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Phylogenetic and Physiological Diversity of Microorganisms Isolated from a Deep Greenland Glacier Ice Core

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We studied a sample from the GISP 2 (Greenland Ice Sheet Project) ice core to determine the diversity and survival of microorganisms trapped in the ice at least 120,000 years ago. Previously, we examined the phylogenetic relationships among 16S ribosomal DNA (rDNA) sequences in a clone library obtained by PCR amplification from genomic DNA extracted from anaerobic enrichments. Here we report the isolation of nearly 800 aerobic organisms that were grouped by morphology and amplified rDNA restriction analysis patterns to select isolates for further study. The phylogenetic analyses of 56 representative rDNA sequences showed that the isolates belonged to four major phylogenetic groups: the high-G+C gram-positives, low-G+C gram-positives, *Proteobacteria*, and the *Cytophaga-Flavobacterium-Bacteroides* group. The most abundant and diverse isolates were within the high-G+C gram-positive cluster that had not been represented in the clone library. The Jukes-Cantor evolutionary distance matrix results suggested that at least 7 isolates represent new species within characterized genera and that 49 are different strains of known species. The isolates were further categorized based on the isolation conditions, temperature range for growth, enzyme activity, antibiotic resistance, presence of plasmids, and strain-specific genomic variations. A significant observation with implications for the development of novel and more effective cultivation methods was that preliminary incubation in anaerobic and aerobic liquid prior to plating on agar media greatly increased the recovery of CFU from the ice core sample.

Interest in the survival of microorganisms in cold environments is increasing, driven in part by the desire to ascertain whether life can exist elsewhere in our solar system. The evidence for ice on Mars and Europa has made the isolation and study of psychrophiles particularly important for determining the types of organisms that can survive in frozen environments and for developing improved methods for their cultivation. In this regard, glacier ice sheets represent possible analogs of extraterrestrial cold habitats. They are also important as long-term, chronological repositories of microorganisms. Despite their relevance, studies of diversity, viability, and physiology of the organisms in glacial ice are just beginning.

In initial studies, Abyzov et al. (3–5) found evidence of diverse microorganisms in the deep glacier above Lake Vostok in Antarctica. The microbial viability was later confirmed by using ^{14}C-labeled organic compounds, and some microorganisms were successfully cultured (2). Skidmore et al. (36) found aerobic chemoheterotrophs, anaerobic nitrate- and sulfate-reducing bacteria, and methanogens in the debris-rich basal ice layers and the surface of a high Arctic glacier, and Lanoil et al. (B. D. Lanoil, M. Sharp, S. P. Anderson, M. T. La Duc, J. Foght, and K. H. Nealson, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. N-208, p. 525, 2001) examined the microbial populations at the bases of two Canadian glaciers. One of the few studies of Greenland ice cores reported a diverse clone library of eukaryotic 18S ribosomal DNA (rDNA), but the viability of the cells was not tested (48). Christner et al. examined glacier ice cores from different geographic locations and used both the analysis of 16S rDNA directly amplified from the melted ice and the recovery of viable isolates to show the presence of a diverse community that included *Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, and gram-positive bacteria (12–14).

The availability of ice cores from GISP 2 (Greenland Ice Sheet Project) provided a special opportunity to examine the microbial diversity and correlate the findings with detailed geochemical measurements. The coring operation drilled through the 3,053-m-deep ice sheet and 1.5 m into the sediment below, and the chemical composition of the core has been examined at several depths (21). Some quantitative anomalies in the greenhouse gases CO_2, CH_4, and N_2O have been attributed to microbial metabolism (40, 42), but this issue has not been yet experimentally addressed (30). In order to better understand the diversity, survival, and possible activity of microorganisms present in ice, we began a study of a sample of the GISP 2 core taken from the "silty" ice at 3,043 m below the surface and about 10 m above the sediment-ice interface. Microorganisms in this sample could have been deposited in the ice more than 120,000 years ago or could have been in the underlying sediment for millions of years, because it has been suggested that the basal ice in central Greenland originated prior to the growth of the current ice sheet (20). Our objective was to investigate the abundance, viability, and diversity of organisms in this sample and make comparisons with studies of geographically different ice cores. In a previous study (33), we

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obtained anaerobic psychrophilic enrichments from the core sample, extracted total genomic DNA, and constructed a clone library of PCR-amplified bacterial 16S rDNA sequences. The phylogenetic analysis of the clone library demonstrated the presence of rRNA genes from several major phylogenetic groups, including α-, β-, and γ-*Proteobacteria*; *Thermus-Deinococcus*; *Bacteroides*; *Eubacterium*; and *Clostridium*.

Our next challenge was to cultivate organisms in order to demonstrate that viable cells existing in the sample can be recovered, to compare their phylogenetic placements with the groupings obtained with the clone library, and to survey their physiological properties. Cultivating and characterizing isolates were of particular importance for providing a more comprehensive view of diversity, because different results can be obtained with molecular and culture-dependent approaches. For example, gram-positive bacteria are often abundant among isolates, but are not well represented in clone libraries (11, 37, 38, 43). Although diversity studies based on amplification of extracted total DNA overcome the inability to cultivate the majority of microorganisms from environmental samples (47), this approach also has limitations. Thus, it is important to combine direct molecular community analysis with the isolation of individual organisms in order to mitigate the pitfalls of each approach. Furthermore, the isolation of pure cultures is required for describing new taxa, for obtaining complete genome sequences, and for assessing the physiological functions of the organisms and their global ecological roles.

Unfortunately, the recovery of culturable organisms from extreme environments such as permanently frozen glacial ice has been difficult, and the development of new methods and conditions that improve the resuscitation and culturability of environmental microorganisms is important. We therefore expanded our previous study to examine the prokaryotic diversity of organisms recovered from the GISP 2 ice core sample. Here we report on the successful isolation of nearly 800 strains that were grouped into categories based on morphological properties and amplified rDNA restriction analysis (ARDRA) patterns and characterized phylogenetically and physiologically.

MATERIALS AND METHODS

Ice core site and sampling. Samples were aseptically removed from a section of the GISP 2 drilled from a depth between 3,042.67 and 3,042.80 m (33). Briefly, the outside ice layer of the core was removed from one end in a laminar flow hood using a platinum wire heated by the current from a 6-V battery. The exposed layer was then treated with ethanol, and a new layer was removed. Samples were then drilled from the ice core by using sterile keyhole bits (20-ml volume each) attached to a sterile drill bit extender and allowed to thaw overnight at 5°C.

Microscopic examination. Microscopic characterization of the melted ice sample was performed by conventional light microscopy. The relative cell sizes and the number of live and dead cells were estimated by staining with propidium iodide and SYTO9 according to the instructions of the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, Oreg.) and examination under an Olympus BX-60 wide-field microscope with a ×40 objective. The same samples were subjected to flow cytometry (XL-MCL; Beckman-Coulter, Miami Lakes, Fla.) in order to enumerate the total number of cells. Counted fluorescent beads were added to the sample in order to estimate the exact volume measured, and 10,000 non-bead events were counted.

Media and cultivation. All new aerobic and anaerobic cultures in different liquid media were started from an aseptically taken subcore and incubated at −2°C with shaking at 150 rpm for 1 year. The aerobic cultures were started by inoculating 0.25 or 0.05 ml of melted ice into 50 ml of R2A broth (full or 1/4-strength R2B [1/4 R2B]), Luria broth (LB), or tryptic soy broth without carbohydrate (TSB). New anaerobic cultures were prepared in the two basal media MM1 and MM2 used in a prior study (33). MM1 contained the following (per liter [wt/vol]): 0.5 g of KH_2PO_4, 0.4 g of NaCl, 0.16 g of NH_4Cl, 0.05 g of $MgCl_2 \cdot 6H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 7.5 g of $NaHCO_3$, 0.01 mg of resazurin, and 1% (vol/vol) each vitamin and trace elements solutions as described previously (41). Na_2S was used as a reducing agent at a 0.075% final concentration, the pH was adjusted to 7, and either Na-acetate or Na-formate was added as a carbon source to a 0.1 M final concentration. Medium MM2 was designed to enrich for fermentative bacteria (MM2.6.1), sulfate reducers (MM2.6.2), and methanogens (MM2.6.3) (18). The anaerobically prepared media in 125-ml bottles were overgassed with N_2-CO_2 (4:1) or H_2-CO_2 (4:1) and inoculated with syringes using 0.25 or 0.05 ml of melted ice per 50 ml. Medium MM1 supplemented with acetate or formate was inoculated and incubated aerobically as well.

Colony isolation. Samples (0.1 ml and 0.25 ml) of either the melted ice or the anaerobic and aerobic cultures were plated periodically onto the following agar media: R2A (32), 1/4 R2A, Trypticase soy agar without carbohydrate (TSA), LB agar, or MM1 agar supplemented with formate or acetate. These plates were incubated aerobically at 5, 10, and 18°C for up to 9 months. Colonies from each morphotype were restreaked several times and checked for purity microscopically.

Characterization of isolates. The temperature growth range of the isolates was tested on TSA or R2A agar media at 2, 10, 18, 25, 29, 33, and 37°C. Broth cultures were grown at 18°C and used as inocula for studying the sensitivity to 14 antibiotics with disks containing the following antibiotics (Becton Dickinson, Boston, Mass.): ampicillin (10 μg), penicillin (10 U), bacitracin (10 U), chloramphenicol (10 μg), tetracycline (30 μg), erythromycin (15 μg), kanamycin (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), novobiocin (30 μg), ciprofloxacin (5 μg), rifampin (5 μg), streptomycin (10 μg), and vancomycin (30 μg). Zones of inhibition were measured after 72 h and evaluated according to the manufacturer's instructions. Enzymatic activities for amylases, proteases, β-galactosidases, and lipases were screened on starch agar, brain heart infusion (BHI)-skim milk agar (39), TSA amended with 0.01% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Sigma, St. Louis, Mo.) and 0.1 mM isopropylthio-β-D-galactoside (IPTG) (Fisher, Pittsburgh, Pa.), and lipase plates (23), respectively. DNase activity was tested on DNA-containing test agar with methyl green prepared according to the Difco manual (Difco Laboratories, Detroit, Mich.).

Plasmid DNA extraction and analysis. The isolates were grown at 18°C in TSB or R2B, and 3 ml was used for purification of plasmid DNA with the Wizard SV-plus kit (Promega, Madison, Wis.). For some isolates showing poor lysis, the cell suspension solution underwent 5 min of bead beating with a MiniBeadbeater-8 cell disrupter (Biospec Products, Inc.). Initially, native uncut plasmids were analyzed on 0.8 or 1% agarose gels with plasmids of known size as references. Restriction digestions were further used for size estimation of the small plasmids (<10 kb).

Repetitive PCR genomic fingerprinting. Comparative genomic fingerprinting was performed with a single-primer ERIC (enterobacterial repetitive intragenic consensus) (5′-AAGTAAGTGACTGGGTGAGCG-3′) according to the protocol previously described (25). Electrophoretic separation of the products was in 1% agarose (OmniPur; Merck, Rahway, N.J.) in Tris-acetate buffer (pH 8) at 5 V/cm.

Isolation of genomic DNA and analysis of 16S rRNA gene sequences. Broth cultures in R2B or TSB grown at 18°C were used for isolation of genomic DNA with a PureGene kit (Gentra Systems, Inc., Minneapolis, Minn.). The cells of isolates 5, 18, and 54 were resistant to lysis, and a 5-min bead-beating step in the cell suspension solution using a MiniBeadbeater-8 cell disrupter (Biospec Products, Inc.) was incorporated in order to obtain genomic DNA. The 16S rRNA genes were amplified with 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) bacterial primers or 515F (5′-GTGCAAGCMGCCGCGGTA-3′) and 1492R universal primers using Ready-to-go PCR beads (Amersham Biosciences, Piscataway, N.J.). The thermal PCR profile was as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 52°C for 1 min, and elongation at 72°C for 1.5 min. The final elongation step was 7 min at 72°C. The 16S rDNA products were analyzed by electrophoresis on 1% agarose gels. ARDRA analysis was performed by treating the 16S rDNA PCR products with one of four base pair-cleaving restriction endonucleases (*Msp*I, *Rsa*I, *Hae*III, or *Alu*I; Promega, Madison, Wis.) at 37°C for 3 to 4 h, and the resulting electrophoretic patterns in 2% agarose gels were used to group the isolates. The 16S rDNA products representing each distinct pattern were further purified and sequenced at the Penn State Nucleic Acid Facility on an ABI 370 sequencer using the 8F, 704F, 907R, or 1492R primer to obtain overlapping sequences.

Phylogenetic analysis. The single-stranded 16S rRNA gene sequences of the Greenland ice core (GIC) isolates were matched with those from both the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/html) and from a BLAST search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The small subunit (SSU) rRNA gene sequences from the 56 GIC isolates were aligned with 69 reference sequences obtained from the RDP and GenBank databases by using the Clustal W program found in the BioEdit platform (version 5.0.6; copyrighted 1997 to 2001 by Tom Hall, Department of Microbiology, North Carolina State University, Raleigh). The BioEdit alignment was used in maximum-likelihood and distance analyses utilizing the PAUP package, version 4.0b10, as described before (33). The tree was rooted by using the sequences of *Taxeobacter gelupurpurascens* and GIC isolate 34.

Nucleotide sequence accession number. GenBank accession numbers for each of the 16S rRNA reference gene sequences are given in Fig. 1. GenBank 16S rRNA gene sequence accession numbers for each of the GIC isolates used in the alignment are given in parentheses after the isolate number: 11 (AY439223), 12 (AY439224), 15 (AY439225), 16 (AY439226), 17 (AY439227), 18 (AY439228), 19 (AY439229), 20 (AY439230), 20or (AY439231), 20y (AY439232), 22 (AY439233), 23 (AY439234), 24or (AY439235), 24y (AY439236), 26 (AY439237), 27or (AY439238), 27y (AY439239), 30 (AY439240), 31 (AY439241), 31w (AY439242), 32 (AY439243), 33 (AY439244), 34 (AY439245), 36 (AY439246), 37 (AY439247), 38 (AY439248), 40y (AY439249), 43 (AY439250), 46 (AY439251), 5 (AY439252), 52 (AY439253), 54 (AY439254), 55 (AY439255), 56 (AY439256), 62 (AY439257), 64 (AY439258), 65 (AY439259), 66 (AY439260), 9 (AY439261), GIC6 (AY439262), R11 (AY439263), R15be (AY439264), R15br (AY439265), R15p (AY439266), R16 (AY439267), R17 (AY439268), R18 (AY439269), R20p (AY439270), R21 (AY439271), R22 (AY439272), R23 (AY439273), R3 (AY439274), R8 (AY439275), T1 (AY439276), T2 (AY439277), and T3 (AY439278).

RESULTS

Isolation of organisms from melted ice and liquid cultures. The ice core sample originated from the "silty" ice of GISP 2, approximately 10 to 11 m above the bedrock. This debris-rich section was chosen because of its unique characteristics: high sediment loads of 0.65% by weight, relatively high gas concentrations with very high concentrations of methane (6,000 ppm by volume [ppmv]) and CO_2 (135,000 ppmv), temperature of −9°C, and ancient origin (21). The initial examination of newly melted ice samples used for the preparation of enrichment cultures by epifluorescent microscopy and flow cytometry indicated the presence of an abundant (6.1×10^7 to 9.1×10^7 cells/ml) and morphologically diverse microbial population in which very small "dwarf" cells (<1 μm) predominated and with a 5:1 ratio of live to dead cells (33).

Our major goal was to maximize the recovery of culturable psychrophilic microorganisms and to compare results from various cultivation approaches. For this purpose, we chose media and incubation conditions relevant to the natural environment based on the assumption that cells in the glacier ice had been subjected to desiccation, low temperature, and limiting nutrient and O_2 concentrations. We also considered that the high levels of CO_2 and methane might be a result of microbial oxidation of organic matter and in situ production, respectively (40). Therefore, for the enrichments and subsequent isolations, we used the common low- and high-nutrient liquid media (R2B, 1/4 R2B, TSB, and LB) for heterotrophic aerobic microorganisms and the minimal medium MM1 and media MM2.6.1, MM2.6.2, and MM2.6.3 designed for the anaerobic growth of metabolically different groups. The anaerobic media contained various substrates and alternative electron acceptors for anaerobic metabolism. Turbidity appeared 20 days after inoculation from a newly melted subsample in the MM1, MM2.6.1, MM2.6.2, and MM2.6.3 anaerobic media incubated at −2°C. Microscopic examination of these cultures revealed morphologically diverse populations. In contrast, the cultures incubated aerobically in the low-nutrient broth R2B and 1/4 R2B became turbid only after 4 and 7 months, respectively, and contained less morphologically diverse cells than those incubated anaerobically. No turbidity was observed in aerobic TSB and LB cultures even after 9 months.

A total of 793 colonies representing diverse morphologies were isolated on aerobically incubated agar media directly from the melted ice and from the anaerobic and aerobic cultures (Table 1 and Fig. 2). Different colony recovery efficiencies were observed after plating onto nutrient-limited or nutrient-rich agar media. Most colonies were recovered on the oligotrophic medium R2A and 1/4 R2A, and a few were recovered on TSA. In contrast, no growth was observed on agar medium MM1 supplemented with acetate or formate or LB agar even after 7 months. We also found that colonies initially isolated from the liquid cultures needed 20 to 60 days before becoming visible; however, the same colonies grew within 1 to 3 days upon subsequent restreaking on agar media.

Phylogenetic analysis of 16S rDNA sequences of isolates. All 793 initially recovered colonies were placed into over 70 groups based on colony and cell morphology and pigmentation, growth patterns, etc., and the chromosomal DNA was extracted from all isolates listed in Table 1 representing each group. The 16S rRNA genes were then amplified with bacterial primers, and the products were purified. In a few cases in which no 16S rDNA product was obtained with bacterial primers, amplification was attempted by using universal primers (515F-1492R). In each case in which amplification products were found with these primers, suggesting that these were possibly eukaryotes, the isolates were excluded from the group of bacterial isolates and were not studied further. In order to avoid sequencing several identical 16S rDNA samples, the PCR products were digested with restriction endonucleases and grouped into different ARDRA patterns (data not shown). The nucleotide sequences for 56 rDNA products representing distinct ARDRA patterns were determined. To increase the coverage of different isolates, a larger number of partial sequences (500 to 1,390 bp) were analyzed instead of a smaller number of full-length sequences. For the majority of isolates, the closest relatives from both databases (RDP II and NCBI) were the same with reliably high similarity values. In cases in which we found low similarity values between the 16S rDNA of the isolate and that of the most closely related organism, a larger portion of the gene was sequenced.

The results of the phylogenetic analysis of the 56 rDNA sequences (Fig. 1) showed that the GIC isolates represented a wide diversity of both gram-positive and gram-negative heterotrophic bacteria belonging to four major phylogenetic groups: the high-G+C gram-positives, low-G+C gram-positives, *Proteobacteria*, and the CFB group. Isolates related to high-G+C gram-positives formed the largest cluster in terms of diversity and high abundance, with 29 phylogenetically distinct organisms. Members of three main subclusters could be distinguished: first, the *Arthrobacter-Micrococcus* lineage; second, several genera of the *Microbacteriaceae*; and third, the *Actinomycetes* group. The isolates belonging to the low-G+C gram-positive bacteria were less diverse and were affiliated with the

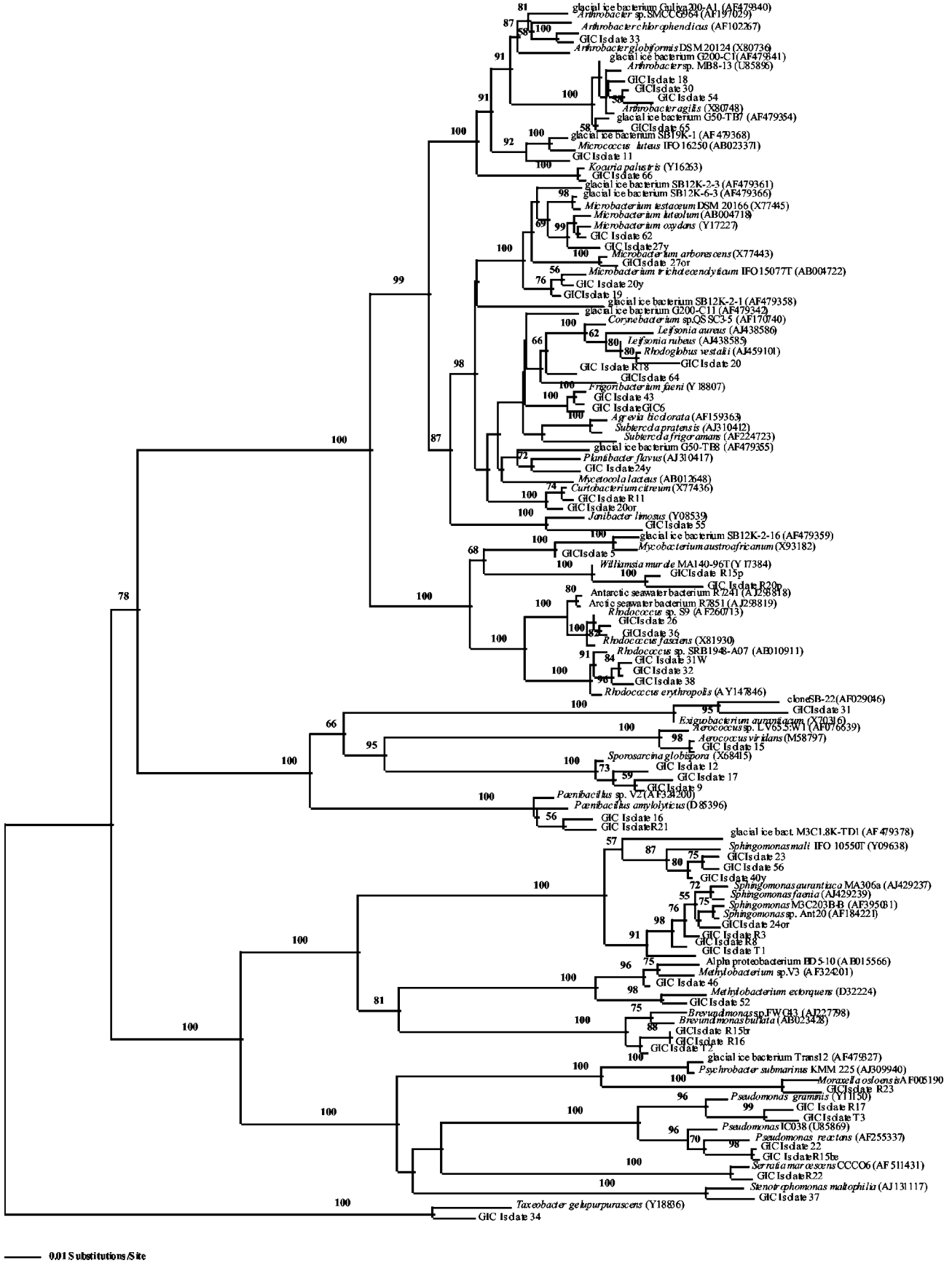

FIG. 1. Phylogenetic relationships of the 56 rRNA gene sequences obtained from the isolates and 69 closely related sequences, based on a distance analysis (neighbor-joining algorithm with Jukes-Cantor model). Bootstrap values generated from 1,000 replicates are shown at the nodes. Accession numbers are provided in Materials and Methods.

TABLE 1. Characteristics of selected GIC isolates

Isolate	Origin or enrichment	Growth temp (°C)	Colony description	Closest relative species in the 16S rDNA sequence database
1y	Direct plating	2–18	Yellow, slimy	*Paenibacillus* sp. strain V2Ant
5	Direct plating	10–18	Orange, small	*Mycobacterium austroafricanum*
55	Direct plating	2–29	White, small	*Janibacter limosus*
56	Direct plating	2–37	Yellow, small	*Sphingomonas mali*
64	Direct plating	2–29	Orange-red, slimy	*Frigoribacterium faeni*
65	Direct plating	2–29	Red, large	*Arthrobacter* sp. strain MB 8–13, Ant
66	Direct plating	18–37	Yellow, medium	*Kocuria palustris (Micrococcaceae)*
19	MM1	7–18	Yellow, small	*Microbacterium trichothecenolyticum*
20or	MM1	2–18	Orange, medium	*Curtobacterium citreum*
20y	MM1	2–18	Yellow, small	*Microbacterium trichothecenolyticum*
12	MM1 + acetate	2–18	Beige, medium	*Sporosarcina globispora*
16	MM1 + acetate	2–33	Whitish, gummy	*Paenibacillus amylolyticus*
18	MM1 + acetate	2–33	Red-pink, medium	*Arthrobacter agilis*
24or	MM1 + acetate	2–25	Dark orange, medium	*Pseudomonas* sp. strain Ant20
24y	MM1 + acetate	2–25	Yellow, small	*Planibacter flavus*
25	MM1 + acetate	2–25	Dark orange, medium	*Pseudomonas* sp. strain Ant20
30	MM1 + acetate	2–25	Red-pink, small	*Arthrobacter agilis*
T1	MM1 + acetate	2–25	Pale yellow, small	*Sphingomonas* sp. strain M3C202B Ant
T2	MM1 + acetate	2–33	Pale yellow, slimy	*Brevundimonas* sp. strain FWC43
T3	MM1 + acetate	2–33	Pale yellow, transparent	*Pseudomonas graminis*
T4	MM1 + acetate	2–33	Pale yellow, transparent	*Pseudomonas graminis*
R3	MM1 + acetate	2–25	Light yellow, medium	*Pseudomonas* sp. strain Ant 20
22	MM1 + formate	2–25	Yellowish, large, jelly	*Pseudomonas* sp. strain ICO38 Ant
23	MM1 + formate	2–37	Yellow, small	*Sphingomonas mali*
36	MM1 + formate	2–37	Orange, medium	*Rhodococcus fascians*
37	MM1 + formate	2–37	Greenish, shiny	*Stenotrophomonas maltophilia*
43	MM1 + formate	2–25	Yellow, large	*Frigoribacterium faeni*
54	MM1 + formate	2–29	Pink, medium	*Arthrobacter agilis*
13	MM2.6.1	2–18	Beige, medium	*Sporosarcina globispora*
14	MM2.6.1	2–18	Beige, medium	*Sporosarcina globispora*
15	MM2.6.1	2–37	White, small	*Aerococcus viridans*
17	MM2.6.1	2–33	White, small	*Sporosarcina globispora*
9	MM2.6.2	2–18	White, shiny	*Sporosarcina globispora*
10	MM2.6.2	2–18	Beige, shiny	*Sporosarcina globispora*
11	MM2.6.2	7–37	Strong yellow, medium	*Micrococcus luteus*
R4	MM2.6.2/9m	2–25	Orange, medium	*Pseudomonas* sp. strain Ant 20
R5	MM2.6.2/9m	2–25	Orange, medium	*Pseudomonas* sp. strain Ant 20
R6	MM2.6.2/9m	2–25	Orange, medium	*Pseudomonas* sp. strain Ant 20
R7	MM2.6.2/9m	2–25	Orange, medium	*Pseudomonas* sp. strain Ant 20
R15p	R2B	2–33	Pink, medium	*Williamsia murale (Actinomyces)*
R15br	R2B	2–33	Yellow, slimy on R2A, brown pigment on TSA	*Brevundimonas* sp. strain FWC43
R15 be	R2B	2–33	Beige, slimy	*Pseudomonas cedrella*
R16	R2B	2–33	Yellow, slimy	*Brevundimonas* sp. strain FWC43
R17	R2B	2–33	Pinky, transparent	*Pseudomonas graminis*
R18	R2B	2–25	Yellowish, slimy	*Rhodoglobus vestalii*
R20p	R2B	2–33	Light pink-orange, medium	*Williamsia murale (Actinomyces)*
R20br	R2B	2–33	Yellow, slimy on R2A brown pigment on TSA	*Brevundimonas* sp. strain FWC43
R21	R2B	2–33	Whitish, large, slimy	*Paenibacillus amylolyticus*
R22	R2B	18–37	White, slimy	*Serratia marcescens*
R23	R2B	18–37	Yellow, small	*Moraxella osloensis*
R8	1/4 R2B	2–37	Orange, medium	*Sphingomonas faenia*
R9	1/4 R2B	2–33	Yellow, gummy	*Sphingomonas faenia*
R11	1/4 R2B	10–37	Whitish, small on R2A, yellow, in depth on TSA	*Curtobacterium citreum*
28	aMM1 + acetate	2–25	Orange-yellow, medium	*Rhodococcus fascians*
40y	aMM1 + acetate	2–29	Yellowish, small	*Sphingomonas mali*
26	aMM1 + formate	2–25	Strong orange-yellow, small	*Rhodococcus fascians*
27or	aMM1 + formate	10–37	Orange, small	*Microbacterium arborescens*
27y	aMM1 + formate	10–37	Lemon yellow, small, transparent	*Microbacterium luteolum*
31or	aMM1 + formate	2–37	Orange, large, shiny	*Exiguobacterium aurantiacum*
31w	aMM1 + formate	2–33	White, very slimy	*Rhodococcus erythropolis*
32	aMM1 + formate	2–25	Pale yellow, large, slimy	*Rhodococcus erythropolis*
33	aMM1 + formate	2–33	Whitish, large, very slimy	*Arthrobacter chlorophenolicus* A6
34	aMM1 + formate	10–25	Pink-orange, medium, shiny, slimy	*Taxeobacter gelupurpurascens*
35w	aMM1 + formate	12–37	White, very slimy	*Rhodococcus erythropolis*
38	aMM1 + formate	2–33	Beige, slimy on R2A, rough, irregular on TSA	*Rhodococcus erythropolis*
46	aMM1 + formate	10–29	Red-pink, small	*Methylobacterium extorquens*
51	aMM1 + formate	2–29	Yellow, irregular on TSA Yellow, very slimy on R2A	*Microsphaera multipartita*
52	aMM1 + formate	2–25	Pink, medium	*Methylobacterium extorquens*
53	aMM1 + formate	10–29	Pink, medium	*Methylobacterium extorquens*
62	aMM1 + formate	2–25	Yellow, small	*Microbacterium oxydans*

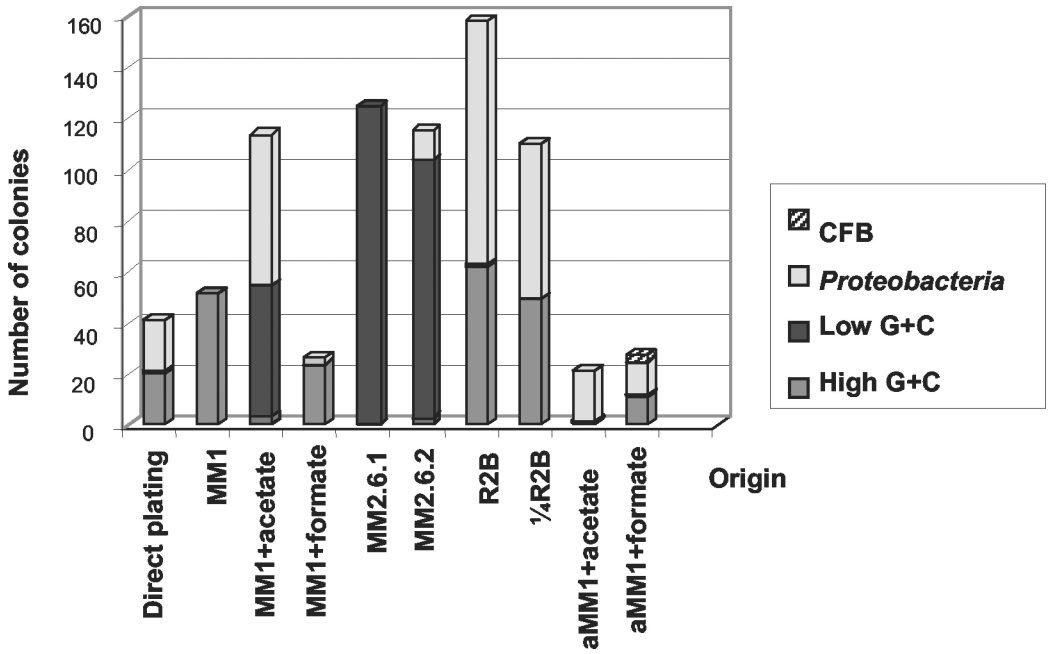

FIG. 2. Distribution of colonies, recovered directly from the melted ice, from anaerobic cultures in MM1, MM1 with acetate, MM1 with formate, MM2.6.1, or MM2.6.2 and from aerobic cultures in R2B, 1/4 R2B, MM1 with acetate, or MM1 with formate according to medium used and major phylogenetic groups.

two sporeforming genera *Sporosarcina* (isolates 9, 12, and 17) and *Paenibacillus* (isolates 1y, 16, and R21) and the nonsporeforming genera *Exiguobacterium* and *Aerococcus*. In many cases (36%), the most closely related sequences in the GenBank and RDP databases appeared to be from psychrophilic organisms isolated from the Arctic and Antarctic polar regions. The examples of these were all *Rhodococcus* and *Arthrobacter* isolates.

The Jukes-Cantor evolutionary distance matrix (data not shown) was calculated in order to determine the degree of relatedness between the ice core isolates and the sequences used in constructing the phylogenetic tree. These results suggested that seven of the isolates may represent new species within characterized genera (>3% distance) (Table 2). The possible novel species belonged to both high-G+C gram-positives and *Proteobacteria*, and three of them (isolates 55, 56, and 64) were recovered directly from the ice. Of special interest was the possible new species within the newly reported genus *Rhodoglobus* (34). Among the novel gram-negative organisms, isolate 34 is likely a new species in the genus *Taxeobacter*, which belongs to the deep-branching *Cytophaga* group. Its 16S rDNA sequence contained the short signature sequence 5′-TGGGTTTAAAGGG-3′ at positions 566 to 578, which has been found to be specific to all CFB representatives (28).

Examination of isolate recovery relative to origin and phylogenetic group. Using the phylogenetic relationships of isolates representative of each of the 70 morphologically different groups to tentatively place all 793 recovered colonies into broad categories, we examined the data to determine which incubation conditions and media gave rise to the different phylogenetic isolate types. Although the identity of isolates within each group could vary from the chosen representative, it is likely that they are sufficiently related to belong within the overall categories of high- or low-G+C gram-positives, *Proteobacteria*, or the CFB group. The distribution of all isolated colonies did differ substantially, depending on the medium used and the conditions of cultivation (Fig. 2). It is seen that a

TABLE 2. Phylogenetic grouping of potentially novel species representatives found among the GIC isolates

Species placement[a]	Isolate no.	Closest relative (accession no.)	% Distance
Novel species in the genus *Taxeobacter*	34	*Taxeobacter gelupurpurascens* (Y18836)	3.40
Novel species in the genus *Methylobacterium*	46	*Methylobacterium extorquens* (D32224)	3.87
Novel species in the genus *Janibacter*	55	*Janibacter limosus* (Y08539)	3.73
Novel species in the genus *Sphingomonas*	56	*Sphingomonas mali* (Y09638)	3.38
Novel species in the genus *Frigoribacterium* or the genus *Subtercola*	64	*Frigoribacterium faeni* (Y18807)	3.40
		Subtercola frigoramans (AF224723)	3.90
Novel species in the genus *Brevundimonas*	R16	*Brevundimonas bullata* (AB023428)	3.14
Novel species in the genus *Rhodoglobus*	R18	*Rhodoglobus vestalii* (AJ459101)	3.46

[a] The assignment of these isolates as novel species is based on distance values from the known closest relative species larger than 3%.

very low number of colonies (41 out of 793) originated from plating samples of the melted ice directly onto agar media without prior incubation in liquid media. In addition to the low recovery, we observed that most of these colonies required 3 to 7 months to become visible, as compared to 20 to 60 days observed for those obtained from the liquid cultures. Based on these results and the data from the flow cytometric enumeration of the cells in the original sample, we estimated that only 0.01 to 0.1% of the cells could be cultivated directly from the melted ice and that the incubation in liquid media substantially improved recovery.

Another observation was that gram-positive bacteria comprised the majority of the colonies (504 colonies) (Fig. 2). The next most abundant (283 isolates) and relatively diverse cluster contained organisms related to *Proteobacteria*. These were isolated from both aerobic and anaerobic cultures and after direct plating from the ice core. A larger number of colonies (437 colonies) was isolated from the anaerobic cultures than from the aerobic ones (318 colonies). The majority of the isolates from the anaerobic cultures in MM2.6.1 and MM2.6.2 belonged to the low-G+C gram-positives, whereas the high-G+C microorganisms and *Proteobacteria* were abundant in the aerobically incubated R2A broth, 1/4 R2A broth, and MM1. The one representative of the CFB group (isolate 34) was found only in the aerobic MM1-plus-formate culture. Isolates 19, 43, R11, R18, 12, 15, R3, and R16 were represented by more than 50 similar colonies/plate, indicating their prevalence in the cultures. Most other isolates were originally recovered as unique single-colony morphotypes, suggesting that they did not grow during incubations.

Morphological and physiological characterization of the isolates. Isolates, representing the different morphotypes and originating from direct plating of melted ice, aerobic and anaerobic liquid medium cultures were also characterized according to their phenotypic properties (Gram staining, colony and cell morphology, pigmentation, sporulation, and growth temperature) (Table 1). The results showed that 79% of all the isolates formed pigmented colonies (red, yellow, orange, pink, or beige). Sporeforming isolates were found to belong to the low-G+C gram-positive clade, some of which (isolates 1y, 16, and R21) had a slimy or gummy appearance. Synthesis of significant extracellular polymers by isolates 31w, 32, 33, 34, 35w, 38, and 64, representing *Rhodococcus*, *Taxeobacter*, and *Frigoribacterium*, was observed as well. Isolate 51, belonging to the genus *Streptomyces*, formed an extracellular matrix on the low-nutrient medium R2A but not on TSA.

Most of the isolates (85%) were psychrophilic according to the definition of Neidhardt et al., which emphasizes an organism's ability to grow at lower temperatures (5°C or below) than mesophiles rather than setting an upper limit (26). Our isolates grew at 2°C with an upper limit between 18 and 37°C (Fig. 3). Among these, isolates 1y, 9, 10, 12, 13, 14, 20or, and 20y grew only at temperatures below 18°C, and some (10, 22, 31w, 32, 33, 35, 36, 38, T1, T3, R6, R7, R17, and R15br) grew rapidly (colony formation within 24 to 48 h) at 2°C. A few isolates (11, 15, 31or, 36, 37, R8, and 56) had a wide temperature range for growth from 2 to 37°C, whereas two others (5 and 19) grew only at temperatures between 10 and 18°C. The only isolates requiring temperatures higher than 18°C for growth were 66, R22, and R23, belonging to the genera *Kocuria*, *Serratia*, and *Moraxella*, respectively. Forty-five isolates had potentially useful cold-active enzymes, including those with proteolytic, glycosidic, amylolytic, lipolytic, and DNA-hydrolyzing activities (Table 3). Eight isolates had four activities, and 10 others were positive for three of them.

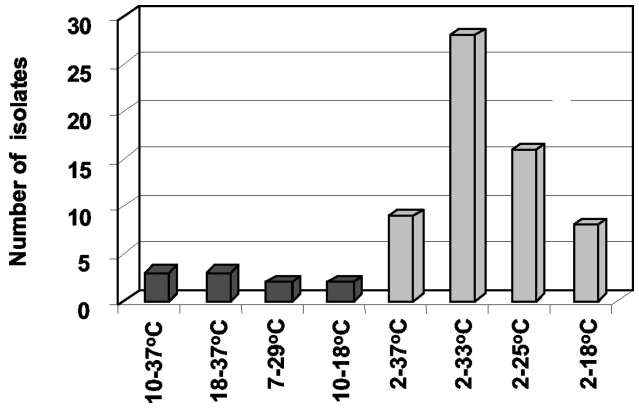

FIG. 3. Distribution of isolates according to growth temperature range. Columns show the number of isolates exhibiting growth only within the indicated temperature range.

The isolation of organisms that had been trapped in the Greenland ice sheet provided the opportunity to examine the levels of antibiotic sensitivity and resistance of organisms in the presumed absence of current antibiotic use. The antibiotic tests showed a wide variability of sensitive and resistant strains (data not shown). It is interesting that only three *Sporosarcina* isolates and one *Rhodococcus* isolate were sensitive to all antibiotics tested. In general, the low-G+C gram-positives were sensitive to more antibiotics than the isolates belonging to the high-G+C gram-positive lineage. Nevertheless, several *Microbacterium* isolates (19, 27y, and 62) and two *Rhodococcus* isolates (31w and 38) were resistant to most of the antibiotics tested. Among the *Proteobacteria*, isolates with multiple resistance belonged to *Pseudomonas* (isolate 22), *Sphingomonas* (isolate 23), and *Methylobacterium* (isolates 46 and 53). These results suggested that antibiotic resistance is a common trait in environments not influenced by human application of antibiotics and is possibly acquired through different mechanisms, such as genetic transfer among members of natural communities, reduced cell wall permeability, or multidrug pumps (22).

Plasmids were detected in 25% of the isolates. Large plasmids (above 40 to 50 kb) were isolated mostly from gram-positive bacteria (11 isolates). Five strains belonging to *Sphingomonas* (isolates T1 and 56), *Arthrobacter* (isolate 54), *Serratia* (isolate R22), and *Moraxella* (isolate R23) possessed one or more small plasmids. Isolate T1 harbored two plasmids with sizes 2.8 and 3.4 kb, respectively, while isolate 54 had a single 2.4-kb plasmid, isolate 56 had a 7.9-kb plasmid, isolate R22 possessed two plasmids with sizes 4.2 and 4.9, and isolate R23 had a single 4.2-kb plasmid. No connection between the presence of plasmids and antibiotic resistance was found, because among the highly resistant isolates, there were both plasmid-carrying and plasmidless strains. Among the *Microbacterium* isolates highly resistant to antibiotics, only isolate 27y harbored one large plasmid, while isolates 19 and 62 had no detectable plasmids. The same was observed for the two *Methylobacterium*

TABLE 3. Enzymatic activities of selected GIC isolates

Isolate	Closest relative species in the 16S rDNA sequence database	Enzymatic activity on:				
		TSA X-Gal	BHI-skim milk	Starch agar	DNase agar	Lipase agar
1y	*Paenibacillus* sp. strain V2 Ant	+	−	+	+	−
5	*Mycobacterium austroafricanum*	+	−	+	−	−
11	*Micrococcus luteus*	−	+	+	(+)	−
16	*Paenibacillus amylolyticus*	+	+	+	+	−
17 (12–14)	*Sporosarcina globispora*	−	−	−	+	−
18	*Arthrobacter agilis*	−	+	−	−	−
19	*Microbacterium trichothecenolyticum*	+	−	+	+	−
20or	*Curtobacterium citreum*	+	+	+	−	−
20y	*Microbacterium trichothecenolyticum*	+	−	+	+	(+)
22	*Pseudomonas* sp. strain ICO38 Ant	−	+	−	−	−
23	*Sphingomonas mali*	−	+	−	ND[a]	−
24or	*Pseudomonas* sp. strain Ant20	(+)	−	−	−	−
24y	*Plantibacter flavus*	+	−	+	+	−
26	*Rhodococcus fascians*	−	−	−	−	+
27or	*Microbacterium arborescens*	−	+	+	−	−
27y	*Microbacterium luteolum*	+	+	+	+	−
30	*Arthrobacter agilis*	−	+	+	−	−
31or	*Exiguobacterium aurantiacum*	−	+	+	++	−
32	*Rhodococcus erythropolis*	+	+	+	+	−
34	*Taxeobacter gelupurpurascens*	−	−	+	+	−
37	*Stenotrophomonas maltophilia*	−	+	−	+	−
38	*Rhodococcus erythropolis*	−	−	−	+	−
40y	*Shingomonas mali*	−	+	−	+	−
43	*Frigoribacterium faeni*	+	+	+	+	−
51	*Microsphaera multipartita*	+	−	+	−	−
53	*Methylobacterium extorquens*	−	+	+	−	−
54	*Arthrobacter agilis*	−	−	−	+	+
55	*Janibacter limosus*	+	+	+	+	−
56	*Sphingomonas mali*	−	+	+	+	−
62	*Microbacterium oxydans*	+	+	−	++	−
64	*Frigoribacterium faeni*	+	−	+	−	−
T1	*Sphingomonas* sp. strain M3C202B	+	−	−	−	−
T2	*Brevundimonas* sp. strain FWC43	−	−	+	+	−
R8	*Sphingomonas faenia*	+	+	+	−	−
R11	*Curtobacterium citreum*	+	+	+	−	−
R15br	*Brevundimonas* sp. strain FWC43	−	−	+	−	−
R15be	*Pseudomonas cedrella*	−	++	−	+	−
R17	*Pseudomonas graminis*	−	−	+	−	−
R18	*Rhodoglobus vestalii*	+	−	−	+	−
R21	*Paenibacillus amylolyticus*	+	−	+	−	−
R22	*Serratia marcescens*	+	+	−	++	+

[a] ND, not determined.

isolates, in which isolate 53 was the most resistant isolate carrying a large plasmid, but isolate 46 did not have any detectable plasmid despite its resistance to 11 of the antibiotics tested.

Repetitive genomic fingerprinting. The *rrn* operons represent only a portion of the genome and high similarity between 16S rRNA gene sequences does not necessarily correspond to high similarity of the genomes of different isolates. Some of our isolates had highly homologous 16S rDNA sequences but different colony and cell morphology and physiological characteristics. In order to determine whether we were repeatedly isolating the same organism and to reveal differences or confirm similarity among closely related organisms, we performed ERIC-PCR genome analysis of our isolates using genomic DNA. This method gives highly specific electrophoretic profiles of PCR-generated fragments, giving a picture of the whole genome, and it has a very high differentiation power at the strain level (45).

We examined each of the 19 isolates affiliated with the high-G+C gram-positive genera *Arthrobacter*, *Micrococcus*, *Microbacterium*, *Corynebacterium*, *Frigoribacterium*, *Curtobacterium*, and *Mycetocola* by ERIC-PCR (Fig. 4A). The finding that 17 isolates had unique profiles (only isolates 19 and 20y were identical) is consistent with the conclusion based on the 16S rDNA sequence analysis that these are different strains of known species, or in the case of isolates 64 and R18, possibly new species. Similarly, seven isolates clustering within the genus *Rhodococcus* all had different ERIC-PCR profiles and are likely different strains and species (data not presented).

Three isolates within the low-G+C genus *Paenibacillus* (1y, 16, and R21) had 16S rDNA sequences that differed by only a few bases. The ERIC-PCR pattern of isolate 16, however, was substantially different from those for isolates 1y and R21, which were identical (Fig. 4B). In contrast, the ERIC-PCR profiles for the six isolates with 16S rDNA sequences matching

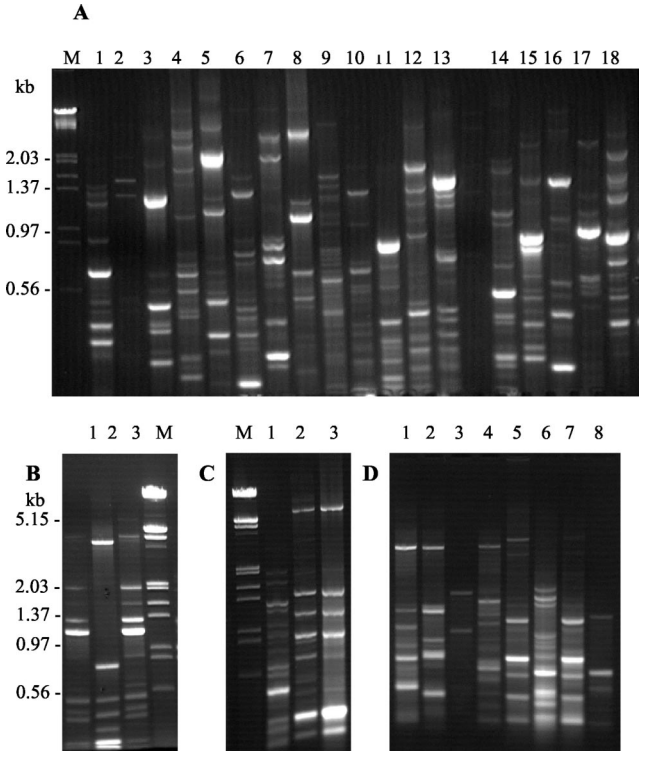

FIG. 4. ERIC-PCR fingerprint patterns of isolates in 1% agarose in Tris-acetate buffer (pH 8). (A) High-G+C gram-positive isolates. Lanes: 1, 20or; 2, R11; 3, 24y; 4, 64; 5, R18; 6, 43; 7, 6; 8, 20y, 9, 27or; 10, 27y; 11, 62; 12, 30; 13, 54; 14, 65; 15, 33; 16, 11; 17, 66; and 18, *Rhodoglobus vestalii*. (B) *Paenibacillus* isolates. Lanes: 1, 1y; 2, 16; and 3, R21. (C) *Methylobacterium* isolates. Lanes: 1, 46; 2, 52; and 3, 53. (D) α-*Proteobacteria* isolates. Lanes: 1, R4; 2, R8; 3, R9; 4, T1; 5, 23; 6, 24or; 7, 40y; and 8, R15br. M, molecular size marker (lambda DNA digested with *Hin*dIII plus *Eco*RI).

Sporosarcina globispora were identical (data not shown) and likely represent the same strain.

The ERIC-PCR analysis was also useful for further characterizing the three pink-pigmented representatives of the genus *Methylobacterium* (isolates 46, 52, and 53) within the cluster of *Proteobacteria*. The finding that the profiles for isolate 46 differed substantially from the identical profiles found for isolates 52 and 53 (Fig. 4C) was congruent with the 16S rDNA analysis, suggesting that isolate 46 was a different *Methylobacterium* species (Table 2). A similar examination of isolates T3, T4, and R17 (γ-*Proteobacteria*) showed that even though they had originated following incubation in different media, these isolates had identical ERIC-PCR profiles and may represent the same organism related to the recently proposed species *Pseudomonas graminis* (1, 6).

Several isolates within the α-*Proteobacteria* were closely related to the genus *Sphingomonas* based on their 16S rDNA sequences. Further examination by ERIC-PCR showed that isolates R3 through R7 had highly similar genomic profiles, whereas isolates R8, R9, T1, and 24or all had unique profiles different from those in the first set (Fig. 4D). Two other isolates, 23 and 40y, were related to *Sphingomonas mali* and had identical ERIC-PCR profiles (Fig. 4D).

These results showed that the combination of 16S rDNA sequence analyses with the strain-specific ERIC-PCR approach, as well as morphological, and physiological surveys, was useful for the characterization of new isolates.

DISCUSSION

This study demonstrates the presence of viable and diverse bacteria in a 120,000-year-old GIC sample. This first recovery of a large collection of phylogenetically diverse organisms from the bottom, most ancient part of the Greenland ice sheet made it possible to compare them with other bacteria from geographically different ice cores and with present day microorganisms from mesophilic and even thermophilic environments. Many (36%) of our isolates were related to genera such as *Methylobacterium*, *Rhodococcus*, *Mycobacterium*, *Sphingomonas*, *Arthrobacter*, and *Frigoribacterium*, representatives of which have been found in other permanently cold environments. The organisms from the GIC were similar to those isolated from glacier ice cores and permafrosts by Christner et al. (13, 14), Brambila et al. (10), Shi et al. (35), and Vorobyova et al. (46). This is consistent with the idea that the occurrence of related phylotypes in geographically diverse cold environments is possibly due to similar strategies to survive freezing and remain active at low temperature (30).

The cell numbers estimated for the GIC sample by flow cytometry were significantly higher than those found in other glacial ice samples. This observation was not surprising, since our sample came from the silty ice close to the bedrock and contained soil particles that may have contributed to the higher abundance of microorganisms. The bacteria in our sample may be of mixed origin, with some originating from airborne deposition (the generally accepted source of organisms in glacial ice), while others may have been entrained from the underlying soil. Thus, the organisms in our sample could have been trapped in the frozen environment 120,000 years ago (estimated by the time of ice deposition), or they could be similar to those found in permafrost, which had been frozen for millions of years.

Interestingly, our results also show a predominance of very small (dwarf) cells, the presence of organisms with various pigments, colonies with extracellular polymers, and sporeforming bacteria, all of which have been suggested as mechanisms for stress adaptation and survival enhancement (7, 16, 19, 26). Our observation that a significant population of very small, but viable, cells exists in the melted ice is also consistent with the assumption that some cells have an adaptive physiological response to environmental stress, including cell size reduction, condensation of the cytoplasm, loss of permeability, decrease in RNA content, reduction of metabolic activity, and inability to divide that leads to the viable but not culturable state (24). Such adaptive responses might explain the difficulty in culturing these cells directly from environmental samples (16).

Predominantly pigmented organisms have been also isolated from other cold environments (9, 12, 14, 15, 38), and Fong et al. (19) found a correlation between the production of carotenoids and the cold adaptation of microorganisms, possibly due to increased rigidity of the membranes. The formation of large quantities of extracellular polymers, observed for 28% of our isolates, has been considered by others as one of the survival responses to desiccation (7, 31), which would have been a

factor during the deposition of microorganisms from the atmospheric airflow into the glacial ice.

Certainly another well-documented survival mechanism is through spore formation (27). Although some isolates were members of sporeforming genera (*Paenibacillus* and *Sporosarcina*), it is perhaps surprising that sporeforming organisms do not dominate, since spores might be expected to survive for thousands of years. There are several possibilities for this result, including the inability of the spores to germinate during our cultivation conditions, since little is known about spore germination from environmental samples (27). It is also possible, however, that sporeforming organisms were not dominant in this ice core population and that many of the isolates have other unknown survival strategies that still need to be investigated.

Two observations from this work are particularly relevant to the broader issues of inefficient cultivation of organisms from environmental samples. The first is reflected by the dramatic difference between the low number of colonies appearing after plating material directly from the melted ice core sample and the increased number found after incubation in liquid media. Although some portion of the increase could result from a proliferation in cell numbers during incubation, this does not account for the simultaneous increase in the diversity of organisms found. More importantly, our microscopic examination using a live/dead staining approach showed that an abundant, viable population of more than 10^7 cells/ml existed in the melted ice sample. In contrast, only a few colonies formed when samples were directly inoculated onto agar media, even after several months of incubation. The incubation in the anaerobic media was particularly effective for the subsequent isolation of the low-G+C gram-positive organisms and some *Proteobacteria*. The aerobic incubation in low-nutrient R2B and 1/4 R2B liquid media enhanced the recovery of the high-G+C gram-positives and *Proteobacteria* on agar media (Fig. 2).

It is clear that the liquid medium incubations were important for the recovery of many isolates from the frozen ice core. Cells deposited from the atmosphere and trapped in the glacier ice have likely been subjected to desiccation, freezing, high pressure, and limiting nutrient and O_2 concentrations. Because of the impact of these multiple and prolonged stress factors, many organisms may have been damaged or persist in a dormant or very low metabolic state. It is possible that the incubation in liquid media permits stressed cells to recover the ability to form colonies. Recently, Bloomfield et al. (8) proposed that the failure to recover cells subjected to starvation or other stress is caused by an oxidative suicide after transfer to a rich medium resulting from the overproduction of superoxide and free radicals. It is possible that our anaerobic incubations provided a period of slow onset of metabolic processes in the absence of oxygen and allowed successful growth and colony formation upon subsequent plating. This is indirectly supported by the observation that cells recover more quickly after the addition of bovine liver catalase, which is a powerful antioxidant (12). Further studies of the survival strategies and the conditions required for cell recovery and improvement of culturability are crucial to the exploration of life in other extreme environments, including extraterrestrial ones.

The second important observation was that many of the colonies recovered from the direct plating of the melted ice core sample did not become visible until after 3 to 7 months of incubation. This is also consistent with the need for cells to repair damage or to recover metabolic functions. The long incubation time was not a result of extremely slow growth rates, since the isolates reformed new colonies within days when restreaked onto new media. It would be interesting to determine if recovery of colonies from other environmental samples would also require such prolonged initial incubation. Development of a better understanding of the processes that occur during the recovery or resuscitation of cells that have had low metabolic activity or have been dormant for extended times is of general importance to the isolation of organisms from environments other than ice cores.

Not surprisingly, the diversity of isolates presented here differs from that found in our prior efforts based on the extraction of DNA from the anaerobic enrichments and the construction and analysis of a clone library (33). In that study, approximately 60 bacterial inserts were screened by restriction endonuclease analysis and grouped into 27 unique restriction fragment length polymorphism (RFLP) types, and 24 representative sequences were compared phylogenetically. Sequences for 16S rDNA genes related to the major phylogenetic groups, including α-, β-, and γ-*Proteobacteria* as well as relatives of the *Thermus* and the anaerobic *Bacteroides*, *Eubacterium*, and *Clostridium* groups, were detected. Sixteen sequences were closely related to those from known organisms, with 4 sequences possibly representing new species, while 7 sequences reflected possible new genera, and 1 may be a representative of a novel bacterial order or family. In addition to the expected absence of strict anaerobes due to the aerobic conditions used for colony isolation, the isolate collection presented here lacked representatives of possible new genera found within the clone library. Thus, as observed by others (11, 37, 38, 43), the clone library of 16S rDNA genes revealed types of bacteria different from the ones found with cultivation methods. Interestingly, some of our isolates had 16S rRNA gene sequences highly related to others found in the clone library belonging to the genera *Methylobacterium*, *Stenotrophomonas*, and *Brevundimonas*, demonstrating the presence of viable representatives in the ice core. Even though the approaches employed produced some common findings, there were also significant differences. Therefore, a more comprehensive view of the microbial community is obtained when the results from multiple approaches are combined and each makes an independent contribution. For example, one of the *Brevundimonas* isolates was selected based on the information from the clone library. Having detected a *Brevundimonas* 16S rRNA gene sequence in the library, we devised media and cultivation conditions specific for *Brevundimonas* species and obtained an isolate with a 16S rDNA sequence corresponding to that found in the clone library. The additional use of information from the clone library should help obtain isolates not yet represented in our collection, such as anaerobes.

The analysis of the community by obtaining and comparing isolates not only contributes to the general knowledge of diversity but also provides a large collection of organisms that can be further characterized and used. In this study, we have obtained isolates that may represent new species or new strains and may have important functions. The results of the morphological characterization and the ERIC-PCR analyses show that

isolates with very similar 16S rDNA sequences may differ in other regions of their genome and may also differ in important physiological properties. In some ecological niches, the presence of different subspecies or strains may be important for establishing the structure of the microbial community. Thus, determination of the genomic and physiological differences among the ice core isolates with close evolutionary relationships increases our understanding of the microbial diversity of this environment.

The strong and often multiple enzymatic activities of many isolates on different substrates (Table 3) was the basis for the conclusion that the versatile metabolic properties of the isolates increase their ability to utilize different substrates. We find this important in the context of the extreme conditions in the environment studied. Our collection of psychrophilic organisms with extracellular enzymes may also be a significant source of novel and potentially useful cold-active enzymes. Some fast-growing psychrophilic isolates containing plasmids may be further developed as hosts for cloning and expression of genes at low temperatures. The first successful attempt of recombinant production of a cold-adapted protein in a psychrophilic host was recently reported (44).

Studies of long-frozen ecosystems are important not only because they provide information about the functional and phylogenetic groups in these environments, but also because they may suggest strategies microorganisms use to survive for thousands to millions of years. In addition, it is not clear whether these organisms have in situ activity or only have been trapped and preserved in the ice. Recent findings including the presence of water channels and brines in frozen matrices, and the results of isotopic fractionation experiments suggest that low rates of metabolism can occur and may be needed for cell maintenance and repair of DNA damages (17, 29). Further studies, including reconstruction experiments with artificial ice cores containing isolates from the natural glacier ice, could help answer questions about the in situ activity and the survival and recovery mechanisms under extreme conditions.

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REFERENCES

1. **Abraham, W. R., C. Strompl, H. Meyer, S. Lindholst, E. R. Moore, R. Christ, M. Vancanneyet, B. J. Tindall, A. Bennasar, J. Smit, and M. Tesar.** 1999. Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. Int. J. Syst. Bacteriol. **49:**1053–1073.
2. **Abyzov, S. S.** 1993. Microorganisms in the Antarctic ice, p. 265–295. *In* E. I. Friedman (ed.), Antarctic microbiology. John Wiley & Sons, Inc., New York, N.Y.
3. **Abyzov, S. S., N. E. Bobin, and B. B. Koudryashov.** 1982. Quantitative assessment of microorganisms in microbiological studies of Antarctic glaciers. Biol. Bull. Acad. Sci. USSR **9:**558–564.
4. **Abyzov, S. S., I. N. Mitskevich, and M. N. Poglazova.** 1998. Microflora of the deep glacier horizons of Central America. Microbiology **67:**451–458.
5. **Abyzov, S. S., I. N. Mitskevich, M. N. Poglazova, N. I. Barkov, V. Y. Lipenkov, N. E. Bobin, B. B. Koudryashov, V. M. Pashkevich, and M. V. Ivanov.** 2001. Microflora of the basal strata at Antarctic ice core above the Vostok Lake. Adv. Space Sci. **28:**701–706.
6. **Behrendt, U., A. Ulrich, P. Schumann, W. Erler, J. Burghardt, and W. Seyfarth.** 1999. A taxonomic study of bacteria isolated from grasses: a proposed new species, *Pseudomonas graminis* sp. nov. Int. J. Syst. Evol. Microbiol. **49:**297–308.
7. **Billi, D., and M. Potts.** 2002. Life and death of dried prokaryotes. Res. Microbiol. **153:**7–12.
8. **Bloomfield, S. F., G. S. A. B. Stewart, C. E. R. Dodd, I. R. Booth, and E. G. M. Power.** 1998. The viable but non-culturable phenomenon explained? Microbiology **144:**1–3.
9. **Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin.** 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. **63:**3068–3078.
10. **Brambilla, E., H. Hippe, A. Hagelstein, B. J. Tindall, and E. Stackebrandt.** 2001. 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. Extremophiles **5:**23–33.
11. **Buckley, D. H., and T. M. Schmidt.** 2002. Exploring the biodiversity of soil. A microbial rain forest, p. 183–208. *In* J. T. Staley and A. L. Reysenbach (ed.), Biodiversity of microbial life. John Wiley & Sons, Inc., New York, N.Y.
12. **Christner, B. C.** 2002. Recovery of bacterial from glacial and subglacial environments. Thesis. Ohio State University, Columbus.
13. **Christner, B. C., E. Mosley-Thompson, L. G. Thompson, and J. N. Reeve.** 2003. Bacterial recovery from ancient glacial ice. Environ. Microbiol. **5:**433–436.
14. **Christner, B. C., E. Mosley-Thompson, L. G. Thompson, V. Zagorodnov, K. Sandman, and J. N. Reeve.** 2000. Recovery and identification of viable bacteria immured in glacial ice. Icarus **144:**479–485.
15. **Dancer, S. J., P. Shears, and D. J. Platt.** 1997. Isolation and characterization of coliforms from glacial ice and water in Canada's High Arctic. J. Appl. Microbiol. **82:**597–609.
16. **De Fede, K. L., and A. J. Sexstone.** 2001. Differential response of size-fractionated soil bacteria in BIOLOG microtitre plates. Soil Biol. Biochem. **33:**1547–1554.
17. **Deming, J. W.** 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. **5:**301–309.
18. **Ferrara-Guerrero, M. J., D. G. Marty, and A. Bianchi.** 1993. Isolation and enumeration of anaerobic and microaerophilic bacteria in aquatic habitats, p. 9–19. *In* P. Kemp (ed.), Handbook of methods in microbial ecology. Lewis Publishers, Boca Raton, Fla.
19. **Fong, N. J. C., M. L. Burgess, K. D. Barrow, and D. R. Glenn.** 2001. Carotenoid accumulation in the psychrotrophic bacterium *Arthrobacter agilis* in response to thermal and salt stress. Appl. Microbiol. Biotechnol. **56:**750–756.
20. **Gow, A. J., and D. A. Meese.** 1996. Nature of basal debris in the GISP2 and Byrd ice cores and its relevance to bed processes. Ann. Glaciol. **22:**134–140.
21. **Gow, A. J., D. A. Meese, R. B. Alley, and J. J. Fitzpatrick.** 1997. Physical and structural properties of the Greenland Ice Sheet Project 2 ice core: a review. J. Geophys. Res. **102:**26,559–26,575.
22. **Hogan, D., and R. Kolter.** 2002. Why are bacteria refractory to antimicrobials? Curr. Opin. Microbiol. **5:**472–477.
23. **Kouker, G., and K.-E. Jaeger.** 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. **53:**211–213.
24. **McDougald, D., S. A. Rice, D. Weichart, and S. Kjelleberg.** 1998. Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. **25:**1–9.
25. **Miteva, V. I., S. Selenska-Pobell, and V. S. Mitev.** 1999. Random and repetitive primer amplified polymorphic DNA analysis of *Bacillus sphaericus*. J. Appl. Microbiol. **86:**928–936.
26. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell, 1st ed. Sinauer Associates, Inc., Sunderland, Mass.
27. **Nicholson, W. L.** 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. **59:**410–416.
28. **O'Sullivan, L. A., A. J. Weightman, and J. C. Fry.** 2002. New degenerate *Cytophaga-Flexibacter-Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. Appl. Environ. Microbiol. **68:**201–210.
29. **Price, P. B.** 2000. A habitat for psychrophiles in deep Antarctic ice. Proc. Natl. Acad. Sci. USA **97:**1247–1251.
30. **Priscu, J. C., and B. C. Christner.** 2003. Earth's icy biosphere, p. 130–145. *In* A. T. Bull (ed.), Microbial diversity and bioprospecting. ASM Press, Washington, D.C.
31. **Ramos, J. L., M.-T. Gallegos, S. Marques, M.-I. Ramos-Gonzalez, M. Espinosa-Urgel, and A. Segura.** 2001. Responses of Gram-negative bacteria to certain environmental stressors. Curr. Opin. Microbiol. **4:**166–171.
32. **Reasoner, D. J., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:**1–7.
33. **Sheridan, P., V. I. Miteva, and J. E. Brenchley.** 2003. Phylogenetic analysis of 16S rDNA of an anaerobic psychrophilic community obtained from a Greenland glacier ice core. Appl. Environ. Microbiol. **69:**2153–2160.

34. **Sheridan, P. P., J. Loveland-Curtze, V. I. Miteva, and J. E. Brenchley.** 2003. *Rhodoglobus vestalii* gen. nov., sp. nov, a novel psychrophilic organism isolated from an Antarctic Dry Valley lake. Int. J. Syst. Evol. Microbiol. **53:** 985–994.
35. **Shi, T., R. H. Reeves, D. A. Gilichinsky, and E. I. Friedmann.** 1997. Characterization of viable bacteria from Siberian permafrost by 16S rDNA sequencing. Microb. Ecol. **33:**169–179.
36. **Skidmore, M. L., J. M. Foght, and M. J. Sharp.** 2000. Microbial life beneath a high Arctic glacier. Appl. Environ. Microbiol. **66:**3214–3220.
37. **Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. van Mil, and K. Wernars.** 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. **67:**2284–2291.
38. **Smith, M. C., J. P. Bowman, F. J. Scott, and M. A. Line.** 2000. Sublithic bacteria associated with Antarctic quartz stones. Antarctic Sci. **12:**177–184.
39. **Sokol, P. A., D. E. Ohman, and B. H. Iglewski.** 1979. A more sensitive plate assay for detection of protease production by *Pseudomonas aeruginosa*. J. Clin. Microbiol. **9:**538–540.
40. **Souchez, R.** 1997. The buildup of the ice sheet in central Greenland. J. Geophys. Res. **102:**26,317–26,323.
41. **Sowers, K. R., M. J. K. Nelson, and J. G. Ferry.** 1984. Growth of acetotrophic, methane-producing bacteria in a pH auxostat. Curr. Microbiol. **11:**227–230.
42. **Sowers, T.** 2003. Evidence for *in situ* metabolic activity in ice sheets based on anomalous trace gas records from the Vostok and other ice cores. Geophys. Res. Abstr. **5:**01994.
43. **Suzuki, M. T., M. S. Rappé, Z. W. Haimberger, H. Winfield, N. Adair, J. Ströbel, and S. Giovannoni.** 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. **63:**983–989.
44. **Tutino, M. L., A. Duilio, E. Parrilli, E. Remaut, G. Aannia, and G. Marino.** 2001. A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature. Extremophiles **5:**257–264.
45. **Versalovic, J., M. Schneider, F. J. de Bruijn, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Methods Mol. Cell Biol. **5:**25–40.
46. **Vorobyova, E., V. Soina, M. Gorlenko, N. Minkovskaya, N. Zalinova, A. Mamukelashvili, D. Gilichinsky, E. Rivkina, and T. Vishnivetskaya.** 1997. The deep cold biosphere: facts and hypothesis. FEMS Microbiol. Rev. **20:** 277–290.
47. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature **345:**63–65.
48. **Willerslev, E., A. J. Hansen, B. Christensen, J. P. Steffensen, and P. Arctander.** 1999. Diversity of Holocene life forms in fossil glacier ice. Proc. Natl. Acad. Sci. USA **96:**8017–8021.