Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods

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Abstract

A combination of culture-dependent and culture-independent methodologies (*Bacteria* and *Archaea* 16S rRNA gene clone library analyses) was used to determine the microbial diversity present within a geographically distinct high Arctic permafrost sample. Culturable *Bacteria* isolates, identified by 16S rRNA gene sequencing, belonged to the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria* with spore-forming *Firmicutes* being the most abundant; the majority of the isolates (19/23) were psychrotolerant, some (11/23) were halotolerant, and three isolates grew at $-5\,^{\circ}C$. A *Bacteria* 16S rRNA gene library containing 101 clones was composed of 42 phylotypes related to diverse phylogenetic groups including the *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Cytophaga – Flavobacteria – Bacteroides*, *Planctomyces* and *Gemmatimonadetes*; the bacterial 16S rRNA gene phylotypes were dominated by *Actinobacteria*- and *Proteobacteria*-related sequences. An *Archaea* 16S rRNA gene clone library containing 56 clones was made up of 11 phylotypes and contained sequences related to both of the major *Archaea* domains (*Euryarchaeota* and *Crenarchaeota*); the majority of sequences in the *Archaea* library were related to halophilic *Archaea*. Characterization of the microbial diversity existing within permafrost environments is important as it will lead to a better understanding of how microorganisms function and survive in such extreme cryoenvironments.

Introduction

A significant development in microbial ecology and evolution has been the realization that microbial life, primarily prokaryotic life, is extremely hardy and can survive and indeed thrive in environments previously thought uninhabitable on Earth. Permafrost is defined as soil that remains at or below $0\,^{\circ}C$ for at least two consecutive years and can extend hundreds of meters to >1000 m into the subsurface (Williams & Smith, 1989). Permafrost represents *c.* 26% of terrestrial soil ecosystems (Williams & Smith, 1989) including 24% of the northern hemisphere landmass (Zhang *et al.*, 1999), yet its biology, essentially microbiology, remains relatively unexplored. Most recent reports of permafrost microbial communities originated from Siberian permafrost samples (Gilichinsky *et al.*, 1989; Khlebnikova *et al.*, 1990; Shi *et al.*, 1997; Vishnivetskaya *et al.*, 2000; Bakermans *et al.*, 2003) and demonstrated that Siberian permafrost harbors a diverse viable microbial community including methanogens, sulfur-reducing bacteria, and both aerobic and anaerobic heterotrophs (Gilichinsky, 2002). Earlier studies have described the recovery of bacteria from Canadian (James & Southerland, 1942) and Alaskan (Boyd & Boyd, 1964) permafrost from southern Arctic latitudes while investigations of Antarctic permafrost identified the predominant culturable isolates (Vorobyova *et al.*, 1997).

Microorganisms isolated from permafrost survive geological time-scales at subzero temperatures, yet the majority of permafrost isolates have optimum growth temperatures at or above $20\,^{\circ}C$ (Rivkina *et al.*, 2004) rather than being true psychrophiles with maximum growth temperatures below $20\,^{\circ}C$ (Morita, 1975). Microscopic investigations of endogenous Siberian permafrost microorganisms revealed bacterial populations dominated by cells with altered ultrastructural characteristics such as thickened capsular layers and intracellular aggregates (Soina *et al.*, 1995, 2004).

A Siberian permafrost microbial population was also characterized by a complete lack of cells undergoing division, and up to 80% of the cells were represented by so-called 'dwarf cells' of 0.1–0.4 μm in diameter (Vorobyova *et al.*, 2001). These investigations have indicated that a large population of bacterial cells may be present in a resting or dormant state (Friedmann, 1994; Vorobyova *et al.*, 2001).

Several recent studies have demonstrated microbial activity in permafrost and other cryoenvironments at ambient subzero temperatures (reviewed by Steven *et al.*, 2006). For example, methane evolution at temperatures as low as $-16.5\,^{\circ}C$ (Rivkina *et al.*, 2004) and incorporation of acetate into lipids at $-10\,^{\circ}C$ (Rivkina *et al.*, 2000) were observed in Siberian permafrost samples. Indirect evidence, such as the presence of 'ancient' viable microorganisms in permafrost up to millions of years old, implies the presence of active DNA repair systems, which at least neutralize the cumulative effects of background terrestrial gamma radiation, which can result in doses as high as $2\,mGy\,year^{-1}$ (Gilichinsky, 2002). These studies suggest that functional microbial ecosystems exist within the permafrost environment, which could have important implications for global biogeochemical processes, including global carbon cycling. For example, permafrost contains up to 30% or more of all the carbon stored in soils worldwide, raising grave concerns about additional emissions of greenhouse gases from thawing permafrost soils (Lawrence & Slater, 2005); as permafrost thaws, it could lead to large-scale emissions of methane or carbon dioxide beyond those produced by fossil fuels (Lawrence & Slater, 2005). Terrestrial permafrost environments are also considered significant astrobiology analogs (Gilichinsky, 2002; Jakosky *et al.*, 2003; Rivkina *et al.*, 2004) with the recent evidence of massive amounts of shallow subsurface ground ice on Mars (Boynton *et al.*, 2002; Gilichinsky *et al.*, 2003; Jakosky *et al.*, 2003); permafrost and ground ice deposits on Mars are primary astrobiology targets for future Mars exploration missions.

Almost all previous studies describing the microbial diversity of permafrost microbial communities have relied on the identification and characterization of culturable cells which may only represent $\leq$ 0.1–1% of the total microbial community (Rappé & Giovannoni, 2003). Culture-independent methods increasingly used in environmental microbiology bypass the shortcomings of studying culturable microorganisms by extracting and analysing total nucleic acids, theoretically representing the entire microbial population from environmental samples (Spiegelman *et al.*, 2005). The objective of this study was to use a combination of culture-dependent and culture-independent methodologies (*Bacteria* and *Archaea* 16S rRNA gene clone library analyses) to determine the microbial diversity present within a geographically distinct high Arctic permafrost sample. A combination of the two approaches should result in a more complete characterization of the microbial diversity existing within permafrost environments and will lead to a better understanding of how microorganisms function and survive in such extreme cryoenvironments.

Materials and methods

Study site and aseptic sampling

A permafrost sample from a depth of 9 m was aseptically collected from Eureka (79°59′41″N, 85°48′48″W), Ellesmere Island, Nunavut Canada in May 2003, as part of a collaboration with the NASA Astrobiology Science and Technology Instrument Development (ASTID) Mars Deep Drill Project. The sample was returned frozen to Montreal, and stored at $-20\,^{\circ}C$ until sampling. A procedure for monitoring contamination of the drilling process and downstream laboratory processing was employed (Juck *et al.*, 2005) and indicated that the 9-m sample was not contaminated during handling and was suitable for both culture-dependent and culture-independent analyses.

Physical and chemical characterization of the 9-m permafrost sample

Total carbon and nitrogen were measured by combustion at $900\,^{\circ}C$ (Lim & Jackson, 1982) with a Carlo Erba Flash EA NC Soils Analyser, and dissolved organic carbon was detected using a Shimadzu TOC-V carbon analyser. The nitrate level was determined by the colorimetric cadmium reduction-diazotization method with a Lachat Quik-Chem AE flow-injection autoanalyser. Permafrost soil pH and electroconductivity were determined using standard methods in a 1:2 soil/deionized water slurry (Rhoades, 1982). Ambient permafrost temperatures were monitored *in situ* for 1 year in the permafrost borehole at depths of 5, 12 and 15 m using Campbell 107B temperature probes (Campbell Scientific Corp.) and a CR10 data logger (Durham Geo.).

Microbial enumeration and characterization

Culturable heterotrophic bacteria were enumerated by the spread plate method. A permafrost suspension was prepared by adding a 5-g composite permafrost sample to a prechilled sterile glass tube (25 × 150 mm) containing 2.5 g of 3-mm-diameter glass beads (Fisher Scientific). The permafrost sample was diluted with 15 mL of 0.1% w/v cold sodium pyrophosphate ($Na_4P_2O_7.10H_2O$, pH 7.0) and vortexed for 2 min. Appropriate dilutions, prepared in cold 0.1% sodium pyrophosphate, were surface spread on R2A agar (Becton, Dickson and Co.), half-strength R2A plates [prepared as R2B (broth) diluted 1:1 with H_2O and supplemented with agar (1.5% final concentration)], trypticase soy agar (TSA; Becton, Dickson), minimal salts medium (MSM) or YTS

(Greer *et al.*, 1993). Plates were incubated at 37, 25 and 5 °C until growth of new colonies was no longer detected (3, 9 and 33 days, respectively). Plate counts were performed in triplicate and the results are presented as the mean of triplicate assays. Colonies from plates with > 100 CFU were differentiated based on colony morphology, pigmentation and growth characteristics (e.g. time of colony appearance, and media for isolation). Genomic DNA was isolated by boiling lysis (Sambrook & Russell, 2001) from representatives of each group and 16S rRNA genes were amplified by PCR, sequenced and analysed as described below; *c.* 60 colonies were originally selected for 16S rRNA gene sequencing and isolates sharing greater than 97% sequence identity were considered to belong to the same taxon. Phenotypic characterization of the strains was determined by subculturing on to R2A, R2A supplemented with 7% NaCl (salt tolerance) or with 7% sucrose for detecting growth at −5 °C. Plates were then incubated at 37 °C for 3 days, 25 °C for 9 days, 5 °C for 1 month and −5 °C for 3 months. Salt tolerance was determined at 5 and 25 °C.

Direct microbial counts were performed on the permafrost sample using 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) using the general method described by Kepner & Pratt (1994). A 1-g permafrost sample was dispersed in a 0.1% solution of $Na_4P_2O_7$ and diluted 10^4-fold. Cells were then stained for 1 h in a 0.5 mg mL^{-1} solution of DTAF in the dark. The soil suspension was then filtered onto 25-mm black polycarbonate 0.22-μm-pore filters (Osmonics Inc.). Ten random fields were counted at × 100 magnification and the total bacterial count is reported as the average count from the 10 fields.

Community DNA extraction and amplification of 16S rRNA genes

Total community DNA was extracted from 10 g of core, using the method of Miller *et al.* (1999); the permafrost sample was collected as a composite sample taken from within the 9-m section of the core. Extracted permafrost DNA was then purified using polyvinylpolypyrrolidone spin columns as previously described (Berthelet *et al.*, 1996). Bacterial 16S rRNA genes were amplified from the permafrost DNA extract by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 758R (5′-CTACCAGGGTATCTAATCC-3′) and archaeal 16S rRNA genes were amplified using primers 333Fa (5′-TCCAGGCCCTACGGG-3′) and A934R (5′-GTGCTCCCCCGCCAATTCCT-3′). Individual reagents and their concentrations or amounts used in a 50-μL PCR reaction were as follows: 5 U of *Taq* polymerase (Invitrogen Canada), 1X PCR buffer, 1.5 mM $MgCl_2$ (both supplied with the *Taq* DNA polymerase), 0.6 μL of 10 mg mL^{-1} bovine serum albumin, 0.2 mM of each deoxynucleoside triphosphate, 0.5 μL of each primer (10 μM concentration), and 5 μL of template DNA (5–50 ng). To minimize nonspecific amplification a touchdown PCR program was used, consisting of an initial denaturation step of 5 min at 96 °C, followed by 10 touchdown cycles (65–55 °C), and 20 further cycles at 55 °C for 1 min, followed by 72 °C for 1 min and a final extension of 72 °C for 5 min.

Construction and analyses of permafrost 16S rRNA gene clone libraries

Amplified 16S rRNA genes were purified using the QIAquick PCR purification kit (Qiagen Inc.) and the cleaned PCR product was ligated into the pGEM-T Easy vector system as described by the supplier (Promega). Plasmid DNA was transformed into *Escherichia coli* strain DH5α with a standard transformation protocol (Sambrook & Russell, 2001). Plasmids containing the ligated 16S rRNA gene inserts were identified using blue/white screening on Luria–Bertani plates containing 100 μg mL^{-1} ampicillin (FisherBiotech) and 80 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; FisherBiotech).

Plasmid DNA from candidate colonies was purified using boiling lysis (Sambrook & Russell, 2001) and used as a template for reamplification of cloned 16S rRNA genes. Amplification of clones was performed using primers T7 and Sp6 targeted to the pGemT plasmid to avoid amplification of the 16S rRNA genes of *E. coli*. PCR reaction mixtures were as described above with the exception that 1 μL of DNA extract (*c.* 50 ng) was used in the reaction. The PCR conditions used were as follows: an initial denaturation of 5 min at 96 °C; 25 cycles of 1 min denaturation at 96 °C, 1 min 30 s annealing at 55 °C, followed by 72 °C for 1 min, and a final extension of 72 °C for 5 min.

Amplified ribosomal DNA restriction analysis (ARDRA) was used to distinguish and classify cloned 16S rRNA gene sequences. Amplified 16S rRNA genes were restricted using the enzymes *RsaI* and *HhaI* (Invitrogen) at 37 °C for 3 h. Restriction digests (20 μL) were analysed by agarose (4% TBE NuSieve 3:1, Mandel Scientific) gel electrophoresis in 1X TAE with ethidium bromide staining (Sambrook & Russell, 2001). Unique restriction patterns were identified visually and representatives of each restriction pattern were used as a template for 16S rRNA gene sequencing. Sequencing was performed at the Genome Québec Innovation Centre (McGill University) using 3730XL DNA analyser systems (Applied Biosystems).

Phylogenetic and statistical analyses of permafrost 16S rRNA gene sequences

16S rRNA gene sequences were compared against the GenBank database using the nucleotide Basic Local Alignment Search Tool (BLASTN) (Altscul *et al.*, 1990) and the

Ribosomal Database Project (RDP) using the Sequence Match software (Cole *et al.*, 2005) to identify the closest relative to the queried 16S rRNA gene clone sequence. Alignments were constructed using the CLUSTAL W program in the MACVECTOR 7.0 software package (Oxford Molecular Ltd). Phylogenetic trees were constructed using a neighbor-joining algorithm with the Jukes-Cantor model; bootstrap values were performed with 1000 replicates. Sequences were checked for chimeras using the Bellerophon server (Huber *et al.*, 2004) and the CHIMERA CHECK program on the RDP II database (Cole *et al.*, 2005). Coverage of the clone libraries was estimated using Good's method with the formula $[1-(n/N)]\times 100$, where n is the number of sequences represented by a single clone and N is the total number of sequences analysed (Good, 1953; Kemp & Aller, 2004).

Nucleotide sequence accession numbers

16S rRNA gene sequences have been deposited in the GenBank database. Accession numbers for *Bacteria* isolates are DQ444972–DQ444994, for *Bacteria*-related clones DQ444995–DQ445037, and *Archaea*-related clones DQ445038–DQ445048. Accession numbers for reference sequences used in phylogenetic studies are indicated in the relevant figures.

Results

Physical and chemical characterization of permafrost

The mean annual air temperature at Eureka, based on 55 years of data, is $-19.7\,^{\circ}$C (W. H. Pollard, personal communication). Ambient *in situ* permafrost temperatures of the borehole from which the 9-m sample was obtained were determined as follows: 15 m, $-17.5\,^{\circ}$C with less than $1\,^{\circ}$C annual variation; 12 m, $-17\,^{\circ}$C with $1.5\,^{\circ}$C annual variation; 5 m, $-18\,^{\circ}$C with $3-4\,^{\circ}$C annual variation. The depth of the active layer (depth of seasonal thaw) at this location is on average 70 cm (Pollard, 1991, 2000). The age and origin of permafrost is related to marine deposition during the late Pleistocene and subsequent Holocene sea-level change. Using the sea-level emergence curve published by Pollard & Bell (1998), the estimated age of permafrost for the Eureka site is between 5000 and 7000–years-old. Physical and chemical parameters of the Eureka permafrost sample are given in Table 1. The electroconductivity measurement was equivalent to a salt concentration of $\sim$14.6 g kg^{-1} (Rhoades, 1982).

Table 1. Physical and chemical characterization of Eur1 9-m permafrost

Permafrost characteristic	Value
Temperature	$\sim -16\,^{\circ}$C*
pH	6.5
Gravimetric water content	21.3%†
Electrical conductivity	11.42 dS m^{-1}
Total carbon	2.19%†
Dissolved organic carbon	12.41 mg kg^{-1}
Total nitrogen	0.14%†
Nitrate	ND‡

*Estimated from thermal profile measurements.
†Percentage of dry weight of permafrost.
‡Not detected. Detection level = 0.5 mg L^{-1}.

Microbial enumeration and characterization of permafrost isolates

Enumeration of viable heterotrophic bacteria was performed with four media and revealed a low number of viable cells in the permafrost, showing a maximum of 6.9×10^{3} CFU g^{-1} on R2A agar incubated at $5\,^{\circ}$C. Incubation at $5\,^{\circ}$C increased viable cell counts about three-fold compared with incubation on R2A at $25\,^{\circ}$C, suggesting that low temperatures aided in the culturing of viable cells. DTAF staining of the sample revealed total bacterial counts of 3.56×10^{7} g^{-1}, indicating that culturable heterotrophs only accounted for *c.* 0.02% of the total bacterial community.

Based on colony morphology, growth characteristics and 16S rRNA gene sequencing, 23/60 isolates were identified as potentially unique and further characterized. The majority (20/23) of the isolates showed $\geq$97% sequence similarity to 16S rRNA gene sequences from cultured organisms in the GenBank database, suggesting they are strains of previously described species (Table 2). Three isolates (Eur1 9.5, 9.15, 9.33) showed sufficient 16S rRNA gene sequence divergence from previously cultured organisms (> 3%; Keswani & Whitman, 2001), suggesting that they may represent novel species, although further work would be required to validate their taxonomic position (Table 2). Sixteen isolates belonged to the phylum *Firmicutes*, with three genera represented: *Bacillus*, *Sporosarcina* and *Paenibacillus* (Table 2). The remaining isolates were related to bacteria from the phyla *Actinobacteria* (genera: *Arthrobacter*, *Micrococcus*, *Kocuria* and *Rhodococcus*) and *Proteobacteria* (genus: *Pseudomonas*).

Most of the Eureka permafrost isolates (19/23) were psychrotolerant, while three of these isolates (Eur1 9.15, Eur1 9.41 and Eur1 9.51) showed growth at subzero temperatures as low as $-5\,^{\circ}$C. Only one true psychrophile (Eur1 9.12) was identified in this study while Eur1 9.1 demonstrated mesophilic growth and could not be grown at temperatures below $20\,^{\circ}$C. Three of the strains (Eur1 9.10, Eur1 9.21, Eur1 9.43) were identified by 16S rRNA gene sequencing but could not be maintained in serial culture for phenotypic characterization. Many of the isolates (11/23) were also halotolerant and able to grow readily on R2A supplemented with 7% NaCl while the growth of all other strains was completely inhibited or significantly retarded (compared with growth in R2A not containing 7% NaCl).

Analyses of the *Bacteria* 16S rRNA gene clone library

The permafrost 16S rRNA gene clone library constructed with *Bacteria*-specific primers consisted of 101 clones and comprised 42 different ARDRA patterns. Representative clones of each unique ARDRA pattern were sequenced and unsequenced clones with identical ARDRA patterns were assigned the same phylotype. The frequency of ARDRA patterns in the clone library was used to calculate coverage of the library, which provides an estimate of the extent to which the sequences in the library represent the total population; coverage of the *Bacteria* clone library was estimated to be 69%. The composition and abundance of the phylogenetic groups identified in the *Bacteria* 16S rRNA gene library are shown in Fig. 1a. However, owing to the biases introduced into molecular surveys from PCR and DNA extraction protocols (Martin-Laurent *et al.*, 2001; Forney *et al.*, 2004), the abundance of phylotypes in the library only represents an estimate of the actual abundance of organisms in the sample (von Wintzingerode *et al.*, 1997). Sequences related to the *Actinobacteria* (38% of the clone library; Fig 1a) clustered into three groups and consisted of six phylotypes (Fig. 2) including the most commonly encountered phylotype in the library, Eur1Bac10 (20% of the clone library). Although *Firmicute*-related bacteria were the most abundant among the cultured isolates, only two of the 16S rRNA gene sequences were identified as members of the *Firmicutes* and these sequences were only distantly related ($\leq$ 85% rRNA gene sequence similarity) to any of the *Firmicute* strains isolated from Eureka permafrost. *Proteobacteria*-related clones were the most heterogeneous group in the library with 23 phylotypes related to the alpha, beta, gamma and delta subclasses (Fig. 3), with the delta subclass being most abundant (43% of *Proteobacteria*-related clones; Fig. 1a). Three of the *Deltaproteobacteria* sequences were highly related ($>$ 97% sequence similarity) to the sulfate-reducing genera *Desulfobulbus* and *Desulfuromonas* (Fig. 3). The *Alphaproteobacteria* showed the highest diversity among the *Proteobacteria*, consisting of eight phylotypes; one clone (Eur1Bac4) was closely related (98% rRNA gene sequence similarity) to *Paracoccus aminophilus*, a facultative sulfur-oxidizing chemolithotroph (Urakami *et al.*, 1990). The *Gammaproteobacteria* represented 20% of *Proteobacteria* clones and consisted of six phylotypes. Clone sequences Eur1Bac9, Eur1Bac56 and Eur1Bac24 were closely related to the genera *Pseudomonas*, *Lysobacter* and *Acidithiobacillus*, respectively. The *Betaproteobacteria* showed the lowest diversity and abundance (Fig. 1a) among the *Proteobacteria* clones, consisting of only a single phylotype (Fig. 3),

Table 2. Phylogenetic relationships of permafrost isolates to cultured organisms in the public database

Isolate	Closest relative	% Similarity	Phylogenetic affiliation
Eur1 9.1	*Micrococcus* sp. Ellin149	99	Act
Eur1 9.2	*Paenibacillus terrae*	99	Fir
Eur1 9.4	*Bacillus* sp. DU	100	Fir
Eur1 9.5	*Bacillus* bacterium Gsoil 1105	95	Fir
Eur1 9.6	*Bacillus muralis*	99	Fir
Eur1 9.8	*Sporosarcina* sp. S11-2	99	Fir
Eur1 9.9	Glacier bacterium FJS42	98	Fir
Eur1 9.10	*Sporosarcina macmurdoensis*	99	Fir
Eur1 9.12	*Bacillus* sp. GD0402	99	Fir
Eur1 9.15	*Arthrobacter* sp. SMCC ZAT262	93	Act
Eur1 9.19	*Bacillus weihenstephanensis*	99	Fir
Eur1 9.21	*Sporosarcina* sp. Tibet-S2a1	99	Fir
Eur1 9.24	*Bacillus baekryungensis*	99	Fir
Eur1 9.25	*Paenibacillus wynnii*	98	Fir
Eur1 9.26	*Paenibacillus* sp. TRO4	98	Fir
Eur1 9.27	*Paenibacillus pabuli*	99	Fir
Eur1 9.33	*Brachybacterium* sp. SKJH-25	94	Act
Eur1 9.35	*Paenibacillus* sp. DSM 1352	98	Fir
Eur1 9.39	*Paenibacillus* sp. 19508	99	Fir
Eur1 9.41	*Pseudomonas reactans*	100	Pro
Eur1 9.43	*Kocuria* sp. JL-75	100	Act
Eur1 9.51	*Arthrobacter citreus*	98	Act
Eur1 9.57	*Rhodococcus* sp. NPO-JL-61	99	Act

Act, Actinobacteria; Fir, Firmicutes; Pro, Proteobacteria.

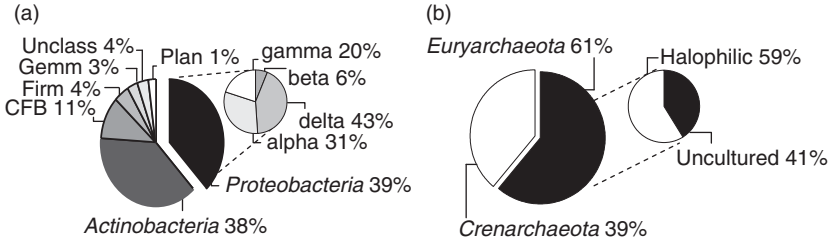

Fig. 1. Phylotype composition of the two clone libraries. (a) *Bacteria* clone library. The larger circle represents the proportion of phylotypes as a percentage of the total library while the smaller inset circle represents the proportion of *Proteobacteria* subdivisions as a percentage of *Proteobacteria*-related clones. (b) *Archaea* clone library. The larger circle represents the proportion of phylotypes as a percentage of the total library while the inset circle indicates the proportion of *Euryarchaeota* that could be identified as being halophilic *Archaea* versus uncultured environmental sequences. Firm, *Firmicutes*; Gemm, *Gemmatimonadetes*; Unclass, unclassified; Plan, *Planctomyces*.

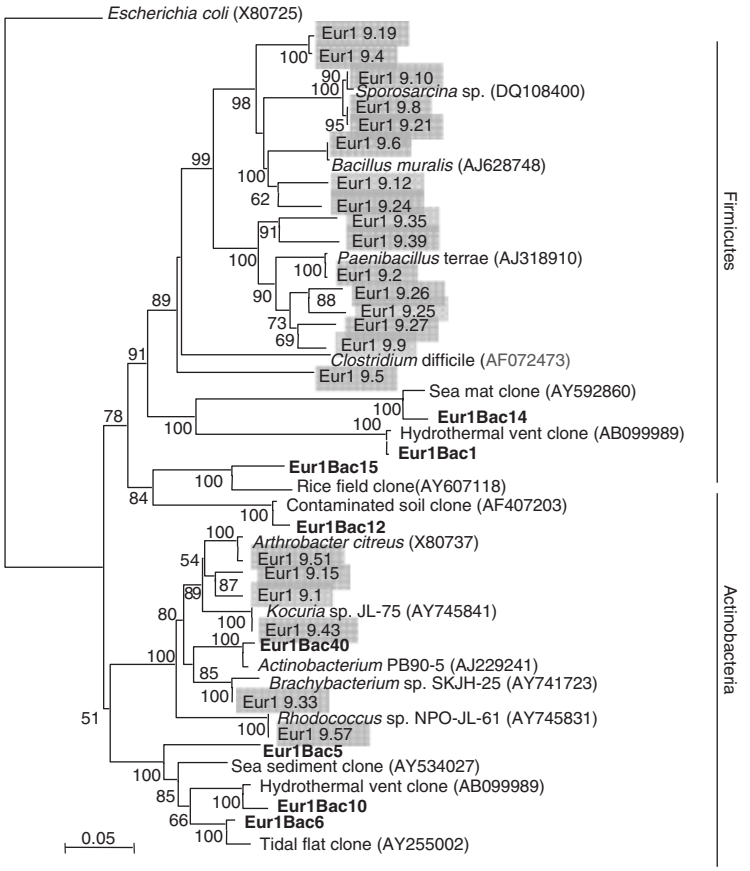

Fig. 2. Phylogenetic relationships of clone sequences and 16S rRNA gene sequences from permafrost isolates related to the *Firmicutes* and *Actinobacteria*. Bootstrap values ≥50% (1000 replicates) are indicated at the nodes. Clone sequences from this study are indicated in bold and 16S rRNA gene sequences from permafrost isolates are highlighted. The scale bar represents the expected number of changes per nucleotide position.

which was only distantly related to described species of *Betaproteobacteria*. The *Cytophaga–Flexibacter–Bacteroides* (CFB) (11% of clones) consisted of six phylotypes; Eur1-Bac50 was closely related (98% rRNA gene sequence identity) to *Gillisia mitskevichiae*, a heterotrophic seawater isolate (Nedashkovskaya *et al.*, 2005). Two phylotypes were related to the phylum *Gemmatimonadetes* and a single phylotype was affiliated with the phylum *Planctomyces* (Fig. 4). Eur1Bac61 did not cluster with any of the other clone sequences and could not be classified into any described bacterial groups (Fig. 4); this is unlikely to be due to a PCR artifact as the phylotype was recovered from the clone library several times (4% of clones; Fig. 1a) and sequencing of independent clones returned similar results. The majority of the 16S rRNA genes were only distantly related to described bacterial species and 28 of the 42 phylotypes had less than 97% sequence similarity to their nearest neighbor in the GenBank database, suggesting the possibility of novel bacterial diversity in Eureka permafrost. Of the 42 phylotypes, only two were related to isolates recovered from the Eureka permafrost: isolate Eur1 9.33 clustered with the *Actinobacteria* and had 88% rRNA gene sequence similarity to Eur1Bac40 (Fig. 2); isolate Eur1 9.41 clustered with the *Gammaproteobacteria* and showed 95% sequence similarity to the clone sequence Eur1Bac9 (Fig. 3).

Permafrost Archaeal clone library

Fifty-six *Archaea* 16S rRNA gene clones were screened by ARDRA and grouped into 11 phylotypes, representing an estimated coverage of 79%. Sequences related to both of the major Archaeal domains were identified, with the *Euryarchaeota* being more abundant (Fig. 1b). The three *Crenarchaeota* phylotypes were all related to uncultured 16S rRNA gene sequences from environmental samples (Fig. 5). Of the eight ARDRA patterns that made up the *Euryarchaeota* clones, two were most closely related to 16S rRNA genes from cultured halophilic *Archaea* of the genera *Halobaculum* and *Haloarcula* (Fig. 5). Seven of the *Archaea* phylotypes had less than 97% sequence similarity to their closest relatives in the public database, suggesting the possibility of novel species.

Discussion

The 9-m permafrost sample from Eureka, Ellesmere Island, represents the most northern latitude from which a

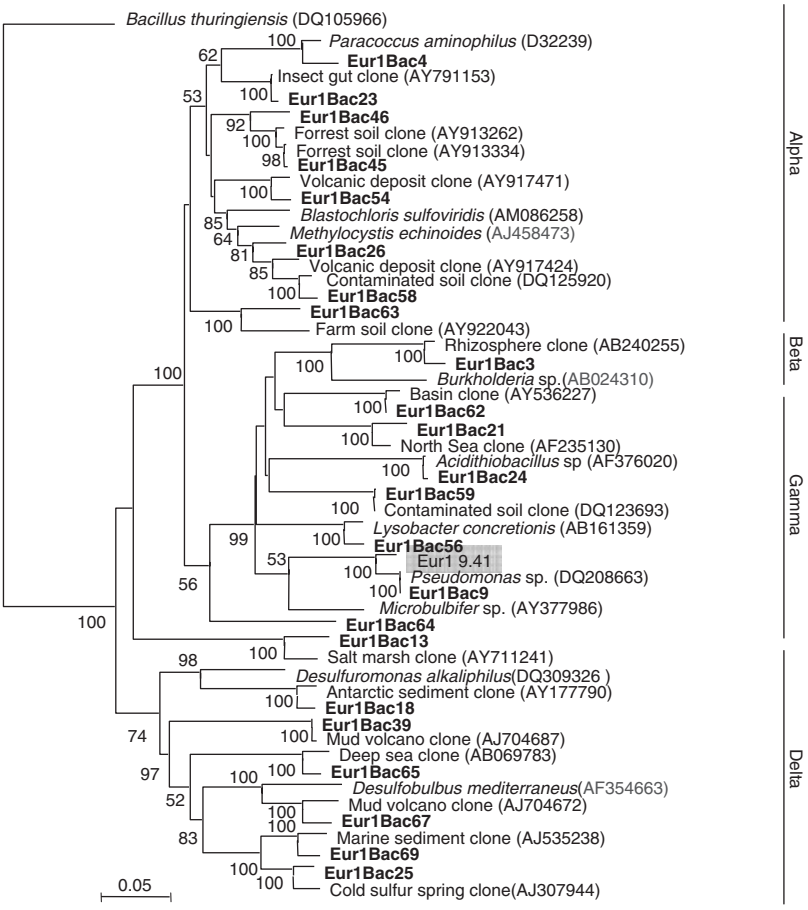

Fig. 3. Phylogenetic relationships of clone sequences and 16S rRNA gene sequences from permafrost isolates related to the *Proteobacteria*. Bootstrap values ≥50% (1000 replicates) are indicated at the nodes. Clone sequences from this study are indicated in bold and 16S rRNA gene sequences from permafrost isolates are highlighted. The scale bar represents the expected number of changes per nucleotide position.

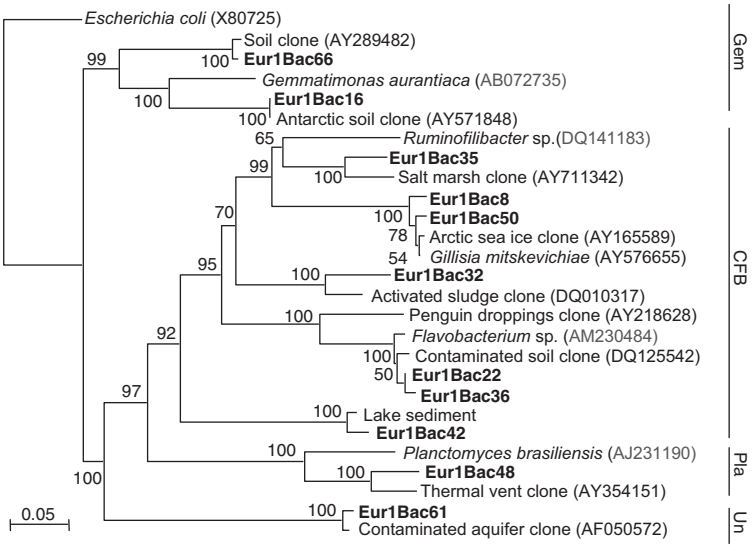

Fig. 4. Phylogenetic relationships of clone sequences related to the CFB, *Gemmatimonadetes* (Gem), *Planctomyces* (Pla) and unclassified (Un) bacterial divisions. Bootstrap values ≥50% (1000 replicates) are indicated at the nodes. Clone sequences from this study are indicated in bold. The scale bar represents the expected number of changes per nucleotide position.

permafrost microbial community has been characterized. The physical/chemical characteristics of Eureka permafrost (Table 1) fall in the range of permafrost samples taken from different geographic regions (reviewed by Steven *et al.*, 2006). As expected, the ambient temperature of the high Arctic permafrost (*c.* −16 °C) from *c.* 80°N was lower than previously characterized Siberian permafrost (*c.* −11 °C) from *c.* 67–70°N but was more similar to Antarctic

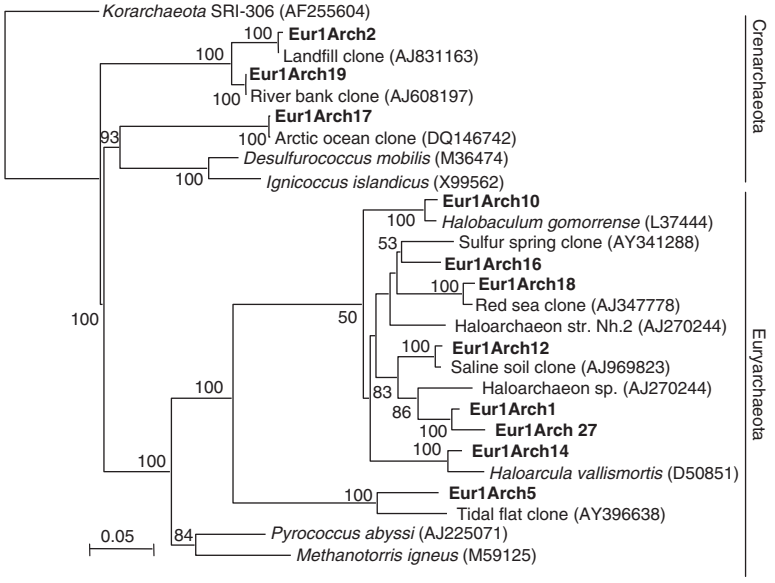

Fig. 5. Phylogenetic relationships of clone sequences related to the *Archaea*. Bootstrap values ≥50% (1000 replicates) are indicated at the nodes. Clone sequences from this study are indicated in bold. The scale bar represents the expected number of changes per nucleotide position.

permafrost (−18 °C to −27 °C; Vorobyova *et al.*, 1997). However, Antarctic permafrost is characterized by relatively low carbon content (total carbon ranged from 0 to 0.43%, Vorobyova *et al.*, 1997). Given the combination of low temperature, relatively high carbon content and northern latitude, this Eureka permafrost sample represents a distinct permafrost environment compared with previously described Siberian and Antarctic permafrost samples.

Heterotrophic cell counts from permafrost can vary over a large range. In Siberian permafrost, viable cell counts ranged from 0 to 10^8 CFU g^{-1} (Gilichinsky, 2002; Steven *et al.*, 2006), while total counts based on fluorescence microscopy were consistently in the range 10^7–10^8 cells g^{-1} (Rivkina *et al.*, 1998). Viable cell counts from Eureka permafrost indicated a low number of viable heterotrophs, with the majority of the isolates related to spore-forming bacteria (*Bacillus*, *Paenibacillus* and *Sporosarcina*; Table 2). These results differ from observations in Siberian permafrost where only *c.* 30% of isolates formed endospores (Shi *et al.*, 1997) and no spores could be detected *in situ* using transmission electron microscopy (Soina *et al.*, 2004). In addition, *Proteobacteria* made up a substantial proportion of Siberian isolates (34%) (Shi *et al.*, 1997), while only one *Proteobacteria*-related isolate was identified from Eureka permafrost. The majority of Eureka permafrost isolates were psychrotolerant rather than psychrophilic, which appears to be a common adaptation among permafrost bacteria; up to 95% of Siberian permafrost isolates also show psychrotolerant growth (Gilichinsky, 2002). Interestingly, three of the Eureka isolates were capable of subzero growth at temperatures of at least −5 °C. Defining the low temperature limits for microbial growth and metabolic activities of these isolates will be important in determining if permafrost organisms are capable of growth and activity at ambient subzero permafrost temperatures.

Owing to the technical and logistical problems as well as the very high costs associated with obtaining deep permafrost cores from such northern latitudes, the results of this study are from a single composite permafrost sample, and therefore only cautious comparisons can be made with other permafrost environments. However, sequencing of 16S rRNA genes from Eureka permafrost isolates has led to the identification of many of the same phylogenetic lineages identified from Siberian permafrost. Siberian permafrost isolates were identified as representing members of the *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Shi *et al.*, 1997), the same phyla as the Eureka permafrost isolates (Table 2). Clone libraries recently constructed from *Bacteria* 16S rRNA genes from Siberian permafrost contained sequences belonging to the *Actinobacteria*, *Firmicutes*, *Proteobacteria* and CFB group (Vishnivetskaya *et al.*, 2006), the same taxonomic groups that dominated the Eureka *Bacteria* clone library. However, the Eureka clone library also contained sequences related to the *Gemmatimonadetes* and *Planctomyces*, the first time these phylogenetic groups have been detected in permafrost.

Recent studies using culture-independent methods have demonstrated that active layer soils at northern latitudes can harbor diverse microbial communities (Zhou *et al.*, 1997; Neufeld & Mohn, 2005). The high coverage that was achieved with a relatively small number of clones in the Eureka and Siberian permafrost clone libraries (Vishnivetskaya *et al.*, 2006) suggests that bacterial diversity in permafrost is low compared with the active layer. Active layer soils are exposed to seasonal variations in temperature that reach above freezing for at least several weeks during the year. Permafrost,

however, is characterized by constant subzero temperatures, which means permafrost microorganisms must survive in an environment characterized by low water activity, extremely low rates of nutrient exchange, and must endure prolonged exposure to subfreezing temperatures and background radiation (Steven *et al.*, 2006). These factors may act to limit bacterial diversity and species composition in permafrost compared with overlying active layer soil.

To our knowledge, this study represents the first comprehensive characterization of the *Bacteria* and *Archaea* microbial communities in permafrost using a culture-independent method. Using 16S rRNA gene clone libraries, halophilic *Archaea* were detected in a permafrost sample for the first time and this result was surprising given that the salinity of the Eureka permafrost ($\sim$14.6 g kg^{-1} soluble salts) would classify it as moderately saline (Rhoades, 1982). By contrast, the presence of halophilic *Archaea* may reflect the marine origins of this 9-m permafrost sample (W.H. Pollard, personal communication). In permafrost, depending on temperature and composition of the soil, 93–99% of water is present as ice (Rivkina *et al.*, 2004). Owing to the exclusion of solutes from the ice fraction during freezing, thin water veins present in permafrost are enriched in dissolved materials (Price, 2000) and it is within these thin brine veins that viable microorganisms are expected to survive. Recently, it was demonstrated that bacterial cells inhabit the brine veins in sea ice (Deming, 2002). The adaptation of the microbial community to high salt concentrations is also reflected in the fact that many of the Eureka permafrost isolates were able to grow with 7% NaCl added to the growth medium.

Several 16S rRNA gene sequences related to the *Crenarchaeota* were also identified. To date, the vast majority of cultured *Crenarchaeota* are extreme thermophiles, although the emerging view of their distribution and role in the environment is shifting given their apparent widespread distribution in culture-independent surveys of microbial diversity (Schleper *et al.*, 2005). Interestingly, none of the 16S rRNA gene sequences in the *Archaea* library was related to known methanogenic *Archaea*. In previous studies, Siberian permafrost was found to harbor substantial numbers of viable methanogens, up 2.5×10^7 cells g^{-1} (Rivkina *et al.*, 1998).

The heterotrophic plate counts and the clone libraries suggested very different community structures for the bacterial population in the Eureka 9-m sample. However, viable aerobic organisms only accounted for a small fraction (*c.* 0.02%) of the total bacterial population as determined by microscopic counts. Increased representation of cultured organisms could be achieved using culturing techniques that are more reflective of the permafrost environment, such as anaerobic culturing or longer incubation times. Improved cell recovery from cryogenic environments such as permafrost and glacier ice has been achieved by long preincubation at low temperatures or through anaerobic enrichment (Vishnivetskaya *et al.*, 2000; Miteva *et al.*, 2004). For example, in Greenland Glacier ice cores, *Firmicute* bacteria were predominant in directly plated ice, but were succeeded by *Proteobacteria* after filtration and enrichment (Miteva *et al.*, 2004). Presumably these techniques facilitate repair of cellular damage accumulated during storage in frozen environments or aid in recovery from a viable but non-culturable state. It is also possible that many bacteria are present in permafrost as dead or dormant cells. The cultured isolates from Eureka permafrost were predominantly related to spore-forming genera of bacteria, indicating that the viable microbial community in this extreme environment is surviving as spores rather than vegetative cells. Lastly, naked DNA persists in soils (England *et al.*, 2004) and may be stable in permafrost for up to 400 000 years (Stokstad, 2003; Willerslev *et al.*, 2004); such exposed DNA molecules may account for at least some of the 16S rRNA gene sequences detected in this study.

We are presently using information from the Eureka permafrost 16S rRNA gene clone libraries to design improved activity assays and culturing conditions to identify the microbial communities that are viable or metabolically active in high Arctic permafrost. Such investigations will be critical in determining if permafrost contains active microbial ecosystems that could have important implications for global nutrient cycling and biogeochemical processes, as well as shedding light on what constitutes the low temperature limit for microbial life. Evidence of *in situ* biogeochemical processes in permafrost could lead to the development of methodologies to detect permafrost microbial activity in the field, including detecting past or extant life on Mars or Europa.

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