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Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice

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Summary

Lake Vostok, the largest subglacial lake in Antarctica, is separated from the surface by ≈4 km of glacial ice. It has been isolated from direct surface input for at least 420 000 years, and the possibility of a novel environment and ecosystem therefore exists. Lake Vostok water has not been sampled, but an ice core has been recovered that extends into the ice accreted below glacial ice by freezing of Lake Vostok water. Here, we report the recovery of bacterial isolates belonging to the *Brachybacteria*, *Methylobacterium*, *Paenibacillus* and *Sphingomonas* lineages from a sample of melt water from this accretion ice that originated 3593 m below the surface. We have also amplified small-subunit ribosomal RNA-encoding DNA molecules (16S rDNAs) directly from this melt water that originated from α- and β-proteobacteria, low- and high-G+C Gram-positive bacteria and a member of the *Cytophaga*/*Flavobacterium*/*Bacteroides* lineage.

Introduction

Lake Vostok is covered by ≈4 km of glacial ice and has apparently been isolated from direct surface input for at least 420 000 years (Petit *et al*., 1999). However, glacial ice melts into Lake Vostok at the northern ice–water interface, and water from the lake freezes and accumulates as accretion ice, below the glacial ice, over the central and southern regions, resulting in water circulating within Lake Vostok (Fig. 1; Kapitsa *et al*., 1996; Jouzel *et al*., 1999; Siegert *et al*., 2000). Glacier movement presumably must transfer sediment from the adjacent bedrock into the lake, and both eukaryotic and prokaryotic microorganisms have been detected in samples of glacial ice collected from above Lake Vostok (Abyzov *et al*., 1998). It seems inevitable, therefore, that microorganisms are seeded into Lake Vostok, but the nature of the environment and ecosystem within Lake Vostok remain uncertain. Concerns for contamination have resulted in a moratorium on direct sampling of Lake Vostok water, and ice core drilling has been terminated above the ice–water interface. An ice core has, nevertheless, been retrieved, in which the bottom ≈150 m are accretion ice (Fig. 1), and this therefore provides a sample of Lake Vostok water (Petit *et al*., 1999). Microbial cells in melt water from sections of this accretion ice core that originated 3590 and 3603 m below the surface (mbs) have been detected by epifluorescence and scanning electron microscopy (Karl *et al*., 1999; Priscu *et al*., 1999), and seven small-subunit bacterial ribosomal RNA-encoding DNAs (16S rDNA) were amplified, from the 3590 melt water, that originated from α- and β-proteobacteria and from an *Actinomyces* (Priscu *et al*., 1999). Evidence for respiration was also obtained by measuring [^{14}C]-CO_2 release during incubations at 3°C and 23°C after the addition of [^{14}C]-acetate or [^{14}C]-glucose to melt water from the 3603 section (Karl *et al*., 1999). However, there was very little, if any, ^{14}C incorporation into macromolecules.

Here, we report the results of experiments undertaken to determine whether viable bacteria could be recovered directly from Lake Vostok accretion ice. Four different isolates have been obtained, and additional 16S rDNAs have been amplified from a section of the accretion ice core that originated at 3593 mbs. The results are consistent with the concept that Lake Vostok is seeded regularly with bacteria initially immured in the overlying glacial ice and is likely to contain bacteria similar to species found in other cold environments.

Results

Enrichment isolates

Melt water that was obtained from inside Vostok deep core section 3593, using the automated ice core sampling system described previously (Christner *et al*., 2000), was used to inoculate a wide range of different growth media (see *Experimental procedures*). After 7 days, growth was observed in M9 glucose-minimal salts (Sambrook *et al*., 1989) and in R2 medium, a low-nutrient medium designed to recover stressed bacteria (Reasoner and Geldreich, 1985), in cultures incubated aerobically at 25°C. Single-colony isolates were obtained from these enrichment cultures by

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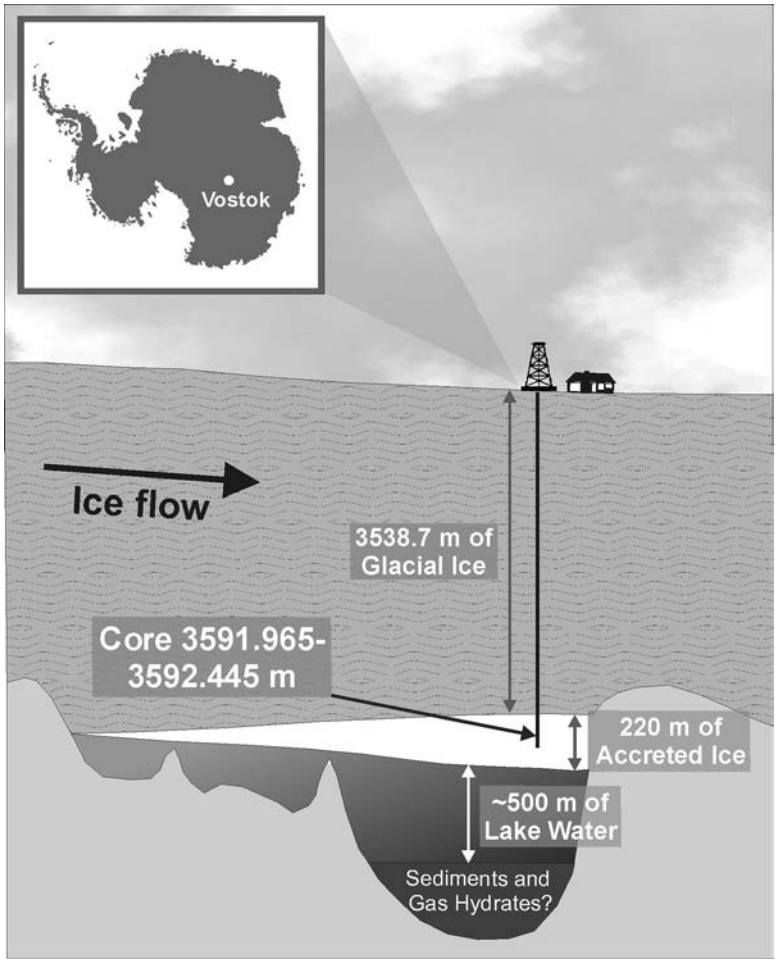

Fig. 1. The origin of deep Vostok ice core section 3593 (based on Bell, 1998; Siegert, 2000). Chemical and isotopic profiles established that the glacial ice–accretion ice interface occurs at ≈3540 m below the surface (mbs; Jouzel *et al*., 1999), and the deep Vostok ice core section used in this study, section 3593, originated from 3591.965 to 3592.445 mbs. As illustrated, glacial ice melts into Lake Vostok at the ice–water interface in the north, and accretion ice accumulates at the base of the glacial ice over the central and southern regions. Radar measurements have detected the presence of a layer of sediment below the lake water (Kapitsa *et al*., 1996), with gas hydrates also predicted to be present (Doran *et al*., 1998).

plating on agar-solidified M9 glucose and R2 media. Three isolates from the M9 enrichment culture, designated V15, V18 and V19, that formed colonies with reproducibly different morphologies were investigated further. They were all resistant to β-lactam antibiotics and sensitive to gentamicin, tetracycline and neomycin, but V19 alone was also resistant to erythromycin. Surprisingly, all three had the same 16S rDNA sequence, indicating a close phylogenetic relationship to *Brachybacterium conglomeratum* (Table 1; Fig. 2), and all the single-colony isolates investigated similarly from the R2 enrichment culture also had this *Brachybacterium*-related 16S rDNA sequence.

Colony isolations

Particulates, collected by filtration from 100–150 ml of melt water, were resuspended at ≈30-fold the original concentration, and aliquots (200 μl) of the resuspended materials were spread on the surface of agar-solidified media. A total of three colonies was obtained, all on agar-solidified R2 medium on plates incubated aerobically at 25°C. The growth of colonies was never observed on any other agar-solidified medium (see *Experimental procedures*), even though the plates were incubated for > 3 months at both 4°C and 25°C. On subculture, all three isolates, designated V21, V22 and V23, grew most rapidly on R2 medium at 25°C, although V22 and V23 also grew at temperatures as low as 4°C, and V21 grew at temperatures as low as 10°C. Based on their 16S rDNA sequences, V21, V22 and V23 are most closely related phylogenetically to *Sphingomonas*, *Paenibacillus and Methylobacterium* species respectively (Table 1; Fig. 2).

16S rDNA amplification and sequencing

Populations of small-subunit rDNA molecules were amplified directly from core 3593 melt water by using universal and *Bacteria*-specific primers, but not when *Archaea*-specific primers were used. Individual DNA molecules were cloned from these populations and sequenced, revealing the presence of bacterial 16S rDNAs from five different phylogenetic lines of descent (Table 2; Fig. 3). Sequence pA419 originated from an

Table 1. Bacteria isolated from deep Vostok ice core section 3593.

Isolate designation	GenBank accession number	Sequence alignment		Nearest phylogenetic neighbour (GenBank accession no.; origin)
		No. of nucleotides[a]	% identity[b]	
V15	AF324202	1454	99.4	*Brachybacterium conglomeratum* (X91030; cheese)
V21	AF324199	1409	99.8	Guliya 500K-14 (AF395037; Guliya ice core)
			99.0	*Sphingomonas* sp. (AB033945; not available)
V22	AF324200	1485	99.2	*Paenibacillus amylolyticus* (D85396; soil)
V23	AF324201	1406	99.3	Unidentified bacterium (AJ223453; not available)
			98.2	*Methylobacterium* sp. (Z23156; biofilm on cooling fan)

a. The number of 16S rDNA nucleotides used for the alignment.
b. The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbour.

α-proteobacterium whose nearest cultured neighbour was isolated from Lake Baikal in Russia, and pA419 is also ≈86% similar to a 16S rDNA sequence retrieved by Benson *et al*. (2000) from an Antarctic lake. Although perhaps not so striking, in terms of such very cold freshwater environments, the other 16S rDNAs amplified from the 3593 melt water also have freshwater isolate relatives. Specifically, sequences pA3178 and pA42B412 are from β-proteobacteria and are most similar to the 16S rDNA sequences of an *Aquabacterium* and a facultative hydrogen autotroph, formerly designated *Pseudomonas saccharophila* respectively. Sequence pA47 is 93.6% identical to that of the 16S rDNA of *Sphingobacterium heparinum*, a member of the *Cytophaga/Flavobacterium/Bacteroides* lineage, and sequences pD12 and pD4 are 93.5% and 98.9% identical to the 16S rDNA sequences of *Alkalibacterium olivoapovlitic* and *Rubrobacter xylanophilus*, respectively, positioning them within the low- and high-G+C Gram-positive groups.

Extensive precautions were taken, but the possibilities of contamination and of DNA molecules being generated artifactually during the polymerase chain reaction (PCR) amplifications were always serious concerns. Infrequently, an amplicon was generated in a negative control reaction and, when these were cloned and sequenced, they had sequences almost identical to a 16S rDNA sequence that has been shown previously to arise in PCR controls (Cisar *et al*., 2000). This sequence (GenBank accession number AF195876) is related to 16S rDNA sequences from γ-proteobacterial pseudomonads and is not closely related to any of the experimental sequences used in constructing Figs 2 and 3.

Discussion

A 48 cm section of the deep Vostok ice core, section 3593, was made available for this study, which accreted over a period of 10–25 years, based on the estimates of Siegert *et al*. (2000). It did not contain any macroscopically visible solid inclusions and, therefore, most probably formed over a relatively deep portion of the lake (Jouzel *et al*., 1999). Epifluorescence microscopy of DNA-stained samples revealed the presence of 2.3×10^3 and $2–3 \times 10^2$ cells ml^{-1} in melt water from flanking Vostok ice core sections 3590 and 3603 respectively (Karl *et al*., 1999; Priscu *et al*., 1999). Presumably, therefore, only a very small percentage of the cells present in core 3593 was recovered, and it is perhaps noteworthy that V21, V22 and V23 are related, although not identical, to species recovered previously from both polar and non-polar glacial ices (Christner *et al*., 2000). These isolates survived repeated cycles of freezing and thawing (B. C. Christner, unpublished), although V22 is the only member of a bacterial group (*Paenibacillus*) that is known to differentiate into cells (endospore) that specifically facilitate airborne transport, resist desiccation and provide long-term survival under non-growth conditions (Cano and Borucki, 1995; Vreeland *et al*., 2000).

The results obtained argue that representatives of at least five bacterial lineages are likely to be present in Lake Vostok, some of which are related, in terms of 16S rDNA sequences, to isolates from other cold, potentially very similar environments. For example, sequence pD12 is ≈92% identical to 16S rDNA sequences from two *Carnobacterium* species that were isolated from ice-covered Antarctic lakes (Franzmann *et al*., 1991; Bratina *et al*., 1998), and sequence pA419 clusters with both the 16S rDNA sequence of an isolate recovered from 400 mbs of Lake Baikal in Siberia and the sequence of an amplicon from a frozen Antarctic lake (Fig. 3). Extrapolations from rDNA sequence similarities to similarities in lifestyle and physiology are clearly very tenuous, but these results do argue that Lake Vostok probably

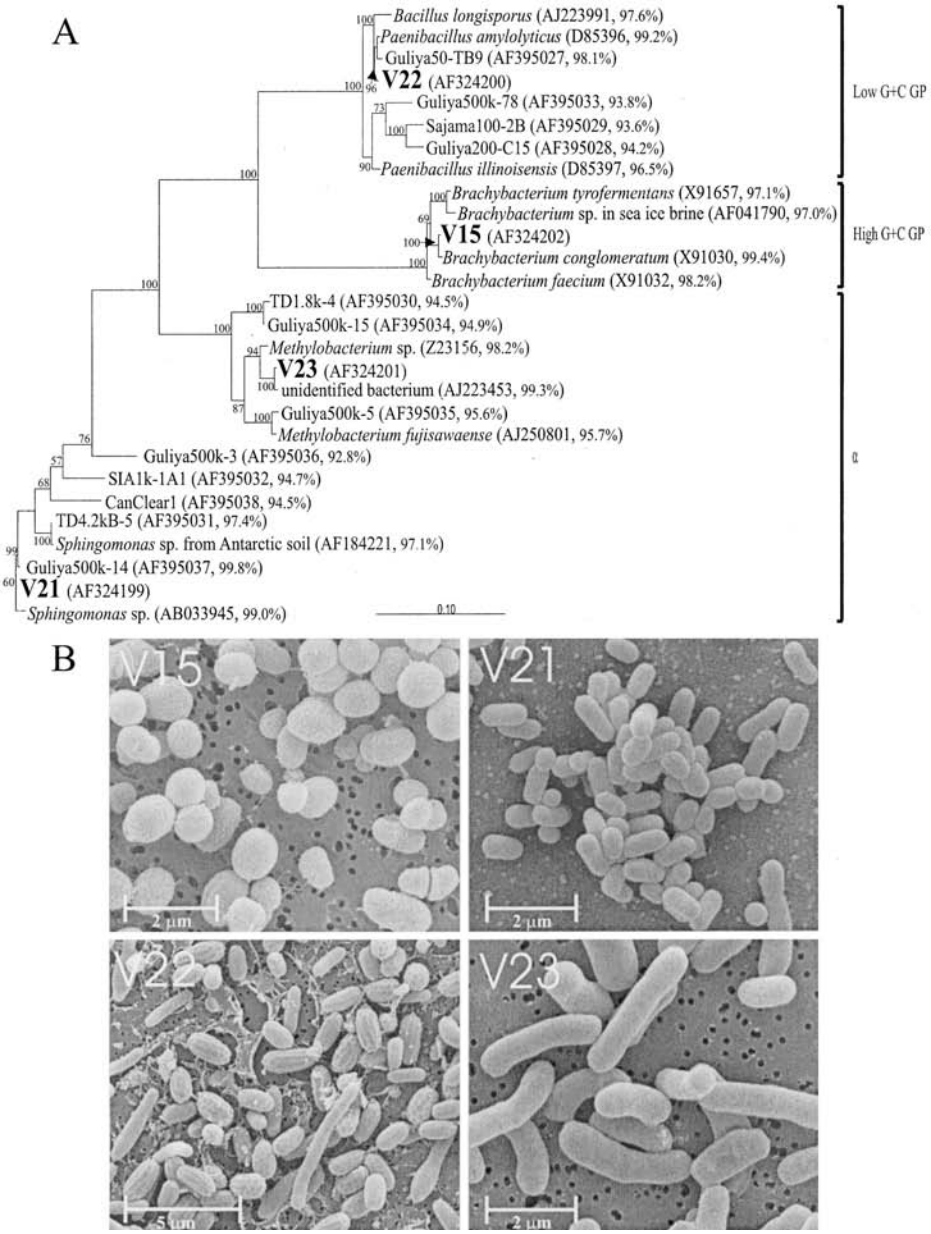

Fig. 2. Phylogenetic analysis and scanning electron micrographs of bacterial isolates from core section 3593.
A. The 16S rDNA sequences obtained from single-colony isolates, corresponding to nucleotides 27–1492 of the *E. coli* 16S rDNA, were aligned based on secondary structure using the ARB software package (Strunk *et al.*, 1998), and a phylogenetic tree was created with maximum likelihood using a 1321 nucleotide mask of unambiguously aligned positions and using FASTDNAML (Olsen *et al.*, 1994). Bootstrap values generated from 100 replicates using the maximum parsimony method are shown at the nodes. Evolutionary distance is defined as the number of fixed nucleotide changes per position. The scale bar indicates 0.1 fixed substitutions per nucleotide position. Isolates V21 and V23 position within the α-subdivision (α) of the proteobacteria, V15 in the high-G+C-containing Gram-positive (GP) group, and V22 in the low-G+C-containing GP group. GenBank accession numbers and the percentage identity of the corresponding 16S sequence with that of the most similar Lake Vostok isolate are listed in parentheses. Ice core isolates from Guliya (China) and Sajama (Bolivia), and from Taylor Dome (TD), Canada glacier (CanClear) and Siple Dome (SIA) in Antarctica are listed. These are designated by their geographic origin, age of the ice in years or thousands (k) of years and strain number, e.g. Guliya500k-78 is strain no. 78 isolated from Guliya glacial ice that was ≈ 500 000 years old.
B. Scanning electron micrographs of cells from cultures of V15, V21, V22 and V23.

Table 2. 16S rDNA molecules amplified from core 3593 melt water.

Sequence designation (no. of clones)[a]	GenBank accession number	Sequence alignment		Nearest phylogenetic neighbour (GenBank accession no.; origin)
		No. of nucleotides[b]	% identity[c]	
pA3178 (4)	AF324205	842	98.7	*Aquabacterium* sp. (AF089858; drinking water biofilm)
pA419 (3)	AF324207	896	96.4	Lake Baikal isolate (AJ001426; 400 mbs in Lake Baikal)
pA42B412 (12)	AF324206	839	99.0	*Pseudomonas saccharophila* (AB021407; not available)
pA47 (1)	AF324208	827	93.6	*Sphingobacterium heparinum* (M11657; not available)
pD4 (2)	AF324203	845	98.9	*Rubrobacter xylanophilus* (AJ243871; hot spring)
pD12 (1)	AF324204	838	93.5	*Alkalibacterium olivoapovlitic* (AF143513; olive wash water)

a. The number of individual clones sequenced that had this sequence.
b. The number of 16S rDNA nucleotides used for the alignment.
c. The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbour.

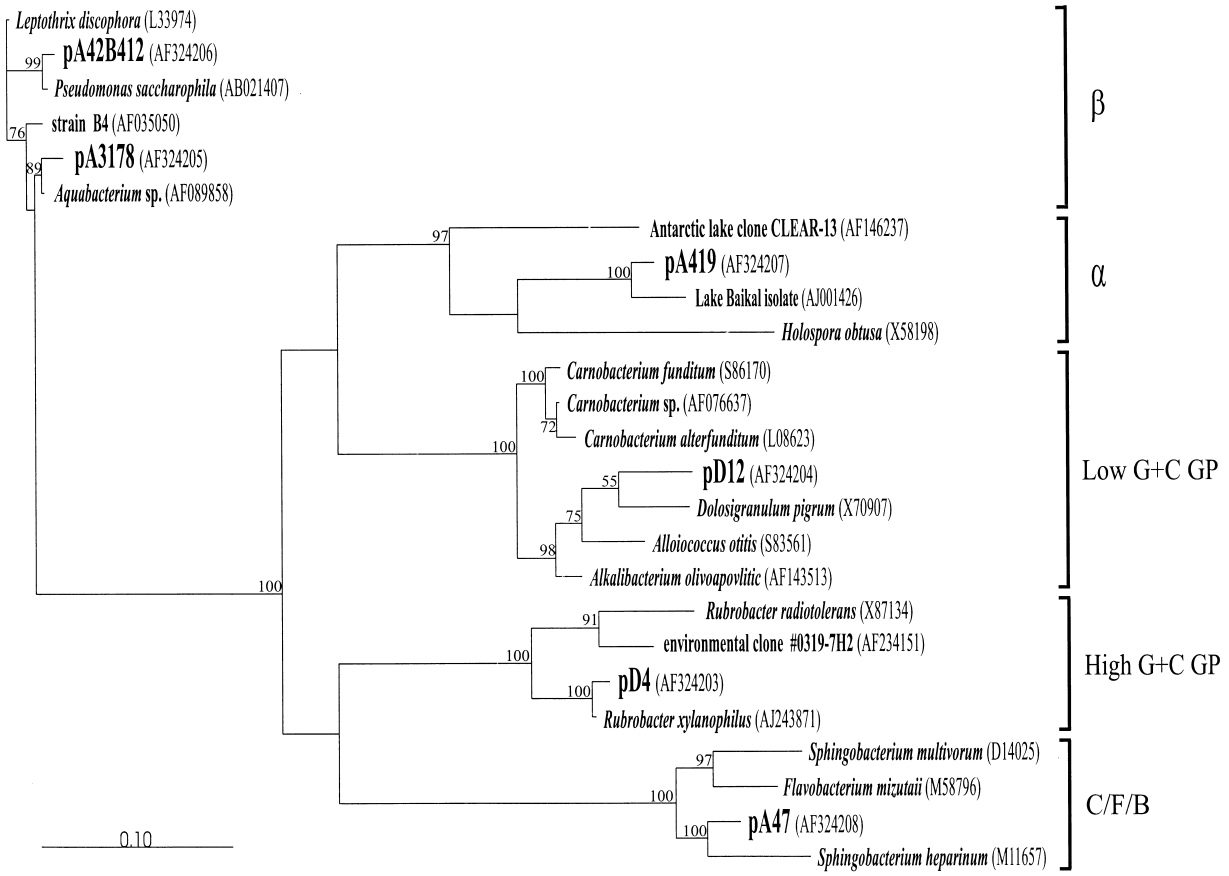

Fig. 3. Phylogenetic analysis of the 16S rDNAs amplified from core section 3593. Sequences, PCR-amplified from melt water, that correspond to nucleotides 515–1392 of the *E. coli* 16S rRNA-encoding gene were designated pA419, pA3178, pA42B412, pA47, pD4 and pD12. The phylogenetic tree was created with maximum likelihood using an 813 nucleotide mask of unambiguously aligned positions (Olsen *et al*., 1994). Bootstrap values generated from 100 replicates using the maximum parsimony method are shown at the nodes. Evolutionary distance is defined as the number of fixed nucleotide changes per position. The scale bar indicates 0.1 fixed substitutions per nucleotide position. GenBank accession numbers are provided in parentheses.

contains bacteria similar to species found in other permanently cold environments.

Experimental procedures

Ice core origin and sampling

A section of the deep Vostok ice core extending from 3591.965 to 3592.445 mbs, here designated core section 3593 (Fig. 1), was obtained from the National Ice Core Laboratory (Denver, CO, USA). Core 3593 was broken in transit, and the longer resulting section (≈33 cm) was subjected to automated melt water sampling. The sampling system melts ice and collects the resulting melt water automatically and aseptically only from the inside of an ice core (Christner *et al.*, 2000). All components of the system were sterilized by autoclaving, dried and then exposed to ethylene oxide for 12 h before the unit was assembled in a class-100 clean bench that was housed inside a walk-in freezer at −5°C. A dust-free band saw was used to cut a small section from one end of core 3593. The newly exposed end was immersed in 95% ethanol for 1 min before the core was positioned vertically in the sampling system with the funnel-shaped, ice-melting head flush against the ethanol-treated end of the core. The melting head was heated and moved up automatically through the interior of the core. The melt water generated was collected through an aperture in the centre of the melting head and was distributed aseptically into sterile collection vessels. The smaller fragment of section 3593 was washed with 95% ethanol and then with sterile, deionized water until the outermost ≈2 cm of ice had been removed. The ice remaining was placed in a sterile sealed container at 4°C to obtain melt water from this section. Measurements made using a Finnigan Mat mass spectrometer (Henderson *et al.*, 1999) revealed that the mean stable isotope ratios in the core were −56.23‰ for $\delta^{18}O$ and −446.24‰ for δD, similar to the values reported for samples from 3540 to 3750 mbs by Jouzel *et al.* (1999) and consistent with a water-freezing-to-ice origin.

Enrichment cultures

All manipulations involving melt water were undertaken inside a BioGard laminar flow hood (Baker Company) maintained at room temperature (≈22°C). Before use, all exposed surfaces of the hood were washed with 0.24% (w/v) sodium hypochlorite and irradiated with germicidal ultraviolet light. Melt water samples were collected over a period of 1–2 h into sterile ice-chilled vessels that were placed in the flow hood. Less than 2 h after melting, aliquots (1 ml) of the melt water were inoculated into nutrient-rich, nutrient-poor and minimal salts growth media (Table 3). Duplicates of these enrichment cultures (10 ml) were incubated aerobically and anaerobically, at 4°C and at 25°C. Included were enrichment culture conditions that are used routinely to grow acetogens, methanogens, methanotrophs, methylotrophs, nitrate and sulphate reducers and denitrifying bacteria. Bovine liver catalase (1 KU; Sigma) was added to enrichment cultures as a known enhancer of the recovery of bacteria from long periods of dormancy (Marthi *et al.*, 1991).

Colony isolations

Samples of melt water (100–150 ml) were filtered at room temperature through 0.2 μm pore size filters (Isopore GTTP04700 filters; Millipore), and the particulates collected were resuspended in 5 ml of phosphate-buffered physiological saline. Aliquots (200 μl) of these suspensions were spread onto the surface of agar-solidified media (Table 3), and duplicate plates were incubated aerobically and anaerobically at 4°C and at 25°C.

Direct amplification, cloning and sequencing of 16S rDNA molecules from melt water

A two-stage PCR procedure was used to amplify 16S rDNA molecules from melt water. All reagent transfers were undertaken within a sterilized laminar flow hood. Before

Table 3. Media[a] inoculated with melt water from ice core section 3593.

Medium	Aerobic (A) and/or anaerobic (AN)	Source/reference
M9 glucose minimal salts	A and AN[b]	Sambrook *et al.* (1989)
R2A	A and AN[b]	Reasoner and Geldreich (1985)
Ammonia minimal salts	A	Patt *et al.* (1974)
Nitrate minimal salts	A	Patt *et al.* (1974)
Basal salts[c] supplemented with H_2/CO_2, methanol, acetate and fructose ± BES	AN	C. M. Plugge; Woods Hole, MA. Microbial diversity course 1999
Basal salts[c] supplemented with H_2/CO_2, lactate, acetate and Na_2SO_4	AN	C. M. Plugge; Woods Hole, MA. Microbial diversity course 1999
Actinomycetes isolation	A	Difco
0.05% tryptone–yeast extract	A	Difco
1% nutrient broth	A and AN[b]	Difco
Tryptose blood	A	Difco

a. When used in plates, media were solidified by the addition of 1.5% (w/v) agar.
b. These anaerobic enrichment cultures also contained 50 mM KNO_3.
c. In 1 l of water: 1.2 g of NaCl, 0.3 g of NH_4Cl, 0.3 g of KCl, 0.4 g of $MgCl_2.6H_20$, 0.1 g of $CaCl_2$, 0.26 g of K_2HPO_4, 3.4 g of $NaHCO_3$, 0.2 g of $Na_2S.9H_2O$, 0.2 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.1 mg of Na_2WO_4, $AlCl_3.6H_2O$, 0.5 mg of resazurin, 0.2 g of cysteine, supplemented with 10 ml of the vitamin solution and 1 ml of the trace metals solution described by Touzel and Albagnac (1983) plus 1 ml of a solution that contained 6 mg of $Na_2SeO_3.5H_2O$, 8 mg of $Na_2WO_4.2H_2O$, 0.5 g of NaOH per 1 l of water. In addition, 0.16 mg of MES and 0.27 μg of $NiCl_2$ or 2.8 g of Na_2SO_4 and 60 μg of $FeSO_4$ were added to facilitate the growth of methanogens and sulphate-reducing bacteria respectively.

use, all reaction tubes and micropipette tips were autoclaved and exposed to ethylene oxide, and all solutions, except for the AmpliTaq Gold DNA polymerase (cat. no. N808-0240; Perkin-Elmer Biosystems), were passed through Microcon YM-100 filters (Millipore). Melt water (1, 10 or 60 μl aliquots) was added directly to PCR mixtures (PE buffer, 4 mM $MgCl_2$) that contained 5 pmol of the forward primers 21F (complementary to archaeal 16S rDNA; Reysenbach and Pace, 1995) or 27F (complementary to bacterial 16S rDNA; Lane, 1991) and 5 pmol of the universal reverse primer 1525R (Lane, 1991). Reaction mixtures were placed at 95°C for 9 min and then subjected to 43 cycles of PCR amplification by incubation for 1 min at 94°C, 1 min at 50°C and 1 min at 60°C, using the chemically modified, thermally activated AmpliTaq Gold DNA polymerase (PE Biosystems). An aliquot (2 μl) of the resulting product was used as the template in the second PCR, with the universal forward primer 515F combined with either reverse primer 1392R (complementary to both archaeal and bacterial 16S rDNAs) or the universal reverse primer 1492R (Lane, 1991; Reysenbach and Pace, 1995). Samples of the PCR products were subjected at each stage to agarose gel electrophoresis and visualized by ethidium bromide staining. DNA molecules of the expected length (≈900 bp) were amplified using the bacterial 16S rDNA primers (27F with 1525R, and 515F with 1392R or 1492R), but not when the archaeal-specific 21F primer was used, consistent with the observations of Priscu *et al*. (1999). Individual DNA molecules were cloned from the ≈900 bp populations into pGEM-Teasy (Promega), and both DNA strands were sequenced using primers that annealed the flanking T7 and SP6 promoter sequences. The sequences obtained were aligned and analysed for phylogenetic relationships using the ARB software package (Strunk *et al*., 1998) and the beta-4a version of PAUP 4.0 (Swofford, 1999).

16S rDNA sequences from bacterial isolates

Cells from a single colony of each isolate were resuspended, lysed and the region of their 16S rDNAs corresponding to nucleotides 27–1525 of the *Escherichia coli* 16S rDNA was amplified and both DNA strands sequenced (Zeng and Kreitman, 1996). Overlapping sequences were obtained using primers 515F, 534R, 907R, 1392R and/or 1492R (Medlin *et al*., 1988; Lane, 1991) to prime the sequencing reactions. The sequences obtained were subjected to phylogenetic evaluation using the ARB software and the beta-4a version of PAUP (Strunk *et al*., 1998; Swofford, 1999).

Electron microscopy

For electron microscopy, cells were concentrated by filtration onto 0.2 μm Isopore filters (Millipore), fixed for 16 h in 3% (v/v) glutaraldehyde in phosphate-buffered saline and then for 1 h in 1% (w/v) osmium tetroxide. The fixed cells were dehydrated by sequential passage through increasing concentrations of ethanol [50–100% (v/v) ethanol in 10% increments], dried in a Pelco CPD-2 critical point dryer (Ted Pella), coated with gold–palladium for 60 s in a Pelco 3 sputter coater and visualized using a Philips XL30 scanning electron microscope.

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