MICROBIAL ECOLOGY

Microb Ecol (1997) 33:169–179

Characterization of Viable Bacteria from Siberian Permafrost by 16S rDNA Sequencing

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Received: 11 April 1996 Accepted: 8 May 1996

Abstract

Viable bacteria were found in permafrost core samples from the Kolyma-Indigirka lowland of northeast Siberia. The samples were obtained at different depths; the deepest was about 3 million years old. The average temperature of the permafrost is −10°C. Twenty-nine bacterial isolates were characterized by 16S rDNA sequencing and phylogenetic analysis, cell morphology, Gram staining, endospore formation, and growth at 30°C. The majority of the bacterial isolates were rod shaped and grew well at 30°C; but two of them did not grow at or above 28°C, and had optimum growth temperatures around 20°C. Thirty percent of the isolates could form endospores. Phylogenetic analysis revealed that the isolates fell into four categories: high-GC Gram-positive bacteria, β-proteobacteria, γ-proteobacteria, and low-GC Gram-positive bacteria. Most high-GC Gram-positive bacteria and β-proteobacteria, and all γ-proteobacteria, came from samples with an estimated age of 1.8–3.0 million years (Olyor suite). Most low-GC Gram-positive bacteria came from samples with an estimated age of 5,000–8,000 years (Alas suite).

Introduction

In 1911, Omelyansky was the first to report the presence of viable microorganisms in permafrost [37]. Kris [32] found low numbers of microorganisms below the interface between the active layer of soil and the permafrost, and fewer or no microorganisms at deeper levels in the Russian arctic on Koluchin and Wrangel Islands. James and Sutherland reported the presence of viable aerobic and anaerobic bacteria in permafrost in northern Canada [25]. (For a detailed review of microbial life in permafrost, see Gilichinsky and Wagener [17]).

From the 1950s to the 1970s, a significant amount of work was done on the microbial flora in both Arctic soils and the underlying permafrost in northern Canada and Alaska [4–8]. A wide variety of species and physiological types of microorganisms, including bacteria (*Bacillus* spp., *Azotobacter* spp., sulfate reducers, and some thermophiles, *Streptomyces* spp.), yeasts, fungi, and protozoa, were found

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in arctic soils [5, 8]. In 1961, Becker and Volkmann [4] reported the isolation of four genera of bacteria from arctic permafrost cores taken at depths of 6–18 m near Fairbanks, Alaska. Shortly afterward, Boyd and Boyd [7] performed the first quantitative investigation of permafrost microorganisms in the western arctic and found only 5–130 cells/g (including mesophilic and thermophilic bacteria) in permafrost at a depth of 1.8 m.

The only report of viable bacteria from Antarctic permafrost is that by Cameron and Morelli in 1974 [10]. Unfortunately, these studies had some methodological and technical deficiencies, and the results have never been confirmed.

Recently, Russian scientists demonstrated the presence of large numbers (up to 10^8 cells/g) of living microorganisms of various functional groups in the Siberian permafrost, using strictly aseptic techniques during their isolation [17, 18, 20, 31, 51, 52]. These microorganisms have survived under conditions of low temperatures, low nutrient concentration, and a closed, and generally reducing, ecosystem (Eh = +40 to −250 mV) for thousands to even millions of years [20].

Recent studies by Russian scientists give us a more accurate picture on the microorganisms in Siberian permafrost [17, 20, 31, 50–52]. Large numbers (10^2–10^8 cells/g) of viable microorganisms, including fungi, yeast, algae, actinomycetes, and bacteria, have been found in most (90%) of the samples studied [50]. The number and diversity of microorganisms varied from sample to sample. Generally, the number of viable microorganisms depends on the age of the permafrost, and decreases with increasing age of the permafrost [17, 20, 31, 51]. The diversity of microorganisms also decreases with age. Fungi were found only in samples younger than 10,000 years [51]. Among bacteria, the Russian researchers studied mostly aerobes. A number of cocci, coryneforms, endospore formers and non–endospore formers, sulfate reducers, nitrifying and denitrifying bacteria, and cellulose decomposers have been identified among the following genera: *Arthrobacter, Brevibacterium, Pseudomonas, Flavobacterium, Rhodococcus, Bacillus, Aeromonas, Acinetobacter, Promicromonospora, Cytophaga, Micrococcus, Deinococcus, Nitrosospira,* and *Nitrobacter* [50]. Only one strict anaerobe, the sulfate reducer *Desulfotomaculum orientalis,* has been isolated to date [46].

The presence of microorganisms in the permafrost has raised several questions. The first, whether permafrost microorganisms are metabolically active or in a state of "anabiosis," has been recently discussed [15–17]. Although a definitive answer based on experimental evidence is still lacking, circumstantial evidence related to permafrost and direct observations from Antarctica suggest that metabolic activity is taking place, although at a very low level [29, 41] at temperatures of −10°C or below. The presence of liquid water in frozen soil is well documented; 0.5–3% of water in the permafrost is unfrozen [12], and the thickness of unfrozen water layers is about 50 nm [1] at temperatures around −10°C.

Investigations, to date, have all been based on traditional morphological and physiological methods. In most cases, isolated bacteria were identified to the level of functional groups. In this study, some basic questions about these microorganisms, such as their identity and phylogenetic relatedness to each other and to known microbes, were addressed mainly through characterization by 16S rDNA sequencing. Partial 16S rDNA sequences for the 29 isolates from Siberian permafrost were determined, and the relationships among these isolates and known species [33] of eubacteria were analyzed by maximum parsimony. In addition, some phenotypic attributes of the isolates were studied.

Materials and Methods

The Siberian Permafrost

Permafrost samples were obtained by means of drilling in northeast Siberia from the Kolyma Indigirka lowland (approximately 158°E, 70°N, Table 1). This area has a cold arctic climate, a mean annual air temperature of −13.4°C, and an annual precipitation of 229 mm (measured in Kolymskoye). The vegetation is tundra dominated by dwarf willow (*Salix polaris*), dwarf birch (*Betula* sp.), berries (*Vaccinium* sp.), grasses, and lichens (*Thamnolia, Cladina,* etc.), or forest-tundra with sparse stands of larch trees (*Larix dahurica*). The deposits of sediment, formed in shallow lake bottoms during the late Pliocene and Pleistocene periods [42] and reaching a thickness of more than 500 m, are the oldest and least disturbed permafrost areas in the Northern Hemisphere, and probably on Earth.

The upper layer of sediment, from 0.5 to 1 m thick, freezes and thaws alternately each year. Below this layer, however, the ground, a soil-and-ice mixture, is permanently frozen. The temperature at a depth of 14 m below the surface is very stable and remains at −10°C for the entire year (Fig. 1). Previous studies have determined that the mean geothermal gradient in the entire frozen layer (400–900 m) is 1.5°C/100 m, but the temperature gradient is close to zero in the upper 200 m [20].

The issue of preservation of the microbiota in frozen permafrost sediments needs to be addressed here. Two questions arise. First, could nonindigenous bacteria have penetrated permafrost layers, either from the surface or from other layers, possibly through vertical movement of water? Second, have the permafrost sediments remained frozen since their original freezing, or could they have been thawed in the past for shorter or longer periods of time during which metabolic activity and cell divisions could have taken place at an increased level? To answer these questions, we must consider the different layers of permafrost separately.

Table 1. Description of wells drilled

Well no.	Date	Location	Suite/age/notes	Sample
6/90	Aug. 1990	Middle part of Bolshaya Chukochya River, right bank	Olyor, 1.8–3 my[a]	All samples inoculated in the field
3/89-35A	Aug. 1989	Middle part of Bolshaya Chukochya River, right bank	Yedoma, 30,000–40,000 y,[b] ice content 38%	1055
1/89-34	Aug. 1989	Middle part of Bolshaya Chukochya River, right bank	Alas, 2,000–5,000 y, ice content 42%	1308
9/89 Kh-yu	Aug. 1989	Khomus-Yuryakh River, right bank	Olyor, 3–5 my, ice content 22.2%	1052A

[a] my, Million years
[b] y, Years

At the drilling site, the Kolyma-Indigirka lowland permafrost comprises three horizons. The upper Alas sediment was deposited during a milder climate. Microorganisms of the Alas which extends to 3 m depth were exposed relatively recently to repeated freezing and thawing cycles during which the upper levels of the underlying Yedoma horizons were also thawed (and thus became incorporated into the Alas). During thawing, bacteria could (and probably did) migrate within the sediment, but the Alas froze 5,000–8,000 years ago and has been frozen since [28].

The next horizon is the Yedoma suite, extending from ca. 3 m to 8 m deep and encompassing a time frame from 10,000 to about 40,000–50,000 years ago. The Yedoma sediment was formed in a cold climate, froze very soon after deposition, and has remained undisturbed since. The presence of vertical ice wedges in the permafrost throughout this horizon demonstrate that it has never thawed [38, 39], so almost certainly, no significant bacterial migration or thawing has occurred in the Yedoma sediment since its formation.

The lowermost horizon is the Olyor suite, which extends from 8 to about 50 m and encompasses the time frame from 0.6 to 3 million years ago. In this study, we used the lower portion of this formation which has an estimated age of 1.8 to 3 million years. No information is available about climatic conditions during deposition or about the history of thawing, so during warmer periods the sediment may have thawed and refrozen, and microbial migration and metabolically more active periods may have occurred. The Olyor sediments have, however, been continuously frozen at least since the time of Yedoma, i.e., 40,000–50,000 years ago [30, 48], so external influence in this horizon was almost certainly limited to the depth of seasonal thawing, and penetration of surface microorganisms to the deeper frozen layers can be virtually ruled out [19].

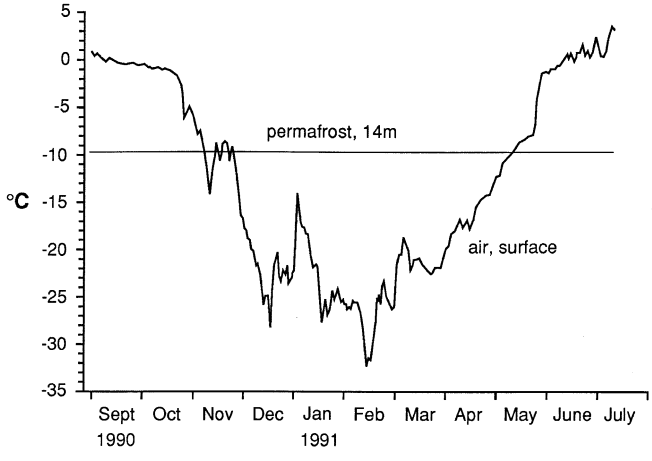

Fig. 1. Soil temperatures at the surface and at 14 m depth (permafrost) at the collecting site from August 1990 to August 1991, measured by thermistors in a drill hole and recorded each day at midnight with a Campbell 21× (Logan, Utah) datalogger. (McKay, Chyba, and Meyer unpublished data).

Sample Collection

Permafrost cores were obtained with a portable gasoline-powered drill that operates without a drilling fluid, which would contaminate biological samples. The engine turns and hammers on the drill rods, which are connected to a coring device. The corer cuts 20- to 30-cm-long cores. After removal from the corer, the surfaces of the cores were cleaned by shaving with an alcohol-sterilized knife. The cores were split into ca. 5-cm-long segments, which were either used immediately for microbiological studies or placed in metal boxes, stored in the field in a cave dug in the permafrost, and transported frozen to Pushchino. There, they were either placed in permanent frozen storage or transported by air to the United States while maintaining them below freezing. To monitor the possibility of contamination during the drilling process, we carried out several tests in Russia. The drilling barrel was seeded with a pure culture of *Serratia marcescens* for 2 h before drilling. In a separate test, drilled frozen core segments were seeded with a pure culture of *S. marcescens* for several hours to several months at −10°C. In tests using the isolation techniques described below, *S. marcescens* was found only on the surface of the frozen sample, never inside the frozen cores. In Tallahassee, permafrost samples were maintained at −23°C in the Antarctic Core Facility at Florida State University.

Bacterial Isolation

For preparation of enrichment cultures in the field, a plexiglas glove box was set up in a tent on site, and the inside of the box

wiped with 70% alcohol between handling of samples. Each freshly obtained permafrost core was placed in a sterile Whirl-pak (Nasco Inc., Santa Fe Springs, CA, USA) plastic bag, split into two with a hammer on a steel anvil, and aseptically opened in the sterilized glove box. Samples were taken aseptically from the center of the freshly opened, fractures permafrost surface and spread on PYGV (peptone, yeast extract, glucose, vitamin) agar plates [47]. The plates were sealed and kept below 0°C until opened in Tallahassee for isolation of strains from the colonies that developed. PYGV, a medium with low nutrient concentration, has been used to isolate bacteria from similar environments, e.g., Antarctic soils and rocks [43].

For isolation in the laboratory, frozen core samples were placed in a sterile hood and split into two pieces with a sterile chisel and hammer. Samples from the center of the freshly opened, fractured permafrost surface were taken aseptically for bacterial isolation by the spread plate technique on both Luria agar (solid version of Luria broth) and PYGV agar plates. Distinct colony types on the spread plates were purified by streaking and restreaking on fresh Luria agar plates. Purified isolates were grown in Luria broth, concentrated by centrifugation, frozen, and stored at −70°C in fresh medium that contained 7% (v/v) sterile dimethyl sulfoxide (DMSO). All the strains beginning with S were isolated and kindly provided by Dr. R. Ocampo-Friedmann, Florida A & M University, Tallahassee, Florida. All strains are kept both in the laboratory of RHR and in the Culture Collection of Microorganisms from Extreme Environments (CCMEE), Department of Biological Science, Florida State University, Tallahassee, Florida 32306-2043.

Phenotypic Characterization of the Isolates

The morphological characteristics of the isolates were examined by phase-contrast light microscopy of wet mounts prepared from 24-h broth cultures. Growth was assayed by incubation of the isolates in Luria broth at 30°C in a waterbath-shaker at 80 rpm for 24–120 h. Gram stain and endospore formation were assayed according to the methods described by Claus [11].

The growth rates of isolates at different temperatures were determined from the changes of turbidities during incubation (Fig. 2). The cultures were incubated in side-arm flasks containing Luria broth at different temperatures, in a waterbath-shaker at 80 rpm. The turbidities of the cultures were measured spectrophotometrically at 620 nm.

16S Ribosomal DNA Sequencing

Genomic DNA was isolated by a standard chloroform–isoamyl alcohol procedure [26]. The DNA amplification by polymerase chain reaction was performed in a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA) with 20 ng of DNA as a template [40]. Two universal primers used in amplification had the sequences 5′-AGAGTTTGATCATGGCTC-3′ (primer F_2C) and 5′-ACGGGCGGTGTGTAC-3′ (primer C), and correspond to positions 8–25 and 1392–1406, respectively, in the 16S rDNA sequence of *Escherichia coli* [9].

A 1,400-bp DNA fragment of the 16S rRNA gene was amplified, and about 900 bases of the amplified fragment, starting at position 522 and ending at position 1,406, were sequenced by the Taq Dye Deoxy terminator cycle sequencing method [2, 34] (Applied Biosystems, Foster City, Calif.), with an Applied Biosystems Model 373A DNA sequencer in the Florida State University Sequencing Facility. Several primers, including 5′-CAGCCGCGGTAATAC-3′ (primer A-C), 5′-AACAGGATTAGATACCCTGG-3′ (primer G-C), and 5′-TGGCTGTCGTCAGCTCGTGT-3′ (primer H-C), corresponding to positions 522–536, 781–800, and 1,056–1,075, respectively, in the 16S rDNA sequence of *Escherichia coli* [9], were used in the sequencing reactions. The GenBank accession numbers for the assembled 16S rDNA sequences are listed in Table 2.

Maximum Parsimony Analysis

The 16S rDNA sequences (about 900 bases) from each of the 29 isolates were manually aligned with the corresponding sequences of eubacteria taken from the Ribosomal Database Project (RDP) [33]. The aligned sequences were analyzed by maximum parsimony with the PAUP 3.1 (Phylogenetic Analysis Using Parsimony, Version 3.1) software package [45]. Initially, a tree containing all 29 Siberian isolates and 15 eubacteria was generated by a heuristic search, using only the phylogenetically informative sites and the alignment gaps. This tree (Fig. 3) is the only one without bootstrap analysis. This is because the analysis would have taken a prohibitive amount of computer time due to the large number of taxa. This tree indicated that the Siberian isolates fell into four major groups. Its reliability was checked by generation of other trees containing fewer taxa, (Figs. 4–7) obtained by maximum parsimony (PAUP) with bootstrapping [13]. We analyzed relationships within each of the four major groups using sequences from all the Siberian isolates from within each group, along with a set of eubacterial sequences from the RDP. The resulting bootstrapped consensus trees (Figs. 5–7) were constructed at the greater-than-50% confidence limit, using 500 replications.

Results

Some Phenotypic Characteristics of the Isolates

Using the isolation methods described above, only bacteria were recovered from the samples. Visible colonies usually appeared 12–48 h after plating. Colony diversity was low, usually with less than three distinguishable colony types per plate. As viable cell numbers were systematically counted by Russian scientists [17, 18, 20, 31, 51, 52], and data from a single drilling core (like the one we studied) are uncharacteristic, we did not perform systematic counting. Instead, we attempted to isolate a range of different colony types.

Of the 29 isolates we studied, 23 were rod-shaped (79.3%), 5 were cocci (17.2%), and 1 was a nonbranching filament (3.5%) (Table 2). This result is consistent with previous reports by Russian scientists that corynebacteria are

Table 2. Phenotypic characteristics of bacteria from Siberian permafrost

CCMEE strain no.	GenBank accession no.	Well no.	Depth (m)	Age of original sample (y)	Soil horizon[a]	Cell shape	Affiliation[b]	Gram stain	Spore	Growth at 30°C[c]
(715)RS5	U31488	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	−	+	+++
(716)RS10	U31472	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	−	−	++
(717)RS13	U31473	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	−	+	+++
(718)RS15	U31474	1/89-34	2.2	$5–8 \times 10^3$	A	Coccus	β-pr	−	−	+++
(719)RS16	U31475	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	+	+	++
(720)RS19	U31476	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	−	+	+++
(721)RS20	U31480	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	+	+	+
(722)RS21	U31481	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	+	+	++
(723)RS21A	U31482	1/89-34	2.2	$5–8 \times 10^3$	A	Rod	LGC	+	+	++
(696)S2	U31495	6/90	4.7	1.8×10^6	O	Coccus	β-pr	−	−	++
(724)RS28	U31485	3/89	6.1	$3–4 \times 10^4$	Y	Rod	HGC	−	−	0
(697)S7	U31484	6/90	8.5	1.8×10^6	O	Short rod	HGC	+	−	+++
(708)S36	U31499	6/90	9.6	$1.8–3.0 \times 10^6$	O	Rod	HGC	+	−	+
(709)S38	U31500	6/90	9.6	$1.8–3.0 \times 10^6$	O	Rod	β-pr	−	−	++
(710)RS1W	U31477	9/89	13.7	$1.8–3.0 \times 10^6$	O	Short rod	HGC	+	−	+
(711)RS1Y	U31478	9/89	13.7	$1.8–3.0 \times 10^6$	O	Filamentous	β-pr	−	−	+
(712)RS2	U31479	9/89	13.7	$1.8–3.0 \times 10^6$	O	Rod	β-pr	−	−	+
(712)RS3	U31486	9/89	13.7	$1.8–3.0 \times 10^6$	O	Rod	LGC	+	+	+++
(714)RS4	U31487	9/89	13.7	$1.8–3.0 \times 10^6$	O	Rod or coccus	HGC	+	−	0
(698)S10	U31489	6/90	14.2	$1.8–3.0 \times 10^6$	O	Coccus	HGC	−	−	+
(699)S11	U31490	6/90	14.2	$1.8–3.0 \times 10^6$	O	Rod	γ-pr	−	−	++
(700)S12	U31491	6/90	14.2	$1.8–3.0 \times 10^6$	O	Rod	LGC	+	+	+++
(701)S13	U31492	6/90	14.2	$1.8–3.0 \times 10^6$	O	Coccus	γ-pr	−	−	++
(702)S13A	U31493	6/90	14.2	$1.8–3.0 \times 10^6$	O	Rod	HGC	+	−	+++
(703)S14	U31494	6/90	14.2	$1.8–3.0 \times 10^6$	O	Short rod	β-pr	−	−	+++
(704)S23	U31496	6/90	28.6	3.0×10^6	O	Rod	HGC	+	−	+++
(705)S25	U31483	6/90	28.6	3.0×10^6	O	Short rod	HGC	+	−	+
(706)S26	U31497	6/90	32.0	3.0×10^6	O	Short rod	γ-pr	−	−	+++
(707)S26R	U31498	6/90	32.0	3.0×10^6	O	Short rod	γ-pr	−	−	+++

[a] O, Olyor; A, Alas; Y, Yedoma
[b] HGC, high-GC Gram-positive; LGC, low-GC Gram-positive; β-pr, β-proteobacteria; γ-pr, γ-proteobacteria
[c] +++, Growth to full density in ~24 h; ++, in ~72 h; +, in ~168 h; 0, no growth

dominant in Siberian permafrost [18, 20], and is similar to that for the deep subsurface [3].

Among the 29 isolates, 16 (55%) were Gram negative and 13 (45%) Gram positive. These results are significantly different from those for deep subsurface isolates, of which 86% were Gram negative [3]. Although the majority of the isolates (70%) were not observed to form endospores (a similar result was reported for Antarctic soil [24]), the percentage of endospore formers (30%) was much higher than that usually found in temperate soils (about 1%) [23] and in the deep subsurface (<0.1%) [3]. Most of the endospore formers came from samples near the surface layer of permafrost (2.2 m), which is relatively younger (5,000–8,000 years) (Table 2).

Both RS4 and RS28 had an optimum growth temperature around 20°C, but did not grow at 30°C (Fig. 2). This result is consistent with the report by Gounot [22], who isolated *Arthrobacter* species from caves (stable temperature about 10°C) that grew slowly at 4°C, and grew appreciably at 10°C, but not at 37°C. The temperature-growth pattern of the type strain of *Arthrobacter globiformis* was used for comparison, because RS4 and RS28 were clustered with *A. globiformis* (among the high-GC Gram-positive bacteria) by the 16S rDNA sequencing analysis (Fig. 3). RS4 and RS28 grew much more slowly than *A. globiformis*.

Phylogenetic Relationships among the Siberian Isolates and Eubacterial Species

We analyzed relationships among the Siberian isolates and known species of eubacteria by maximum parsimony with

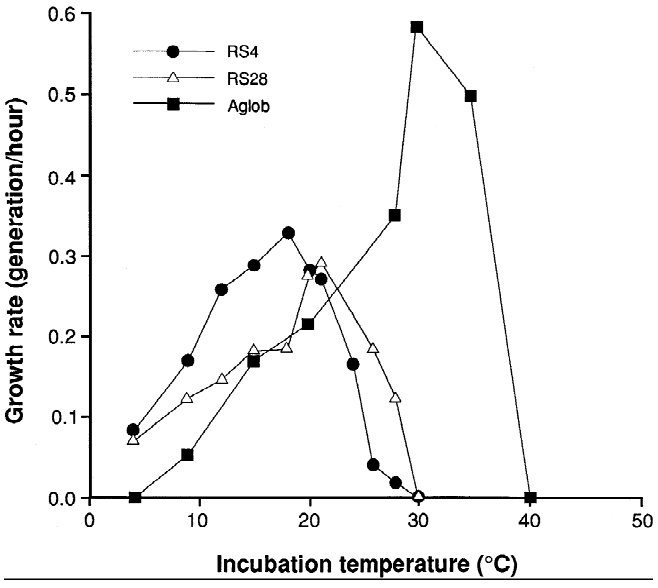

Fig. 2. Temperature growth patterns for RS4 and RS28. Aglob, *Arthrobacter globiformis.*

bootstrapping, using the PAUP software package [45]. The phylogenetic tree in Fig. 3 was one of 11 trees of shortest overall branch length generated by a heuristic search using PAUP with default settings. This tree is shown to establish the relationships among all 29 Siberian isolates and 15 known eubacterial species. The overall topologies of all 11 trees were very similar, and all defined the same groups for the Siberian isolates. A consensus bootstrapped tree with a reduced set of taxa (12 of the 29 Siberian isolates) is shown in Fig. 4. The two maximum parsimony trees, with and without bootstrapping, infer the same overall relationships among the Siberian isolates. These trees placed the Siberian isolates in four major groups: high-GC Gram-positive bacteria, β-proteobacteria, γ-proteobacteria, and low-GC Gram-positive bacteria. A rough correlation between phylogenetic affiliation and permafrost strata is indicated by the data shown in Table 2, but the limited number of isolates, and the fact that all isolates were from a single core hole, do not permit valid statistical comparisons. However, Russian scientists made some comparisons on the basis of their data and found that the younger strata generally contain more diverse bacteria than do older ones [52].

Nine Siberian isolates were assigned to the high-GC Gram-positive group (Fig. 5). All except RS28 came from samples of the lower Olyor suite, and all except S13A fell into a large group with *A. globiformis* (79% confidence limit). These isolates were closely related to *A. globiformis* (90–98% sequence identities). Six of them formed three closely related pairs (S10 and RS28, S23 and S36, RS4 and RS1W). S13A was closely related to *Rhodococcus fascians.*

Six Siberian isolates were assigned to the β-proteobacteria. All except RS15 came from samples of the lower Olyor suite. Four of them (S2, S14, RS15, and S38) formed a clade that, in comparison with current RDP was most closely related to *Alcaligenes faecalis* (Fig. 6). The other two, RS2 and RS1Y from samples of 13.7 m depth, were closely related to each other and did not seem to be closely related to any characterized species included in the β-proteobacteria.

Four Siberian isolates were assigned to the γ-proteobacteria, all of them from samples of the lower Olyor suite, and they formed tight clusters closely related to members of the Enterobacteriaceae (92% confidence limit) (Fig. 7). S26 and S26R were very closely related to *Serratia marcescens,* and S11 was most closely related to *E. coli* (98% sequence identity).

Ten Siberian isolates were assigned to the low-GC Gram-positive bacteria, and all except S12 and RS3 came from samples of the Alas suite. All but one (RS10) could form endospores. All the Siberian isolates, except RS3, formed a large group (56% confidence limit) related to *Bacillus subtilis* and other *Bacillus* spp. RS16 was closely related to *B. psychrophilus* (86% confidence limit). The detailed relationships among the strains within this group could not be resolved (tree not shown).

Discussion

This study has demonstrated the presence of viable bacteria in the Siberian permafrost, and thus confirmed the work of Russian scientists [17, 18, 20, 31, 50, 51]. Although phylogenetic analysis of bacterial isolates from Siberia indicated that they were diverse, many types of bacteria such as *Pseudomonas, Flavobacterium, Nitrobacter, Clostridium, Micrococcus, Mycobacteria,* and actinomycetes, present in the arctic soils of the northern USSR [35], were not isolated from our permafrost samples. Nutrient concentration and availability, an open ecosystem, and the influence of human activity are the major factors contributing to the abundance and diversity of the microbiota in arctic soils. In contrast, permafrost conditions (extreme low temperature, low nutrient concentration, and a generally reduced environment) might have imposed a strong selective pressure on the microbes preserved there, and thus have led to the elimination or reduction of certain types of bacteria and to the decrease of microbial diversity. Presumably, only bacteria able to maintain a certain level of metabolism at −10°C have survived.

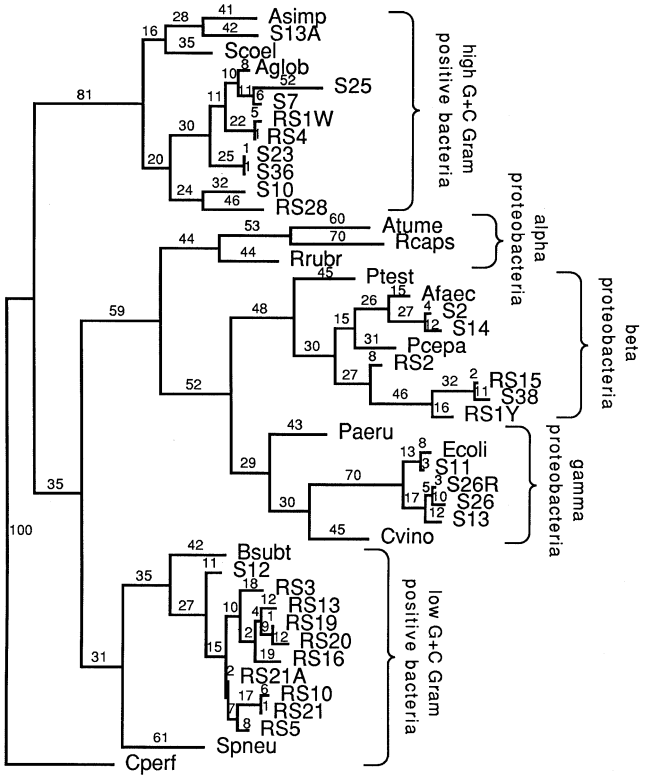

Fig. 3. Phylogenetic tree of 29 Siberian isolates and 15 species of eubacteria [33] generated by a heuristic search using PAUP 3.1. PAUP 3.1 was used to analyze approximately 1,011 characters of aligned nucleotide sequences for a total of 44 taxa. Eleven trees were retained in a heuristic search with a minimum length of 2,108 steps, and 454 informative sites were used in the heuristic search. One of the 11 trees is shown here. The number above each horizontal line corresponds to the branch length. Abbreviations for eubacterial species: Aacti, *Actinobacillus actinomycetemcomitans;* Acaps, *Actinobacillus capsulatus;* Align, *Actinobacillus lignieresii;* Ahydr, *Aeromanas hydrophila;* Aeryt, *Aeromicrobium erythreum;* Atume, *Agrobacterium tumfaciens;* Afaec, *Alcaligenes faecalis;* Axylo, *Alcaligenes xylosoxidans;* Anaso, *Arsenophonus nasoniae;* Aglob, *Arthrobacter globiformis;* Asimp, *Arthrobacter simplex;* Bsubt, *Bacillus subtilis;* Bbifi, *Bifidobacterium bifidum;* Bbron, *Bordetella bronchiseptica;* Bcary, *Burkholderia caryophylli;* Cvino, *Chromatium vinosum;* Cfreu, *Citrobacter freundii;* Cperf, *Clostridium perfringens;* Cvari, *Corynebacterium variabilis;* Ecorr, *Eikenella corrodens;* Ecaro, *Erwinia carotovora;* Eherb, *Erwinia herbicola;* Ecoli, *Escherichia coli;* Halve, *Hafnia alvei;* Mlute, *Micrococcus luteus;* Mglyc, *Methylobacillus glycogenes;* Mbovi, *Mycobacterium bovis;* Nmult, *Nitrosolobus multiformis;* Neuro, *Nitrosomonas europaea;* Ntenu, *Nitrosovibrio tenuis;* Pvola, *Pasteurella volantium;* Pvulg, *Proteus vulgaris;* Paeru, *Pseudomonas aeruginosa;* Pcepa, *Pseudomanas cepacia;* Ptest, *Pseudomonas testosteroni;* Rsalm, *Renibacterium salmoninarum;* Rcaps, *Rhodobacter capsulatus;* Rfasc, *Rhodococcus fascians;* Rpurp, *Rhodocyclus purpureus;* Rrubr, *Rhodospirillum rubrum;* Rdent, *Rothia dentocariosa;* Rgela, *Rubrivivax gelatinosus;* Smarc, *Serratia marcescens;* Spneu, *Streptococcus pneumoniae;* Scoel, *Streptomyces coelicolor;* Ttume, *Terrabacter tumescens;* Vharv, *Vibrio harveyi;* Yente, *Yersinia enterocolitica;* Ypest, *Yersinia pestis;* Zrami, *Zoogloea ramigera.*

Results from this study (the prevalence of non–endospore formers in the permafrost, and the fact that visible colonies were usually seen within 12–48 h after plating) and recent work by Gilichinsky et al., which demonstrated the slow growth of microbes from Siberian permafrost at −8°C to −10°C over 6–18 months [21], suggest that these microorganisms are in a metabolically active state. Active metabolism, although at a very low level, would allow the organisms to perform certain basic functions necessary for survival, including repair of DNA damage caused by radiation and maintenance of membrane integrity [15]. Electron microscopic observations by Soina and Vorobyova [44] showed that cell structures of both Gram-positive and Gram-negative bacteria in permafrost remained undamaged in situ.

Although the stable low temperature in permafrost is likely to be the strongest selection factor, no true psychro-

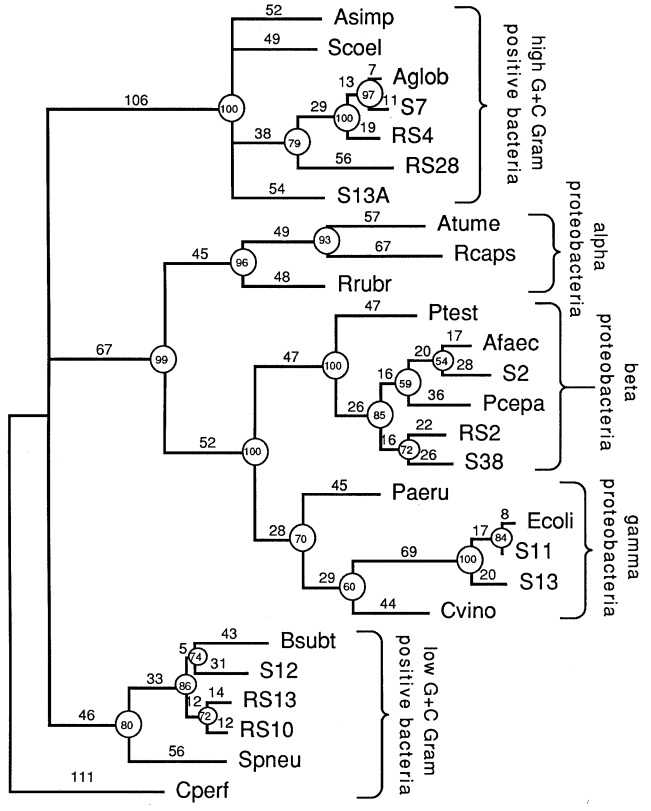

Fig. 4. Bootstrap consensus tree for 12 Siberian isolates and 15 species of eubacteria [33] generated by bootstrapping using PAUP 3.1. PAUP 3.1 was used to analyze approximately 1,011 characters of aligned nucleotide sequences for the 27 stains. The bootstrap consensus tree shown here was generated from 397 informative sites at the greater than 50% confidence limit, with 500 replications. The number above each horizontal line corresponds to the branch length, and the numbers in circles are the confidence limits of branch points. Abbreviations for eubacterial species are as in Fig. 3.

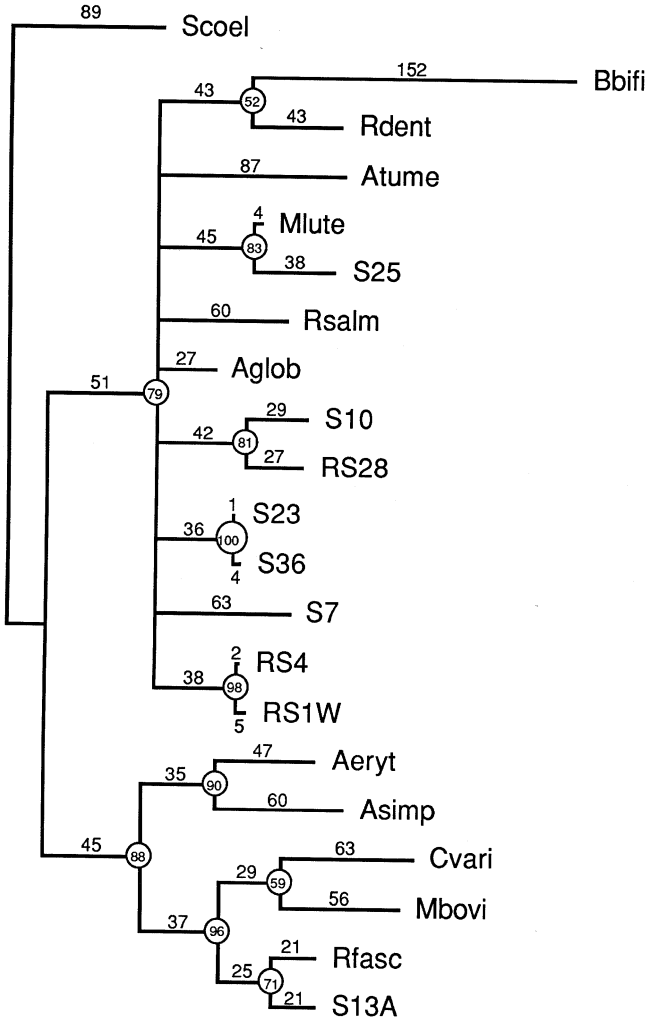

Fig. 5. Bootstrap consensus tree for nine Siberian isolates and 12 species of high-GC Gram-positive bacteria [33] generated by bootstrapping using PAUP 3.1. The PAUP 3.1 was used to analyze approximately 1,598 characters of aligned nucleotide sequences for the 21 strains. The bootstrap consensus tree shown here was generated from 1,032 informative sites at the greater than 50% confidence limit, with 500 replications. The number above each horizontal line corresponds to the branch length, and the numbers in circles are the confidence limits of branch points. Abbreviations for eubacterial species are as in Fig. 3.

philes were found in our study. It is a common and significant phenomenon that psychrophiles are absent or very rare in terrestrial habitats, even in Antarctic rocks and soils [27, 36, 49]. The majority of true psychrophiles has been isolated from marine environments. Compared with permafrost, marine environments provide bacteria with stable, yet higher, temperature and an easy access to water and nutrients. Bacteria in such an environment have a higher metabolic activity and division rate, and thus may have evolved more rapidly. In contrast, because of their very low metabolic activity and division rate, microorganisms in permafrost do not seem to have evolved significantly during the past several million years to adapt to their environment. Therefore, the permafrost microbiota is affected more by selection than by evolution.

All the isolates in this study grew well at atmospheric oxygen concentration, despite the fact that the Siberian permafrost is a nearly closed and generally reduced habitat. This result suggests that many of the microbes in the permafrost

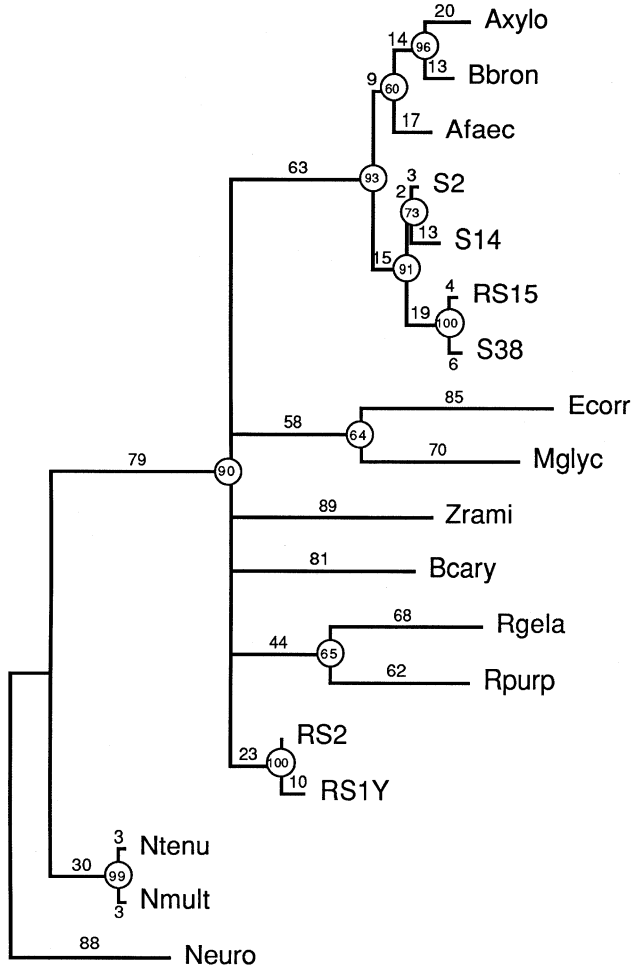

Fig. 6. Bootstrap consensus tree for 6 Siberian isolates and 11 species of β-proteobacteria [33] generated by bootstrapping using PAUP 3.1. PAUP 3.1 was used to analyze approximately 1,562 characters of aligned nucleotide sequences for the 17 strains. The bootstrap consensus tree shown here was generated from 1,227 informative sites at the greater than 50% confidence limit, with 500 replications. The number above each horizontal line corresponds to the branch length, and the numbers in circles are the confidence limits of branch points. Abbreviations for eubacterial species are as in Fig. 3.

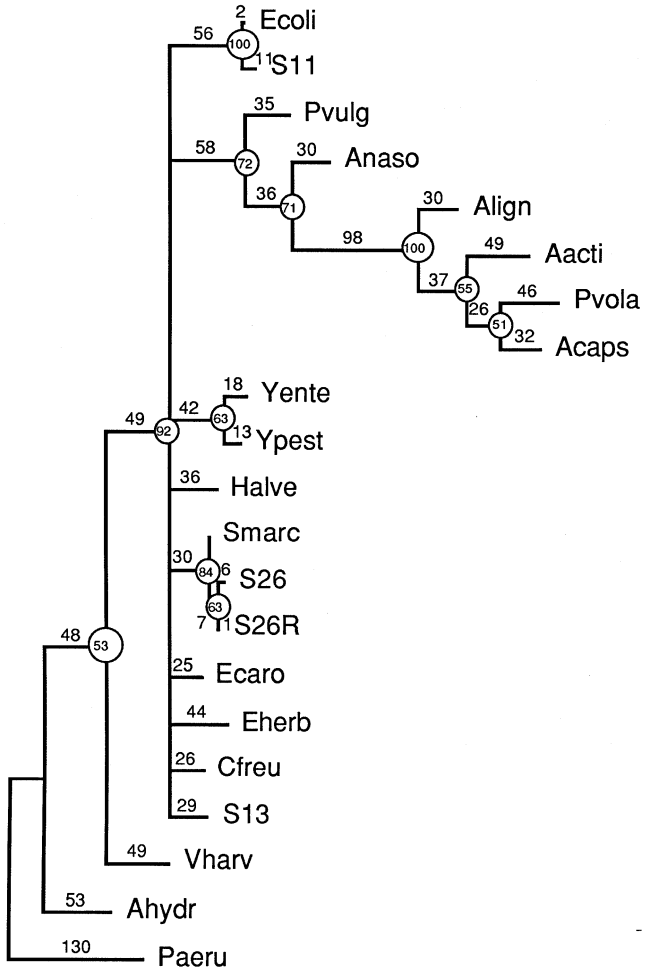

Fig. 7. Bootstrap consensus tree for 4 Siberian isolates and 17 species of γ-proteobacteria [33] generated by bootstrapping using PAUP 3.1. The PAUP 3.1 was used to analyze approximately 1,576 characters of aligned nucleotide sequences for the 21 strains. The bootstrap consensus tree shown here was generated from 817 informative sites at the greater than 50% confidence limit, with 500 replications. The number above each horizontal line corresponds to the branch length, and the numbers in circles are the confidence limits of branch points. Abbreviations for eubacterial species are as in Fig. 3.

were aerobic, and probably facultatively anaerobic, at the time they were frozen. Because of the very low metabolic activity, and, thus, low demand on oxygen and the alternative availability of microenvironments with different levels of oxygen supply, the generally reduced environment may not be an important selection factor. Russian scientists did isolate a few strict anaerobes, such as sulfate-reducing bacteria [46], which indicated the existence of strictly anaerobic microenvironments within the generally reduced environments in permafrost.

The discovery of the four isolates related to the Enterobacteriaceae was unexpected, and their origin is unknown. Although contamination cannot be ruled out, it is unlikely because the isolates originate from two different samples. The four isolates were from the lower Olyor suite, of which the climatic history is largely unknown. It is therefore possible that these organisms are coeval with the permafrost sediment or that they were trapped from the underlying soils during a period of thawing. Geological evidence indicates, however, that they have been frozen for at least 40,000–50,000 years. Even if the Enterobacteriaceae in Siberian permafrost are only 40,000–50,000 years old, their survival contradicts the report that coliform bacteria die very rapidly in arctic soil [14].

Acknowledgments

This work was supported by DOE grant DE-FG05-91ER61159 to RHR, NASA grants NAGW 1871 and NAGW 4044 to EIF, and a grant from the Russian Ministry of Science and Technology entitled "Viable Microorganisms in Permafrost" to DAG. We thank Ms. Jane Reeves for her technical help; Ms. Ellyn Whitehouse for her help in sequencing DNA fragments; Dr. Christopher C. McKay (NASA-Ames Research Center, Moffett Field, Calif.), Dr. Christopher F. Chyba (National Security Council, Washington, D.C.), and Dr. Michael A. Meyer (NASA, Washington, D.C.) for their kind permission to use the unpublished permafrost temperature data in Fig. 1; and Dr. Anne B. Thistle for critical editing of the manuscript.

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