	48	6.7	1.6	0.5	1.2×10^7	ND	4.4×10^3	25[f]

ND, not identified.

[a]Site map showing these locations in Vishnivetskaya *et al.* (2000).

[b]Age of frozen soil layer in years before present (YBP). K = 1,000 years; M = 10^6 years.

[c]From DNA yielding by calculation using an average of 4×10^{-15} g DNA per cell (Ellenbroek and Cappenberg, 1991).

[d]Staining with DTAF.

[e]Plate cultivation method. CFU, colony-forming units.

[f]Data obtained from V.S. Soina (personal communication).

positions 49–68 (5′-TNA NAC ATG CAA GTC GRR CG-3′) and 1510 to 1492 (5′-RGY TAC CTT GTT ACG ACT T-3′) where degenerate R positions were accommodated by purine analog dK, and Y positions were accommodated by pyrimidine analog dP or N positions where there was an equal mixture of both analogs at a single position (Glen Research, Sterling, VA). The 5′ ends of both primers contained a phosphalink phosphoramidite (Applied Biosystems, Foster City, CA) that aids in cloning efficiency (Moyer *et al.*, 1994; Emerson and Moyer, 2002).

Amplifications were performed in a reaction mixture containing ~50 ng of total-community genomic DNA, 2.5 U of *Taq* DNA polymerase (Applied Biosystems), 5 μl of 10× PCR buffer, 2.5 m*M* $MgCl_2$, 200 μ*M* each deoxynucleotide triphosphate, 150 ng/μl T4 gene 32 protein (Roche Molecular Biochemicals, Indianapolis, IN), 1 μ*M* each forward and reverse primers, and sterile water to a total volume of 50 μl. Reaction mixtures were irradiated with ultraviolet light for 5 min at 254 nm (1,000 J/m^2) before the addition of template, T4 gene 32 protein, and *Taq* DNA polymerase to eliminate potential contamination (Sarkar and Sommer, 1993). T4 gene 32 protein was used to enhance the efficiency of PCR in the presence of residual amounts of humic acids (Tebbe and Vahjen, 1993; Kreader, 1996) and to keep the cycle numbers at 30. All negative controls with water instead of DNA template showed no amplification. We have also tested several of the reagents we used and confirmed that no contamination came from the proteinase K, lysozyme, or the achromopeptidase. The results of PCR were reproducible.

Genomic DNA from the 49 strains was isolated (Ausubel, 1994) and stored at −20°C until analysis. The 16S rRNA genes were amplified using a previously described forward primer (designated here LFP1) (Zhou *et al.*, 1997) targeting positions 10–27 of E. coli 16S rRNA, and 1510–1492 for the reverse primer (above). Primers were obtained from the Macromolecular Synthesis Facility, Michigan State University (East Lansing, MI). The standard PCR mix (50 μl) contained 5 μl of 10× PCR buffer, 1.5 m*M* $MgCl_2$, 30–50 ng of DNA, 2.5 m*M* each deoxynucleotide triphosphate, 1 μ*M* each primer, 0.5 U of *Taq* DNA polymerase, and 200 ng/ml bovine serum albumin. The temperature profile included an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Screening of environmental clone libraries

The amplified PCR products from the total-community genomic DNA were directly ligated into the pCR™ 2.1 vector using an Original TA cloning kit (Invitrogen, Carlsbad, CA) and transformed into *E. coli* INVαF′ competent cells (Invitrogen), according to the manufacturer's instructions. Positive clones were confirmed by PCR with primers TA106 and TA187 (Invitrogen) specific to the polylinker of the vector pCR 2.1. The re-amplified PCR products were analyzed for 16S rRNA gene inserts of the correct size range (1.5 kilobases) by electrophoresis in 1.2% agarose gel. Four environmental clone libraries were constructed from the four samples indicated above, and were called A14, B22, C20, and F45 representing the sites and sample numbers from which they originated.

Amplified ribosomal 16S rRNA restriction analysis (ARDRA) of environmental clones was used to detect clones with discrete operational taxonomic units (OTUs). Plasmid DNA was isolated from every putative positive clone with the QIA prep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Plasmids were then used as templates for an additional PCR (using the same conditions as for clone library generation). Fifteen microliters of the PCR-amplified products were then double-digested with 10 units of each tetrameric restriction enzyme *Msp*I plus *Rsa*I, or *Hha*I plus *Hae*III (Invitrogen) at 37°C overnight. The resulting restriction fragment length polymorphism patterns, created by using tandem tetrameric restriction endonucleases, were analyzed by gel electrophoresis in 3.5% Metaphor agarose (Cambrex, East Rutherford, NJ). The gel was stained with 0.5 μg/ml ethidium bromide and visualized by ultraviolet transillumination. Gel images were digitized, and ARDRA patterns were normalized with molecular size markers and clustered as previously described (Rademaker *et al.*, 1999). The cluster analysis of these data was done using the GelCompar software program (Applied Maths, Austin, TX).

16S rRNA sequencing of environmental clones and bacterial isolates

Sequences of the 16S rRNA genes from environmental DNA clones were determined using either purified plasmid or PCR-amplified plasmid inserts as the sequencing template. The PCR products were purified with the Wizard PCR

Preps DNA Purification System (Promega, Madison, WI). Nearly full-length sequences from 11 representative clones (a clone from each major OTU containing five or more clones plus a clone from two phylogenetically divergent Gram-positive, high G+C OTUs, each containing two clones) were constructed using standard primers (Lane *et al.*, 1985). Complete 16S rRNA sequences were manually aligned using both primary and secondary structure. All sequences were checked for chimera formation using CHIMERA_CHECK version 2.7 software of the Ribosomal Database Project II (Cole *et al.*, 2003).

The 16S rRNA genes from bacterial cultured isolates were partially sequenced using LFPI and a reverse primer targeting positions 515–529 of *E. coli* 16S rRNA (Lane *et al.*, 1985) using an automated DNA sequencer.

Phylogenetic analyses

Sequences were initially aligned against the most similar sequences in the ARB small subunit rRNA database using the alignment algorithm in ARB (http://www.mikro.biologie.tu-muenchen.de). Additional 16S rRNA gene sequences from closely related bacteria were retrieved from GenBank following identification by BLAST search (Altschul *et al.*, 1990). Sequence alignments were then adjusted manually based upon elements of primary sequence and secondary structure. Because of the broad phylogenetic diversity of the permafrost isolates and because local analyses are more accurate than global ones, phylogenetic inferences were divided into five separate analyses. Ultimately, each phylogenetic analysis was based upon nucleotide positions near the 5′ end of the molecule, which were present in all sequences compared and could be unambiguously aligned (*Arthrobacter*, 333 positions; Microbacteriaceae, 507 positions; Firmicutes, 380 positions; Bacteroidetes, 486 positions; Proteobacteria, 356 positions). Phylogenetic analyses were performed by the neighbor-joining (Saitou and Nei, 1987) and distance (DeSoete, 1983) methods from within the ARB environment (data not shown), and by a maximum likelihood method using the program fastDNAml. Bootstrap values were based on 100 trees generated using the program fastDNAml_boot (Olsen *et al.*, 1994). All maximum likelihood analyses included a jumbled order of sequence addition. GenBank accession numbers of previously published sequences are displayed in the phylogenetic trees (Fig. 1).

GenBank accession numbers

Sequences generated in the present study were deposited in GenBank (accession numbers AY444816 through AY444863 for cultured isolates and AY539810 through AY539820 for representative clones).

RESULTS

DNA recovery from permafrost and screening of bacterial 16S rRNA clones

A standard method of DNA recovery from soils (Zhou *et al.*, 1996) gave poor DNA yields from permafrost. Instead, after grinding of the samples frozen in liquid nitrogen, achromopeptidase was added to the DNA extraction buffer to maximize the community genomic DNA yield from Gram-positive bacteria. The yield of DNA ranged from 0.5 to 2.8 μg/g of sediment dry weight and did not correlate with pH or the organic carbon content of the samples (Table 1). Theoretical calculations of bacterial cells from total DNA recovered were close to that of the total direct counts. The data suggest that most of the indigenous permafrost microbes are being lysed and their DNA is being efficiently extracted. Thus, differences between theoretical/total cell counts and culturable colony-forming units suggest that the bulk of the DNA recovered belonged to mostly uncultivated members of the permafrost community (Table 1).

A total of 49, 47, 70, and 99 putative positive 16S rRNA gene clones were detected from the A14, B22, C20, and F45 clone libraries, respectively, based on correct size of insert and successfully passing the CHIMERA_CHECK analysis using partial sequence data (5′- and 3′-ends) from all clones examined. CHIMERA_CHECK detected between 6% to 8% chimeras for each of the libraries; these clones were dropped from further analysis. Most chimeras shared similarity with sequences of the most predominant OTUs. No chimeras were detected from any OTU that contained more than a single clone as determined by ARDRA. A final total of 265 16S rRNA genes were successfully cloned from total-community genomic DNA recovered from four permafrost samples.

Cluster analysis showed that the A14, B22, and C20 clone libraries were each composed of 17, 17, and 16 OTUs (Table 2), respectively, dominated

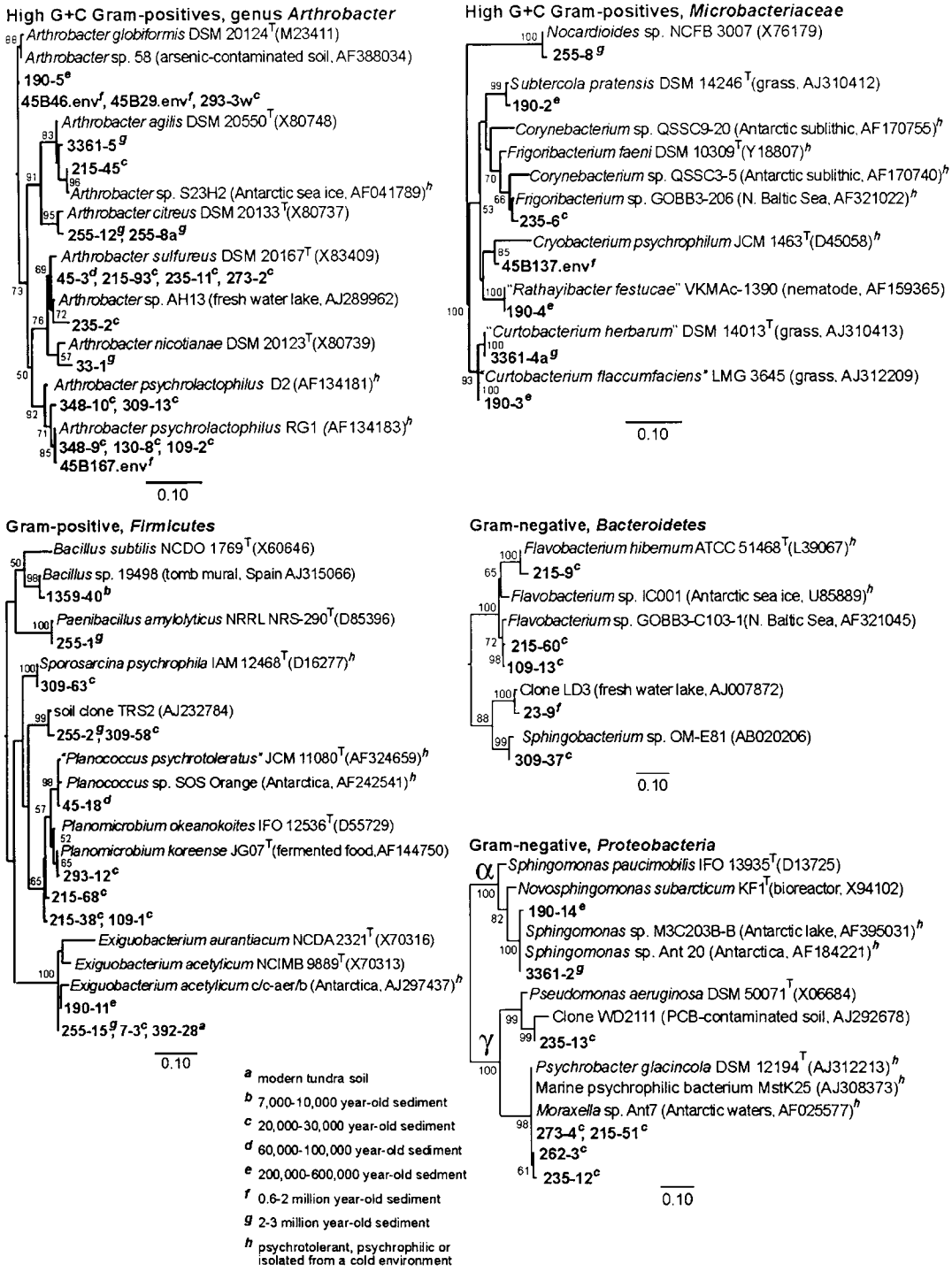

FIG. 1. Phylogenetic relationships of Siberian permafrost isolates and selected relatives. Trees were produced by a maximum-likelihood method using fastDNAml_loop (Olsen *et al.*, 1994). Bootstrap values are based upon 100 bootstrap trees calculated using fastDNAml_boot (Olsen *et al.*, 1994). Nodes without bootstrap values represent either branching orders that were inconsistent between the phylogenetic tree and the consensus tree generated from the bootstrap trees or bootstrap values less than 50%. The scale bars represent 0.1 changes per nucleotide position. *Nocardioides* strain NCFB 3007 and isolate 255-8 were incorporated in the Microbacteriaceae tree as an outgroup to root the tree. Outgroup sequences used to root the other trees were removed for aesthetic considerations. The Proteobacteria tree is unrooted.

TABLE 2. COMPARISON OF THE DIVERSITY AMONG 16S rRNA CLONES FROM SIBERIAN PERMAFROST SITES

Clone library sample site[a]	*Origin*	*Number of total positive clones*[b]	*Number of total OTUs*[c]	Lysobacter, *ES-1 phylotypes*[d]	*Gram-positive, high G+C phylotypes*[d]	*Estimated coverage (%)*[e]
A14	Marine	49	17	13	0	80
B22	Lake-swamp	47	17	9	2	75
C20	Lake-alluvium	70	16	7	0	84
F45	Buried soil	99	31	5	25	82

[a]Site locations and sample numbers from Table 1 from which the total community genomic DNA was extracted.
[b]Clones containing and entire ~1.5-kilobase 16S rRNA insert with 5'- and 3'-ends passing CHIMERA_CHECK analysis.
[c]OTUs as determined by ARDRA using two pairs of tetrameric restriction enzyme digests.
[d]As determined by either full or partial (*e.g.*, 5'- and 3'-ends) sequencing.
[e]As defined by Good (1953), estimating the fraction of a clone library of infinite size successfully sampled by our clone library with the same polymerase chain reaction and primer parameters.

by at least two major OTUs whose sequences were similar to both the putative lithotroph ES-1 and *Lysobacter* sp. C3 (AY074793) within the Xanthomonadaceae (Emerson and Moyer, 1997; Sullivan *et al.*, 2003). Nearly twice as many OTU patterns ($n = 31$) were found in the F45 clone library (Table 2). This library was dominated by OTUs contained within two taxonomic groups, which accounted for 57% and 39% of the total clones (Fig. 2). These two taxonomic groups were affiliated with high G+C Gram-positives such as *Arthrobacter, Micrococcus, Renibacterium,* and *Clavibacter,* and the Xanthomonadaceae (ES-1 and *Lysobacter* sp.), respectively. Minor OTUs across these four clone libraries were affiliated with other gamma-Proteobacteria including many Enterobacteriaceae such as *Aeromonas, Yersinia, Serratia,* and *Citrobacter* and Pseudomonadaceae such as *Pseudomonas* (0–9%) or Firmicutes such as *Clostridium* and *Bacillus* (ranging from 0% to 17%) or both (Fig. 2). A comparison of the diversity exhibited among these clone libraries is shown and based on the dominant phylotypes detected among OTUs along with the estimated coverage for each library, which ranged from 75% to 84% (Table 2).

16S rRNA phylogenetic analysis

The 16S rRNA sequences of clones from each major OTU group were compared with sequences for viable bacteria (Table 3 and Fig. 1). Four environmental clones had a high percentage of similarity with isolates. All other environmental clones studied had low similarity with viable isolates and were not included in the final phylogenetic trees. Sequences were grouped into five diverse bacterial assemblages (Fig. 1).

The vast majority of the 16S rRNA gene sequences of the isolates (37 sequences or 76%) were affiliated with the Gram-positive bacteria, and 23 of those (47% of all sequences) belonged to the family Micrococcaceae in the phylum Actinobacteria. Over one-third of all sequences of permafrost strains belonged to a single genus, *Arthrobacter* (17 sequences or 35%), and six (12%) belonged to the closely related Microbacteriaceae.

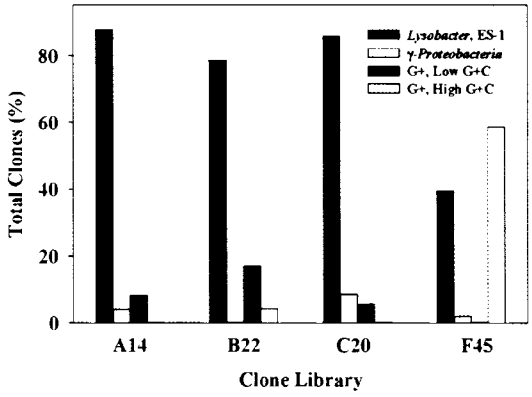

FIG. 2. The diversity, represented by four different permafrost samples, revealed by the rank abundance and phylogenetic affiliation of all putative positive 16S rRNA clones. Phylotype designations are as follows: *Lysobacter*, ES-1, Xanthomonadacea group of gamma-Proteobacteria; gamma-Proteobacteria, "other" gamma-Proteobacteria, including many Enterobacteraceae and Pseudomonadaceae; G+, Low G+C, Firmicutes, including mostly *Clostridium* and *Bacillus* spp.; G+, High G+C, Actinomycetales and Microbacteriacea, including mostly *Arthrobacter* spp.

TABLE 3. FEATURES OF MICROORGANISMS CULTIVATED FROM SIBERIAN PERMAFROST

Affiliation	*Number of strains*	*Oldest age (YBP)*[a]	*Depth (m)*	*Medium type*[b]	*Growth at −2.5°C*[c]	*Pigment*[d]	*Morphology*	*Resistance*[e]		*Catalase positive*[c]
								Ampicillin (100 μg/ml)	*Streptomycin (50 μg/ml)*	
Gram-positive, *Actinobacteria*, (high G+C)										
Arthrobacter	17	3 M	3.0–68.6	F/D/DSu/DS	15:17	w/c/y/p	Rod-to-coccus/ cocci	0:2:4	1:2:4	15:15
Microbacteriaceae	5	3 M	5.5–55.8	F/DS	5:5	y/o	Ovoid rods/cocci	0:0:2	0:2:2	4:4
Nocardioidaceae	1	3 M	68.6	F	1:1	n	Irregular rods	ND	ND	1:1
Gram-positive, Firmicutes, (low G+C)										
Exiguobacterium	4	3 M	0.15–68.6	F/DS	4:4	y/o	Rods/rods pairs	0:2:4	1:3:4	4:4
Planococcus	5	100 K	8.0–40.0	F/D/DS	3:5	y/o	Cocci/cocci pairs	0:0:3	2:1:3	3:3
Sporosarcina	3	3 M	8.0–68.6	D	3:3	y/w/n	Rods	ND	ND	3:3
Bacillus	1	10 K	1.2	D	0:1	c	Rods	ND	ND	1:1
Paenibacillus	1	3 M	68.6	DSu	1:1	n	Rods	ND	ND	1:1
Gram-negative, Bacteroides										
Flavobacterium	3	30 K	5.5–13.0	D	0:3	y	Pleomorphic rods	1:0:2	1:0:2	2:2
Sphingobacterium	2	2 M	7.2–8.0	F/D	1:2	y	Pleomorphic rods	ND	ND	1:1
Gram-negative, gamma-Proteobacteria										
Psychrobacter	4	30 K	12.0–13.5	F/D/DSu	3:4	c/n	Rods	0:0:2	0:0:2	4:4
Pseudomonas	1	30 K	13.5	F/DSu	1:1	c	Rods	ND	ND	1:1
Gram-negative, alpha-Proteobacteria										
Sphingomonas	2	3 M	5.5–55.8	F/D	2:2	y/o	Rods/rods chain	0:0:2	2:0:2	2:2

[a]Age of frozen soil layer in years before present (YBP). K = 1,000 years; M = 10^6 years.
[b]Medium: F, full TSA; D, $\frac{1}{2}$ or 1:10 diluted TSA; DSu, $\frac{1}{2}$ or 1:10 diluted TSA with 0.1 *M* sucrose; DS, $\frac{1}{2}$ or 1:10 diluted TSA with 2% NaCl.
[c]Positive versus total.
[d]w, white; c, creamy; y, yellow; p, pink; o, orange; n, colorless.
[e]Antibiotic resistance as resistant:intermediate:total.

The 14 remaining Gram-positives belonged to the Firmicutes. Fewer Gram-negative bacteria were found in the sequenced set (12 or 24% of total). Five of those were affiliated with the Bacteriodetes, with the remaining seven belonging to the Proteobacteria assemblages. Within the Proteobacteria, two belonged to the genus *Sphingomonas* of the alpha-subdivision, one was a pseudomonad of the gamma-subdivision, and the remaining four belonged to the Moraxellaceae, also in the gamma-subdivision. When the 49 permafrost sequences were compared with GenBank 16S rRNA gene sequences, 22 (45%) had nearest relatives that were either psychrophilic or psychrotolerant or were isolated from cold environments, most from the Antarctic. All were from taxa that had members from cold environments.

Characteristics of permafrost isolates

Features of isolates for which 16S rRNA gene sequences were determined are summarized in Table 3. Bacterial strains isolated from permafrost were subsequently checked for their ability to grow at −2.5°C. Sixty-seven percent of the 462 strains grew at −2.5°C and formed visible colonies within the 3-week incubation period (*cf.* Table 3). Nineteen different permafrost isolates were tested for resistance to five antibiotics. Permafrost bacteria were more resistant or intermediate in resistance to streptomycin than to ampicillin or the other antibiotics tested (Table 3). In a more detailed study of a few of these isolates, four out of six showed a trend of increased antibiotic sensitivity at 4°C versus 24°C with all classes of antibiotics except erythromycin (Ponder *et al.*, 2005).

Actinobacteria

Nearly 55% of the 462 isolated bacterial strains were Gram-positive; over half of them had the rod-to-coccus growth cycle that is a characteristic of Actinomycetales. A high sequence similarity was found between environmental clones and high G+C Gram-positive bacteria. The cultivable bacteria and clones were obtained from samples collected at different depths. Thus, clones 45B29.env, 45B46.env, 45B167.env, and 45B137.env were from the F45 clone library, obtained from sample 45 of 40 m in depth, and were affiliated with the Actinomycetales (Fig. 1). Three clones belonged to the genus *Arthrobacter* and one clone to Microbacteriaceae. Clone 45B167.env was closely related to isolates 130-8, 109-2, 348-10, 348-9, and 309-13, from 4.5, 6.5, 3.0, and 8.0 m, respectively, which in turn are related to *Arthrobacter psychrolactophilus* RG1, a psychrophile isolated from a limestone quarry. Clones 45B46.env and 45B29.env both had a high percentage of similarity to isolates 190-5 and 293-3w, from 5.5 and 8.0 m, respectively, which were most closely related to *Arthrobacter* sp. strain 58, isolated from an arsenic-contaminated soil. One clone, 45B137.env, had high similarity to *Cryobacterium psychrophilum*. Isolates that belong to the genus *Arthrobacter* were coccoidal rods or spherical in shape; colonies were creamy or yellow pigmented. Microbacteria were yellow, orange, or pink pigmented colonies composed of cocci. High G+C Gram-positives were isolated from young Holocene to old Late Pliocene sediments and were able to grow on full, diluted trypticase soy broth supplemented with 1.5% agar (Difco, Detroit, MI) (designated here as TSA) and TSA supplemented with 0.1 *M* sucrose or 2% NaCl as cryoprotectants.

Firmicutes

The remaining 14 Gram-positive bacteria (29% of the isolates) belonged to the genera *Bacillus*, *Sporosarcina*, *Exiguobacterium*, or *Planomicrobium*, all of them in the order Bacillales. Only one isolate was related to spore-forming bacteria of the genus *Bacillus* (Fig. 1). One non-pigmented bacterial strain with rod-shaped cells was found to be closely related to *Paenibacillus amylolyticus* (99.4% similarity). Four isolates (8%) with creamy-yellow-orange pigmentation were related to *Exiguobacterium*. They shared 98.4–99.7% 16S rRNA sequence similarity with each other and 96.7–98.8% similarity to *Exiguobacterium acetylicum*. They were isolated from young and old permafrost sediments on full-strength or dilute TSA media supplemented with 2% NaCl. Five strains (20%) were affiliated with the Planococcoaceae. Orange-pigmented strains with coccoidal cells belonged to the genera *Planococcus*, as well as *Planomicrobium*, were isolated from sediments up to 100,00 years old. They were isolated on full-strength and diluted TSA and on diluted TSA supplemented with 2% NaCl (but not sucrose). Strain 215-68 was able to grow on medium supplemented with 10% NaCl.

Bacteroidetes

Five (10%) bright- to dark-yellow pigmented isolates belonged to the Bacteroidetes, mostly to

the class Flavobacteria (Fig. 1). Strains that belonged to this assemblage were composed from filamentous rods, which could grow on full-strength and diluted TSA. Four of the strains were isolated from 20,000–30,000-year-old sediments with only one isolated from the 0.6–2-million-year-old layer.

Gamma-Proteobacteria

The isolates formed two groups within the gamma-subdivision of Proteobacteria (Fig. 1). Four strains, isolated from different samples, had a high percentage of sequence similarity between them (96.9–99.2%) and were members of the genus *Psychrobacter* with 98.8–99.5% similarity. These strains produced colorless to creamy colonies and were motile rods, which could grow on full-strength and diluted TSA and on diluted TSA supplemented with 0.1 *M* sucrose. Strain 235-13 was affiliated with *Pseudomonas* and was most closely related to an environmental clone WD2111 from a PCB-contaminated soil (99.0% sequence similarity). It had rod-shaped cells that were able to grow and produced creamy colonies on full-strength and diluted TSA supplemented with 0.1 *M* sucrose. All gamma-Proteobacteria were isolated exclusively from relatively young late Pleistocene permafrost (20,000–30,000 years old).

Alpha-Proteobacteria

Strains 190-14 and 3361-2 were affiliated with the genus *Sphingomonas* of the alpha-Proteobacteria (sequence similarity 99.8–100%). Strain 190-14 isolated from sediments of 200,000–600,000-year-old had yellow pigmented colonies; its non-motile rods occured singly or in chains. Strain 3361-2, from 2–3-million-year-old permafrost, formed orange colonies composed from weakly motile rods. Both strains were able to grow on full-strength and diluted TSA. Their nearest relative, also psychrotolerant, was isolated from Antarctic soil.

DISCUSSION

In this study, permafrost communities were analyzed by culture and culture-independent approaches in an effort to assess their cultivable component and their total biodiversity. Bacterial communities in the permafrost contain culturable cells as well as viable but non-culturable cells, *e.g.*, deep resting cells and dead cells (for review, see Vorobyova *et al.*, 1997). Epifluorescence microscopy revealed from 2.1×10^7 to 4.6×10^8 cells/g of sediment. The comparative analysis showed that an average from <0.01–0.3% (this study) to 0.1–10% (Vorobyova *et al.*, 1997) of cells seen by microscopy with DTAF or acridine orange, respectively, grew on nutrient media. The high percentage of high G+C Gram-positive bacteria among isolates suggests that they are particularly well adapted to the frozen environments, and are more easily cultured. Possibilities for the high survivability of non–spore-forming bacteria in the permafrost may involve their ability to form dormant cells. Cyst-like refractive cells have been shown for *Arthrobacter* sp. and *Micrococcus* sp. (Soina *et al.*, 2004). In the case of other non–spore-forming bacteria, alternative mechanisms may contribute to an efficient minimal physiology and to survival strategy in the permafrost sediments.

Indigenous bacterial communities from Siberian permafrost have been shown to have slow but continuous metabolic activity down to −20°C (Rivkina *et al.*, 2000). The other studies of permafrost strains, a *Psychrobacter* sp. (Bakermans *et al.*, 2003), a *Psychrobacter* strain 273-4, and an *Exiguobacterium* strain 255-15 (Ponder *et al.*, 2005), showed that these strains could grow at temperatures below 0°C. Growth at low temperatures (4°C) was accompanied by changes in cell membranes that could affect membrane transport and, hence, explain the differences in carbon source utilization and antibiotic sensitivity (Ponder *et al.*, 2005). The changes in membrane composition with decreasing temperature may reflect additional adaptations that improve survival. These data suggest that permafrost microorganisms may be active in their native habitat. Recently, Price and Sowers (2004) analyzed data indicating that microbes can survive at negative temperatures as well as maintain low metabolic activity. This suggests that cells retain protein synthesis machinery and a DNA replication system at subzero temperatures. Comparing data on rates of aspartic acid racemization and DNA depurination with data on metabolic rates for survival, Price and Sowers (2004) assumed that "rates of repair of DNA and protein damage in living microbes are similar to rates of damage," consistent with viability.

Regardless of the fact that permafrost contains substantial densities of live bacteria, Willerslev *et*

al. (2004) were able to reproducibly amplify only short 16S rRNA gene sequences of 120 basepairs and 600 basepairs from permafrost samples up to 400,000–600,00 years, and stated that these fragments belong to ancient DNA. Our method of DNA isolation targeted DNA isolation from viable bacterial cells and amplification of nearly full-length 16S rRNA gene sequences. The results of Willerslev *et al.* (2004) contradict previous reports on the isolation of viable bacteria from permafrost samples of several million years (Gilichinsky *et al.*, 1995; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000) and findings described in this paper. The differences may be caused by different approaches and methods.

Both culture and culture-independent approaches revealed the presence of gamma-Proteobacteria and Gram-positive bacteria with high and low G+C content. By culture-independent approach, we found that one phylotype, which is closely related to *Lysobacter* sp. of the Xanthomonadaceae in the gamma-Proteobacteria, was a major part of the total bacterial community. This phylotype has also been found to share a nearly identical 16S rRNA gene sequence with that of ES-1, a microaerophilic iron-oxidizing bacterium that grows at neutral pH (Emerson and Moyer, 1997). Multiple strains of iron-oxidizing bacteria that exhibit this novel physiology have now been isolated from the rhizosphere of wetland plants (Emerson *et al.*, 1999) and have been shown to account for over half of the Fe(II) oxidation that occurs in these wetland systems (Neubauer *et al.*, 2002). Here conditions may be biogeochemically similar to the Kolyma soils and sediments prior to their becoming permanently frozen. The ES-1/*Lysobacter*-like phylotype was detected in permafrost of all ages, whereas high G+C Gram-positives were a minor component at site B22 and the dominant phylotypes at the oldest site (F45). The low G+C Gram-positive phylotypes were found in the 100,000–600,000-year-old sites (A14, B22, and C20) but not in the 1.5–2-million-year-old permafrost site (F45). What is perhaps more striking about our analysis of the permafrost 16S rRNA clones is the absence of *Acidobacterium* found in almost all soils examined by this approach (cf. Kuske *et al.*, 1997; Barns *et al.*, 1999; Zhou *et al.*, 2003).

An interesting phylogenetic pattern has also emerged during our comparison of the seven different *Lysobacter*-like phylotypes that underwent nearly full 16S rRNA gene sequence analysis (cf. Tables 1 and 2). A single representative sequence, which came from library F45 (clone F45B39), contains the consensus of each of the other six sequences. Therefore, clone F45B39, which originates from the oldest permafrost sediment-derived clone library at 1.5–2 million years old, could be the common denominator for every other sequence variation observed across the remaining younger sediment-derived libraries (which account for a total of 14 unique point mutations, presumably fixed at a later time). This pattern was perpetuated by the pair of representative clones from library B22 (clones B22B17 and B22B20) and library C20 (clones C20B1 and C20B9) having acquired either single- or double-point mutations, with these libraries representing permafrost sediments that are either 600,000 or 200,000–600,000 years old, respectively. Finally, the two representative clones (A14B9 and A14B6) from the youngest site at 100,000–120,000 years old, A14, have the most point mutations with three and four each, respectively. From a parsimony standpoint this would imply that, if these clones did originate at approximately the same time as these ancient permafrost sediments were deposited, then an average of 2.45 fixed mutations per 10^6 years can be estimated, which translates into ~0.17% per 10^6 years for this lineage of 16S rRNA genes.

The phylogenetic diversity of 16S rRNA clones from permafrost is much lower than described for tundra (surface) soil of the same region for which no dominant clones were found (Zhou *et al.*, 1997) and rarefaction analysis showed the diversity to be as high as for temperate soils (Tiedje *et al.*, 1997). Furthermore, the phylogenetic affiliation of the tundra clones was different from the permafrost clones. The tundra soil clones were affiliated with the delta- and alpha-Proteobacteria, *Bacteroidetes*, *Planctomyces*, Spirochaetes, *Chlorobium*, and *Acidobacterium* (Zhou *et al.*, 1997). The tundra soil thaws every summer, plant productivity generates new carbon resources, and moisture levels are high, which creates an environment very different from that of the underlying permafrost.

A high degree of similarity between 16S rRNA gene sequences of a few environmental clones and cultivable permafrost isolates was found for two groups within the genus *Arthrobacter* and one in the Microbacteriaceae. This correspondence between soil and isolate 16S rRNA gene sequences strongly suggests that these bacterial

groups are both resident in and survive in permafrost sediments.

Our study showed that the cultured permafrost bacteria were dominated by high G+C Gram-positive bacteria, especially *Arthrobacter* and Microbacteriaceae; followed by the Firmicutes, especially *Exiguobacterium* and *Planomicrobium*; the Bacteroidetes, especially *Flavobacterium*; the gamma-Proteobacteria, especially *Psychrobacter*; and the alpha-Proteobacteria, especially *Sphingomonas*. In a previous phylogenetic study of cultured isolates from Siberian permafrost (Shi *et al.*, 1997), four major assemblages were detected: high G+C and low G+C Gram-positives and the beta- and gamma-Proteobacteria. Our phylogenetic data are similar in some features: the abundance of high G+C Gram-positives in permafrost of different ages, isolation of low G+C Gram-positives from young and old permafrost, and detection of gamma-Proteobacteria. The two studies differ, however, with regard to the isolation of Gram-negative bacteria. Shi *et al.* (1997) isolated gamma-Proteobacteria only from old strata (1.8–3.0 million years old), while we isolated them only from young sediments (20,000–30,000 years old), though we found their signature in the community genomic DNA from permafrost of different ages. Shi *et al.* (1997) isolated beta-Proteobacteria, but not alpha-Proteobacteria or Bacteroidetes. In contrast, we isolated members of the latter two groups. Similar to our study, the same bacterial groups were discovered in late Holocene glacial ice of 5,000–20,000 years old (Christner *et al.*, 2000). Although isolates from ancient cold environments such as glacial ice and permafrost belonged to the same phylogenetic assemblages, they are of different eras. It is striking that about half of our permafrost isolates are closely related to species isolated from other cold environments. Furthermore, the majority of our isolates grew well at −2.5°C, which indicates their enhanced psychroactivity. These patterns suggest that adaptation to the stresses of these frozen environments is a phylogenetically conserved trait. The dominant viable bacteria demonstrate their considerable resistance to the stress factors of long-term frozen permafrost.

A more comprehensive assessment of microbial diversity in subsurface permafrost environments can be obtained by using a combination of culture- and molecular-based techniques than by using either method alone, since these two methods yield very different perspectives about the bacterial community structure. The viable members of the permafrost bacterial community may harbor special mechanisms that have allowed their adaptation to the harsh environment. We are currently exploring these adaptation mechanisms through genomic and proteomic approaches.

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ABBREVIATIONS

ARDRA, amplified ribosomal 16S rRNA restriction analysis; DTAF, 5-(4,6-dichlorotriazin-2-yl)-aminofluorescein; OTU, operational taxonomic unit; PCR, polymerase chain reaction; TSA, trypticase soy broth supplemented with 1.5% agar.

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