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ORIGINAL ARTICLE

Dilution-to-Extinction Culturing of Psychrotolerant Planktonic Bacteria from Permanently Ice-covered Lakes in the McMurdo Dry Valleys, Antarctica

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Abstract Lakes in the McMurdo Dry Valleys of Antarctica are characterized by a permanent ice cover and little or no anthropogenic influence. Although bacterial cultures have been obtained from these habitats, recent culture-independent studies indicate that the most abundant microbes in these systems are not yet cultivated. By using dilution-to-extinction cultivation methods with sterilized and nutrient-amended lake water as media, we isolated 148 chemotrophic psychrotolerant bacterial cultures from fresh surface water of Lake Fryxell and the east lobe of Lake Bonney and the hypersaline, suboxic bottom water from the west lobes of Lake Bonney. Screening of the 16S ribosomal ribonucleic acid (rRNA) genes of the cultures by restriction fragment length polymorphism (RFLP) yielded 57 putatively pure psychrotolerant, slow growing cultures grouped into 18 clusters. The sequencing of 16S rRNA genes of randomly selected representatives of each RFLP cluster revealed that the corresponding isolates belong to the *Alphaproteobacteria* (six RFLP patterns), *Betaproteobacteria* (six RFLP patterns), *Bacteroidetes* (four RFLP patterns), and *Actinobacteria* (two RFLP patterns). Phylogenetic analysis of the sequences showed that the vast majority of the isolates were not closely related to previously described species. Thirteen of 18 RFLP patterns shared a 16S ribosomal deoxyribonucleic acid sequence similarity of 97% or less with the closest described species, and four isolates had a sequence similarity of 93% or less with the nearest described species. Phylogenetic analysis showed that these sequences were representatives of deeply branching organisms in the respective phylum. A comparison of the isolates with 16S rRNA clone libraries prepared from the same environments showed substantial overlap, indicating that dilution-to-extinction culturing in natural lake water media can help isolate some of the most abundant organisms in these perennially ice-covered lakes.

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Introduction

The McMurdo Dry Valleys of Antarctica (MCM) are the sites of the only perennially ice-covered lakes on Earth. The perennial ice covers prevent wind-driven mixing and inhibit gas exchange, light penetration, and allochthonous sediment deposition [28, 29, 36, 39]. As a result, the water columns of these lakes are highly stable. Lake Bonney (LB) and Lake Fryxell (FRX) are two such lakes located in the Taylor Valley. Both LB and FRX have chemoclines separating oxygen-rich, fresh surface waters from oxygen-depleted, saline deep waters. LB, which has a maximum depth of 38 m, has two lobes that are separated by a sill at 13 m depth. The surface waters of east lobe LB (ELB) and west lobe LB (WLB) are chemically similar; however because of a strong chemocline at ~15 m depth, the deep waters are ancient, do not interact with each other, and their solutes are derived from different origins [21]. Thus, we treat ELB and WLB as discrete lake systems. FRX has a

maximum depth of 18 m with a weak chemocline at ~10 m depth. FRX possesses highly sulfidic, brackish deep waters. The lakes have been the subject of extensive studies of the paleohistory, biogeochemical dynamics, and ecosystem interactions (e.g., [21] and references therein).

Whereas broad-scale biogeochemical studies have indicated the presence of an abundant and active microbial community in the water columns of these lakes [37], relatively little is known about the diversity of these microbes. Cultivation of psychrophilic organisms and the search for novel cold-stable enzymes is of major importance for biotechnology and was one of the driving forces behind exploration of freshwater lakes in Antarctica [2]. A variety of heterotrophic, phototropic, and chemolithotrophic bacteria have been isolated from Antarctic lakes, including MCM lakes [3, 4, 18, 19, 22, 23, 32, 33, 38] by using liquid or solidified complex media and incubation temperatures allowing for rapid growth of the organisms (5–15°C). However, recent culture-independent studies have indicated that the most abundant phylotypes in the water column remain uncultured (Foo and Lanoil, unpublished; [15]).

High-throughput culturing of marine oligotrophic organisms using dilution-to-extinction methods and natural seawater as basis for the medium [6, 9] has resulted in the cultivation of *Candidatus* Pelagibacter ubique, the most abundant oligotrophic organism on earth [30]; *Lentisphaerae*, a hitherto unrecognized phylum of bacteria [8], and many other previously uncultured organisms (e.g., [7]). Similar to marine environments, dilution-to-extinction culturing in limnetic systems yielded abundant hitherto uncultured bacteria [14, 27].

In this study, we explore the use of dilution-to-extinction high-throughput culturing on oligotrophic bacteria from water samples of three perennially ice-covered MCM lake systems: ELB, WLB, and FRX. The goal of this project was to obtain numerically abundant not-yet-cultivated organisms for future studies of their activities and physiology to better understand their interactions with the unusual geochemistry of the MCM lakes.

Materials and Methods

Site Description and Sample Collection

LB is located at the southwest end of the Taylor Valley proximal to the Taylor Glacier at 77.43°S, 162.20°E [36]. The lake possesses two glacially scoured basins that are separated by a sill at 13 m depth. The lake is 38 m deep, with a chemocline at approximately 15 m depth. Thus, ELB and WLB are chemically similar in their surface waters but are distinct below the chemocline and thus are treated as separate systems in this and previous studies [21]. There are no surface outflows from LB, and the only known water loss occurs from the ablation of ice from the surface of the lake. LB is fed primarily by meltwater from the Taylor Glacier but also from other mountain glaciers in the surrounding valleys. FRX is located toward the northeastern end of the Taylor Valley at 77.37°S, 163.09°E, between the Commonwealth and Canada Glaciers [36]. The lake is 18 m deep with a chemocline at approximately 10 m, below which the water is brackish and sulfidic. Meltwater sources include the Commonwealth and Canada Glaciers and surrounding mountain glaciers. Like LB, FRX has no surface outflows, and the only source of water loss is through ablation of surface ice.

Water was collected with a Niskin bottle from FRX at a depth of 6 m on November 17, 2003 and from ELB at a depth of 6 m and WLB at a depth of 38 m on November 26, 2003. Table 1 shows the dominant chemical and physical features of these samples. Samples from FRX and ELB are both fresh, fully oxygenated water with low nutrient and organic carbon content, whereas the WLB sample is from the suboxic, nutrient-rich, hypersaline bottom water [16, 21, 36]. These specific depths were chosen to allow a cross-lake comparison between equivalent depth samples (ELB and FRX 6 m) and including a sample from the hypersaline bottom waters (WLB 38 m). The depths were also chosen because they match some of the depths sampled for a cultivation-independent study (Foo and Lanoil, unpublished). Samples for inoculation were amended with 15% glycerol (v/v), frozen, and shipped to Oregon State University on dry ice.

Media Preparation and Sample Handling

Triplicate 1-mL samples from all three locations were fixed with 2% formalin, stained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min, and subsequently filtered on polycarbonate membranes, and microscopic-direct cell counts were determined as previously described [9]. Media preparation followed the protocol for low-nutrient heterotrophic media [9]. Briefly, the water was filtered through a tangential flow filtration system (Millipore) using a 30-kDa molecular cutoff ultrafilter to remove cells. The media was then autoclaved for 3 h in 20-L carboys, followed by CO_2 sparging for 16 h and aeration for 48 h. For FRX and WLB samples, ammonium chloride (1 μM), sodium phosphate (0.1 μM), 0.001% (w/v) of a mixed carbon solution "MC" [7], and a 10^4 dilution of a vitamin mixture [30] were added to the medium [27]. For ELB samples, ammonium chloride (10 μM), sodium phosphate (1 μM), and a modified carbon solution mixture was added ("MC2," containing 0.001% [w/v] each of formic acid, ethanol, pyruvic acid, glycerol, succinic acid, ribose, glucose, *n*-acetyl glucosamine, glycolate, and glutamine).

Table 1 Physical and chemical data for samples studied here

	ELB 6 m	FRX 6 m	WLB 38 m
Temperature (°C)	1.7	0.7	−4.2
Conductivity (mS cm^{-1})	2.5	1.6	79.4
PH	8.5	7.8	5.9
Dissolved Oxygen (mg L^{-1})[a]	>20[b]	>20[b]	0.95[c]
DIC (mM)	1.9	8.85	67.55
DIN (μm)	11.8	1.36	319.3
DOC (g C L^{-1})	1.02	3.18	21.42
Na^{+}(mg L^{-1})[d]	480	251	39,178
K^{+}(mg L^{-1})[d]	24	26	1,386
Mg^{2+}(mg L^{-1})[d]	74	29	9,127
Ca^{2+}(mg L^{-1})[d]	90	51	2,311
Si^{+}(mg L^{-1})[d]	2.4	4.6	4.3
SO_4^{2-}(mg L^{-1})[d]	204	71	4,912
NH_4^{+}(μM)	0.6	ND	223
SRP (μM)	0.01	0.29	0.6
NO_3^{-} (μM)	7.5	ND	1.2
POC (μM)[e]	22	16	13
PON (μM)[e]	268	195	151
C/N ration (particulate)	13	21	88
TDR (nM TDR day^{-1})	0.013348	0.029908	0.000023
CHL (μg chl-a L^{-1})	2.5	6.6	0.2
PPR (μg C L^{-1} day^{-1})	1.1	2.5	ND

Data is from the MCM LTER (http://www.mcmlter.org). All data is from the 2003–2004 season unless otherwise indicated.
DIC Dissolved inorganic carbon, *DIN* dissolved inorganic nitrogen, *DOC* dissolved organic carbon, *SRP* soluble reactive phosphate, *POC* particulate organic carbon, *TDR* thymidine incorporation, *CHL* chlorophyll a concentration, *PPR* primary productivity, *ND* not detected
[a] Data from 2000–2001 season
[b] Data collected with an oxygen meter that has a maximum of 20 ppm
[c] Because of high salinity, this value should be considered an estimate rather than an exact value.
[d] Data from 2004–2005 season
[e] Data from 2002–2003 season

Inoculation and Cultivation

Dilutions of the inocula were done in chilled media (4°C). Forty-eight-well polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ) were filled with 2 mL media, inoculated, and incubated at 4°C in the dark. Final inoculation densities were approximately 3 (two plates) and 10 cells mL^{-1} (two plates) for FRX. For WLB, inocula were approximately 3 (one plate) and 10 cells mL^{-1} (one plate). For ELB, inocula were approximately 20 cells mL^{-1} (four plates).

Screening and Storage of Cultures

After incubation for 24 weeks, 200 μL of each well was stained with DAPI, fixed with formalin, and transferred to a polycarbonate membrane, according to Connon and Giovannoni [9]. Membranes were screened using an epifluorescence microscope (DMRB, Leica, Germany) and scored as positive if the cell concentrations were greater than 1×10^5 cells mL^{-1}. Duplicate 200-μL aliquots of positive cultures were amended with 10% glycerol (v/v) and frozen in liquid nitrogen for storage.

Molecular Analysis

Deoxyribonucleic acid (DNA) was extracted from 200-μL samples of positive wells with a DNeasy extraction kit according to manufacturer's instructions (Qiagen, Valencia, CA). 16S ribosomal ribonucleic acid (rRNA) gene sequences were amplified by polymerase chain reaction (PCR) using 1 μL of the extracted DNA as template as described by Connon and Giovannoni [9].

Positive PCR products were digested with *Hae*III restriction endonuclease for 2 h at 37°C. Digests were electrophoretically separated on 3% agarose gels, stained with SYBR Safe (Invitrogen, Carlsbad, CA), and visualized under UV light. If the sum of the length of the bands was equal to or less than one 16S rRNA gene (~1,500 bp), then the culture was considered pure. Strains with identical RFLPs isolated from the same environment were grouped together. PCR products of two randomly chosen strains of

Table 2 Summary of dilution-to-extinction results

	FRX	ELB	WLB	Total
Positive wells	42	103	3	148
Axenic	9	46	2	57
Mixed	15	38	1	54
No PCR product	18	19	0	37

each RFLP pattern (if applicable) were sequenced with primer 27F on an ABI 111 sequencer (Applied Biosystems, Foster City, CA). ABI traces were manually checked using DNAStar (GATC, Konstanz, Germany). The length of the partial sequences was between 700 and 1,000 bp. Pairwise distance similarity matrices as integrated in ARB were used to calculate the identity to closest-related sequences. Chimera checks were performed with the online tool available at the Ribosomal Database Project at Michigan State University (http://www.rdp8.cme.msu.edu/). No obvious chimeric sequences were detected.

Phylogenetic Analysis

Sequence data were analyzed with the ARB software package (http://www.arb-home.de). The new sequences were added to the ARB database and aligned with the Fast Aligner tool. Alignments were checked and corrected manually where necessary. 16S rRNA gene sequences from the isolates were compared to sequences in public databases with nucleotide basic local alignment search tool (BLASTn) [1]; 16S rRNA gene sequences with high similarities to those determined in this study were retrieved and added to the alignment. Highly variable regions of the 16S rRNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences.

Framework trees were calculated with fastDNAmL [26], a maximum-likelihood method implemented in ARB, using only sequences greater than 1,400 bases. Shorter sequences were added to these trees with the ARB parsimony tool, which allows the addition of short sequences to existing phylogenetic trees without changing global tree topologies [20]. The stability of the branching pattern was tested with the neighbor-joining and maximum-parsimony (DNA-PARS) methods included in the PHYLIP package [10] implemented in ARB. The reproducibility of the branching pattern was confirmed by bootstrap analysis with 1,000 replicates with the maximum-parsimony algorithm and the program Seqboot implemented in PHYLIP.

Accession Numbers

Sequences were submitted to GenBank under accession numbers EF628475–EF628504.

Results and Discussion

Total prokaryotic cell counts of lake water samples were in a similar range as previous cell counts in these systems [37]. Cell concentrations were highest in FRX with $1.6\times10^6\pm2.4\times10^5$ cells mL^{-1} and nearly an order of magnitude lower in both WLB ($3.2\times10^5\pm7.2\times10^4$ cells mL^{-1}) and ELB ($2.3\times10^5\pm5.7\times10^4$ cells mL^{-1}).

In an initial screen, no growth was observed after 1 month. Final screening of the cultures was performed after 24 weeks incubation in the dark at 4°C. In total, dilution-to-extinction culturing yielded 148 cultures: 103 from ELB, three from WLB, and 42 from FRX. Screening of the cultures by RFLP of 16S rRNA genes revealed that 57 were putatively pure cultures, whereas 54 had a RFLP pattern indicative of mixed cultures. No PCR products, either with bacterial primers or with primers specific for *Archaea*, were obtained for 37 cultures (Table 2). Presumably, the lack of amplification was due to the difficulty of lysing the cells or the presence of compounds interfering with PCR. These cultures were not pursued further. Forty-six of the pure cultures presented in this study were isolated from ELB, nine from FRX, and only two were from WLB (Table 2). Based on the dissolved carbon concentration (DOC) content of the media (Table 1), isolates from ELB and FRX were oligotrophic according to the definition of Fry (i.e., growth at 6 g C L^{-1}; [12]). However, the WLB isolates grew in media containing significantly higher DOC and thus are mesotrophic. Based on their growth temperature and incubation time (i.e., 4°C and 24 weeks, respectively), all isolates are psychrotolerant and slow growing.

Table 3 Distribution of bacteria by major phylogenetic group and similarity to nearest validly described species

Phylum/division	Number of RFLPs	16S rRNA gene similarity (≥99%)	16S rRNA gene similarity (≥97%)	16S rRNA gene similarity (≥93%)	16S rRNA gene similarity (≥90%)
Alphaproteobacteria	6	3	3	0	0
Betaproteobacteria	6	2	3	0	1
Bacteroidetes	4	0	3	1	0
Actinobacteria	2	0	0	1	1

Pure cultures were grouped according to their RFLP pattern into 18 groups containing between 1 and 12 strains (Table 3). There was no overlap of patterns from the three different locations, except for RFLP groups FRX1 and ELB7 (Table 4). 16S rRNA genes of two randomly selected strains from each RFLP group were partially sequenced. 16S rRNA gene sequences from isolates from the same group and location were 99–100% identical; therefore, the groups were considered homogenous. BLAST searches of the sequences in GenBank revealed that the strains belonged to the *Alphaproteobacteria* (six groups), *Betaproteobacteria* (six groups), *Bacteroidetes* (four groups), and *Actinobacteria* (two groups; Table 3).

Detailed phylogenetic analysis revealed that the isolates shared 88–99% 16S rRNA gene sequence identity with the most closely related validly described species. Whereas six groups had a sequence similarity of 99% or more to the closest described species, 12 of them shared less than 97% sequence similarity, and four of these had a sequence similarity of 93% or less (Table 3).

Phylogenetic trees are shown for sequences that were less than 99% related to those of validly described bacterial species (Figs. 1, 2, 3, and 4). RFLP groups with greater than 99% sequence similarity to described species were in all cases related to common soil bacteria (Table 4) and were omitted from further phylogenetic analysis.

Table 4 Taxonomic affiliation of isolates determined by BLAST similarities to nearest validly described neighbors

Habitat	RFLP group	Strains isolated	Sequenced strains (HTCC numbers)	Accession numbers	Phylum/division	Closest described species	Percent 16S rDNA similarity
FRX	1	1	4019	EF628476	*Alphaproteobacteria*	*Sphingopyxis witflariensis*	94
	2	2	4034	EF628478	*Alphaproteobacteria*	*Agrobacterium sanguineum*	99
	3	1	4029	EF628477	*Betaproteobacteria*	*Ralstonia picketti*	99
	4	2	4013, 4042	EF628475, EF628481	*Betaproteobacteria*	*Polaromonas aquatica*	97
	5	2	4037, 4045	EF628479, EF628482	*Betaproteobacteria*	*Aminomonas aminovorus*	92
	6	1	4038	EF628480	*Betaproteobacteria*	*Alcaligenes xylosoxidans*	95
ELB	7	11	4052, 4111	EF628483, EF628493	*Alphaproteobacteria*	*Sphingomonas natatoria*	96
	8	5	4081, 4096, 4103, 4120, 4125	EF628486, EF628489, EF628491, EF628496, EF628499	*Alphaproteobacteria*	*Erythrobacter luteolus*	95
	9	1	4110	EF628492	*Alphaproteobacteria*	*Bradyrhizobium elkanii*	99
	10	2	4077, 4112	EF628485, EF628494	*Betaproteobacteria*	*Polaromonas vacuolata*	97
	11	2	4118, 4126	EF628495, EF628500	*Bacteroidetes*	*Algoriphagus yeomjeoni*	97
	12	9	4072, 4101	EF628484, EF628490	*Bacteroidetes*	*Pedobacter caeni*	93
	13	12	4086, 4123	EF628487, EF628498	*Bacteroidetes*	*Cellulophaga pacifica*	94–95
	14	1	4122	EF628497	*Bacteroidetes*	*Flavobacterium frigoris*	95
	15	1	4091	EF628488	*Actinobacteria*	*Acidimicrobium ferrooxidans*	88
	16	2	4138, 4145	EF628501, EF628502	*Actinobacteria*	*Sporichthya polymorpha*	92
WLB	17	1	4155	EF628504	*Alphaproteobacteria*	*Sphingomonas rhizogenes*	99
	18	1	4153	EF628503	*Betaproteobacteria*	*Pedomonas saccharophila*	99

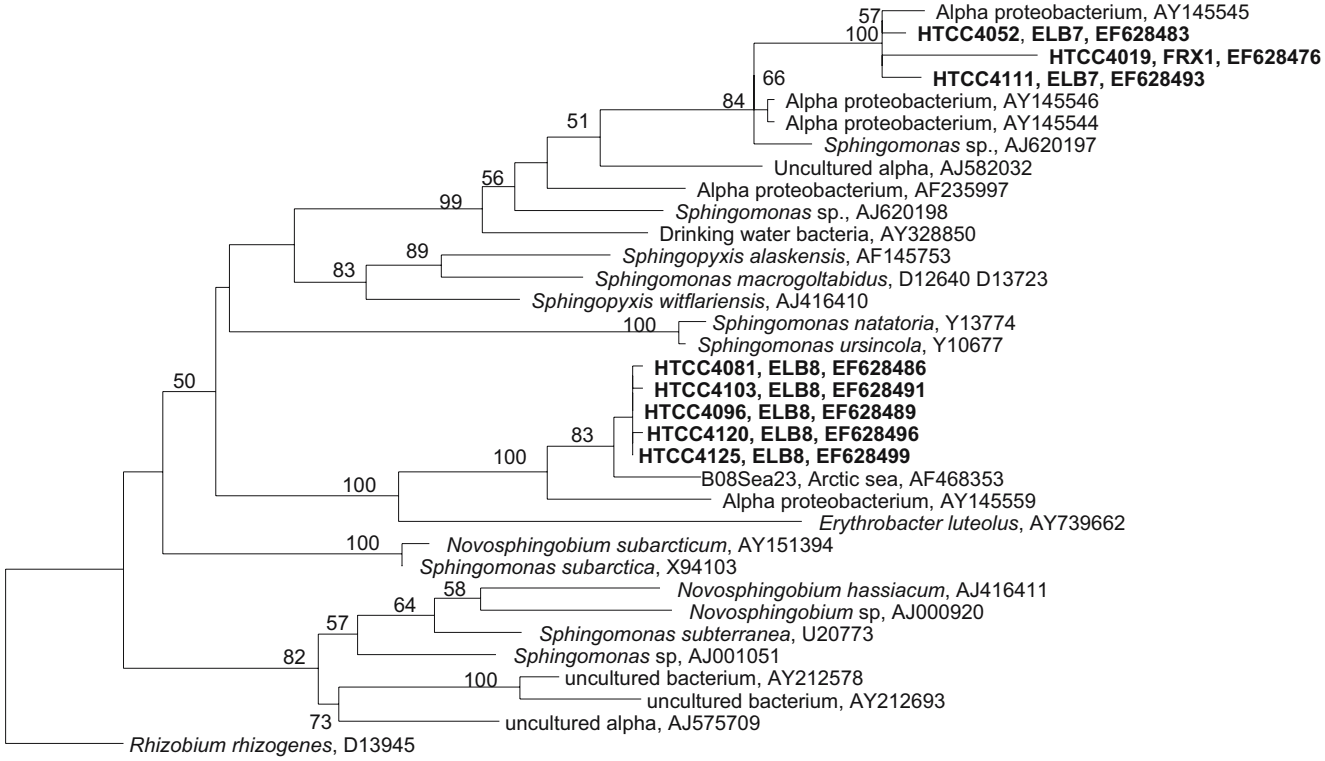

Figure 1 Phylogenetic tree showing relationships between the isolated *Alphaproteobacteria* (*bold*, including strain designation, RFLP group, and accession number), closely related environmental sequences, and cultured representatives, inferred from 16S rRNA gene sequence analyses. Bootstrap values greater than 50% are shown

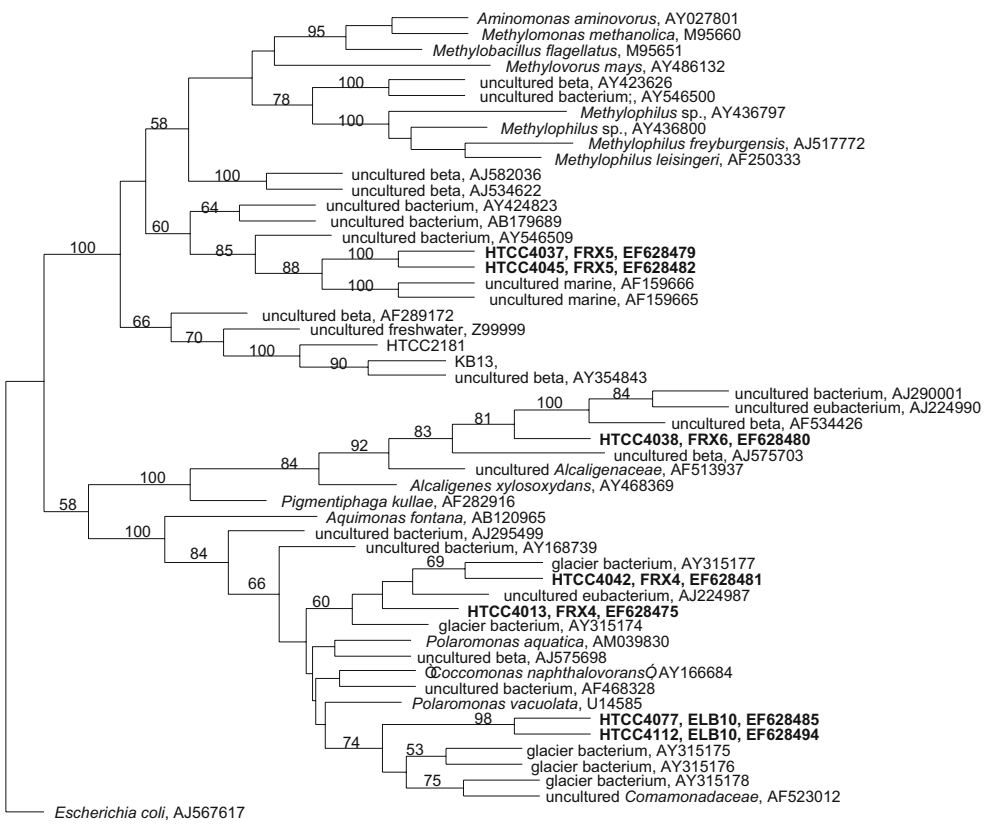

Figure 2 Phylogenetic tree showing relationships between the isolated *Betaproteobacteria* (*bold*, including strain designation, RFLP group, and accession number), closely related environmental sequences, and cultured representatives, inferred from 16S rRNA gene sequence analyses. Bootstrap values greater than 50% are shown

Cultured Alphaproteobacteria

Among the *Alphaproteobacteria*, all RFLP groups clustered within the *Sphingomonadales* and represent members of the families *Erythrobacteraceae* (RFLP group ELB8) or *Sphinomonadaceae* (RFLP groups FRX1 and ELB7) and were closely related to uncultured bacteria found in marine and freshwater habitats (Fig. 1). ELB8 was closely related to bacterial sequences obtained from sea ice [5]. FRX1 and ELB7 were closely related to a bacterium isolated from the ultraoligotrophic Crater Lake, using the same techniques as applied in this study [27].

Cultured Betaproteobacteria

Isolates in the *Betaproteobacteria* came from two different phylogenetic groups. RFLP groups FRX4 and ELB10 cluster within *Comamonadaceae* and were closely related to the genus *Polaromonas* (Fig. 2, [17]). This group of organisms has been commonly seen in subglacial and other ice-covered environments [11, 13, 34]. Sequences from RFLP group FRX5 clustered within the marine OM43 clade of the *Betaproteobacteria*. Members of the OM43 group were cultivated from marine environments using dilution-to-extinction culturing [9], but so far, no validly described species exist. They were recently reported as one of the dominant components of the bacterial community during a diatom bloom at the Oregon coast [24].

Cultured Actinobacteria

Isolates from the phylum *Actinobacteria* were recovered exclusively from ELB and were only distantly related to validly described species (Fig. 3). The sequences clustered together with uncultured bacteria in two unclassified groups within the order *Actinomycetales* (RFLP groups ELB15 and ELB16). The most closely related sequences to ELB 15 came from uncultured bacteria from Arctic and Antarctic pack ice [5]. ELB 16 clustered together with a sequence obtained from the anoxic sediment of an Antarctic lake in the Vestfold Hills [3].

Cultured Bacteroidetes

Isolates from the phylum *Bacteroidetes* were exclusively recovered from ELB (Fig. 4). RFLP groups associated with this phylum represent two different phylogenetic lineages: *Flavobacteriaceae*, containing isolates from the genera *Cellulophaga* (RFLP group ELB13) and *Flavobacterium* (RFLP group ELB14) and from the order *Sphingobacteriales* with the genera *Algoriphagus* (RFLP group ELB11) and *Pedobacter* (RFLP group ELB12). Sequences closely related to these isolates were retrieved from marine environments [25] and glaciers [11]. Unpublished sequences closely related to ELB14 were recovered from freshwater environments (AF493663, AF493637, DQ017917).

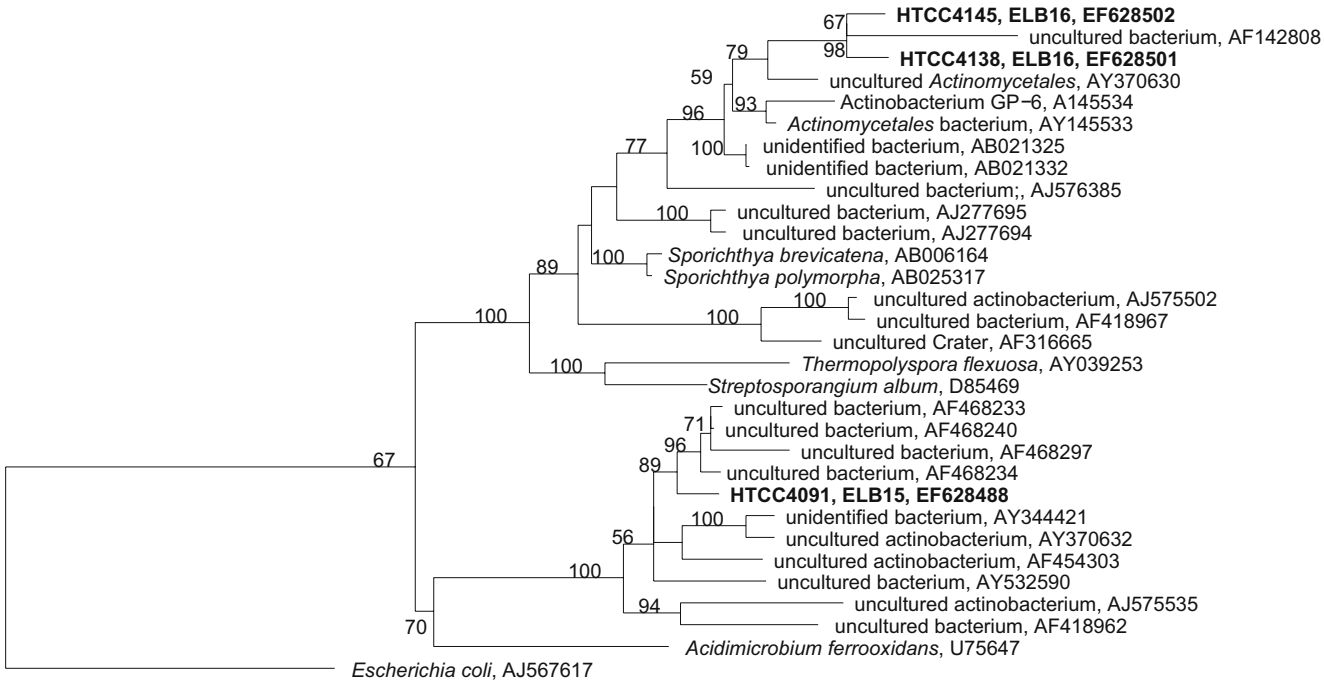

Figure 3 Phylogenetic tree showing relationships between the isolated *Actinobacteria* (*bold*, including strain designation, RFLP group, and accession number), closely related environmental sequences, and cultured representatives, inferred from 16S rRNA gene sequence analyses. Bootstrap values greater than 50% are shown

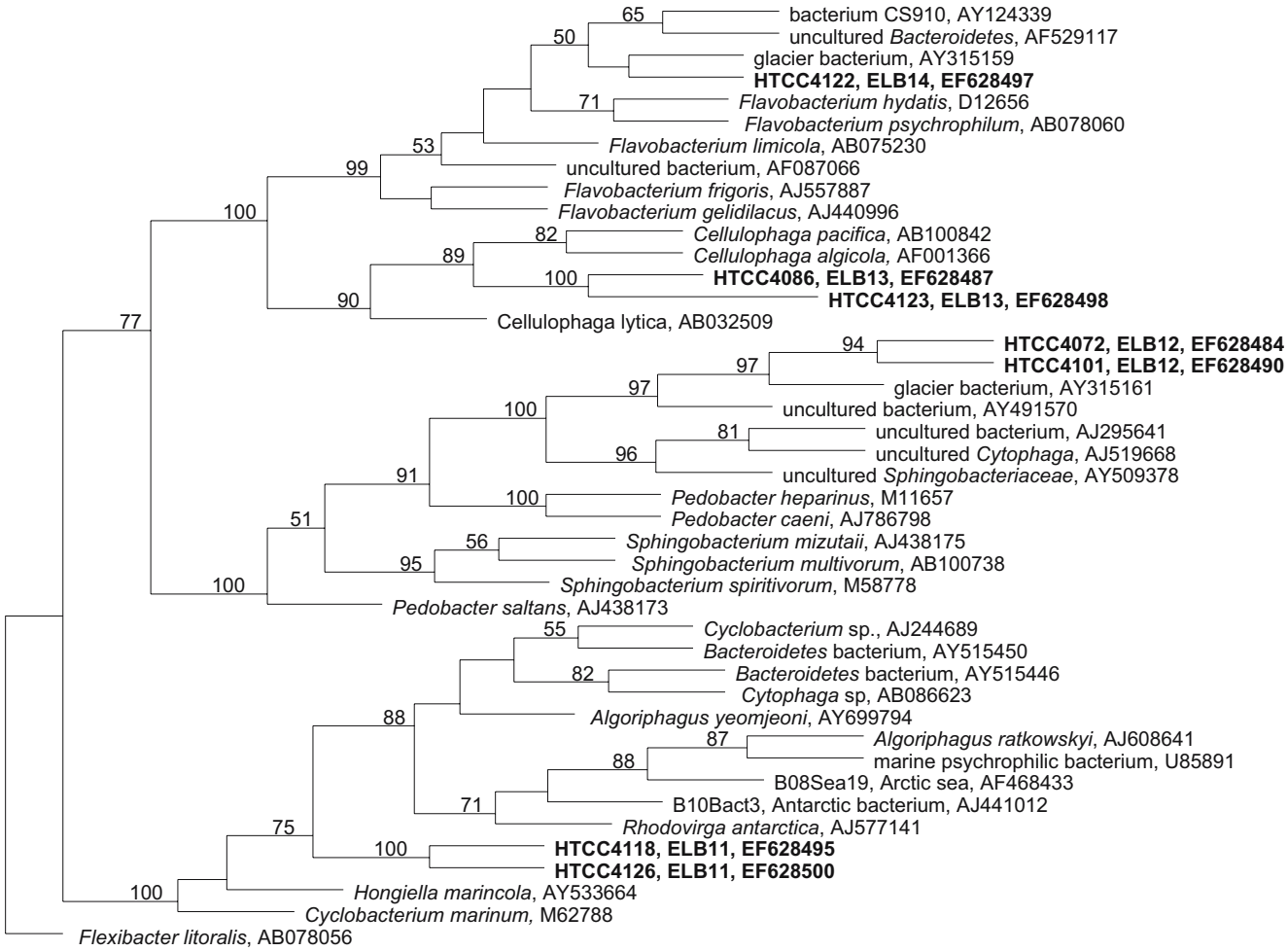

Figure 4 Phylogenetic tree showing relationships between the isolated *Bacteroidetes* (*bold*, including strain designation, RFLP group, and accession number), closely related environmental sequences, and cultured representatives, inferred from 16S rRNA gene sequence analyses. Bootstrap values greater than 50% are shown

Comparison of Isolates with rRNA Gene Clone Libraries from the Same Samples

One half (15 of 30) of the isolates show high 16S rRNA gene sequence similarity (>97%) to clones from environmental DNA extracted from the same environments, including ten isolates with greater than 99% similarity to clones from these libraries (Table 5; Foo and Lanoil, unpublished). *Cellulophaga pacifica* is the closest relative of two and *Agrobacterium sanguineum* of one of these isolates; the remaining are most closely related to uncultured sequences obtained from diverse environments including estuarine, lacustrine, and marine environments. Another six isolates show a sequence similarity between 93 and 97% to clones obtained from the same libraries. It is surprising to note that the remaining isolates are distinct from clones obtained from these environments and are primarily from the *Alphaproteobacteria* group, which represents less than 10% of the total clone libraries from each of the three systems (Foo and Lanoil, unpublished). Planned fluorescence *in situ* hybridization studies will determine whether these isolates are underrepresented in the clone library or overrepresented in the culture collection.

Conclusions

Previous culturing studies of permanently ice-covered lakes in Antarctica do not show satisfactory overlap with culture-independent studies (Foo and Lanoil, unpublished; [4, 15]). In this study, we show that by using dilution-to-extinction culturing in media based on natural lake water, abundant phylotypes can be cultured from these extreme environments. Although throughput in this study was low compared to high-temperature combustion in marine envi-

Table 5 Similarity of isolates to 16S rRNA gene clones retrieved from the same lakes and same depths [12]

RFLP group*	Culture source	Nearest Blast Neighbor	Similarity to BLAST neighbor	Closest clone match	Similarity to clone†	Clone location (number of clones)
1	FRX	Alpha proteobacterium LH1D (DQ535022)	99%	W6-131	93%	WLB (1)
2	FRX	Antarctic bacterium R-9478 (AJ441013)	99%	W6-68	99%	WLB (1)
3	FRX	*Ralstonia* sp. Q3-8/14 (AY216798)	99%	E6-131	91%	ELB (4), FRX (2), WLB (8)
4	FRX	Uncultured bacterium clone ANTLV7_H07 (DQ521547)	99%	W6-7	97–98%	ELB (1), FRX (1)
5	FRX	Uncultured beta proteobacterium clone C319a-R8C-F6 (AY678527)	97%	F6-79	99–100%	FRX (1), WLB (1)
6	FRX	Beta proteobacterium QLW-P2DMWB-4 (AJ938031)	98%	E6-131	99%	ELB (4), FRX (2), WLB (8)
7	ELB	Alpha proteobacterium LH1D (DQ535022)	99%	W6-131	93%	WLB (1)
8	ELB	Arctic sea ice bacterium ARK10016 (AF468353)	99%	W18-3	86%	WLB (10)
9	ELB	Uncultured bacterium clone KSC2-39 (DQ532286)	99%	W38-82	89%	WLB (3)
10	ELB	Glacier bacterium FXS33 (AY315176)	99%	W6-7	97%	ELB (1), WLB (1)
11	ELB	*Algoriphagus yeomjeoni* strain MSS-161 (AY699795)	97%	W18-16	96%	ELB (7), FRX (2), WLB (11)
12	ELB	*Sphingobacteriaceae* bacterium SOC A20 (36) (DQ628953)	99%	F9-101	99%	ELB (2), FRX (1), WLB (1)
13	ELB	*Cellulophaga pacifica* (AB100842)	95%	W6-60	99%	ELB (9), FRX (3), WLB (12)
14	ELB	Uncultured bacterium clone ANTLV2_D10 (DQ521513)	99%	F9-50	99%	FRX (1), WLB (1)
15	ELB	Uncultured bacterium clone ANTLV2_D07 (DQ521512)	99%	E6-82	90%	ELB (1), FRX (21)
16	ELB	Uncultured bacterium clone sponge_clone11 (AY948359)	99%	F9-55	98–100%	ELB (6), FRX (4), WLB (2)
17	WLB	*Sphingomonas* sp. oral clone AV069 (AF385529)	99%	W6-131	92%	WLB (1)
18	WLB	Uncultured beta proteobacterium clone SM1G08 (AF445700)	99%	W6-7	93%	ELB (1), FRX (1)

*See Table 4 for more details.
† If there are multiple isolates with different similarities in the same RFLP group, a range of similarities is given.

ronments [9], we showed that the technique is applicable to these specific environments and that some abundant bacteria can be cultured including novel strains of groups that were hitherto uncultured. The most likely explanation for the success of modified lake water as a medium is that no artificial media can replicate all of the relevant environmental conditions, especially in environments that are highly stable, stratified, and gradient dominated like the MCM lakes [31, 35, 38].

Culturing efficiency between the three locations varied. This can be explained to a certain degree by differences in inoculum density. The ELB plates, which yielded most of the cultures, were inoculated with the highest densities (20 cells mL^{-1}), whereas FRX and WLB plates were inoculated with fewer cells (3–10 cells mL^{-1}). The fact that the culturing efficiency of WLB was lower than from FRX can be explained by the depth from which the inoculum was taken: Samples from FRX came from the oxic epilimnion, whereas the samples from WLB were collected from the suboxic hypolimnion, close to the sediment where almost no heterotrophic activity is present [37]. Culture conditions were not optimized for the suboxic to anoxic conditions found in this environment.

In many environments, the most abundant groups of bacteria remain uncultured. Dilution-to-extinction culturing in media prepared directly from the environment can improve culturing efficiencies. Future studies will concentrate on the spatial and temporal distribution of the isolated bacteria in the MCM lakes and on their metabolic properties and functions.

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References

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
2. Ashbolt NJ (1990) Antarctic biotechnology—what is the potential? Aust J Biotechnol 4:103–105, 108
3. Bowman JP, Rea SM, McCammon SA, McMeekin TA (2000) Diversity and community structure within anoxic sediment from marine salinity meromictic lakes and a coastal meromictic marine basin, Vestfold Hills, Eastern Antarctica. Environ Microbiol 2:227–237
4. Brambilla E, Hippe H, Hagelstein A, Tindall BJ, Stackebrandt E (2001) 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. Extremophiles 5:23–33
5. Brinkmeyer R, Glockner FO, Helmke E, Amann R (2004) Predominance of beta-proteobacteria in summer melt pools on Arctic pack ice. Limnol Oceanogr 49:1013–1021
6. Button DK (1993) Nutrient-limited microbial growth kinetics: overview and recent advances. Antonie van Leeuwenhoek 63:225–235
7. Cho JC, Giovannoni SJ (2004) Cultivation and growth characteristics of a diverse group of oligotrophic marine *Gammaproteobacteria*. Appl Environ Microbiol 70:432–440
8. Cho JC, Vergin KL, Morris RM, Giovannoni SJ (2004) *Lentisphaera araneosa* gen nov, sp nov, a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, *Lentisphaerae.* Environ Microbiol 6:611–621
9. Connon SA, Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68:3878–3885
10. Felsenstein J (1989) PHYLIP—Phylogeny Inference Package (Version 3.2). Cladistics 5:164–166
11. Foght J, Aislabie J, Turner S, Brown CE, Ryburn J, Saul DJ, Lawson W (2004) Culturable bacteria in subglacial sediments and ice from two Southern Hemisphere glaciers. Microb Ecol 47:329–340
12. Fry JC (1990) Oligotrophs. In: Edwards C (ed) Microbiology of extreme environments. Open Univ. Press, Milton Keynes, UK, pp 93–116
13. Gaidos E, Lanoil B, Thorsteinsson T, Graham A, Skidmore M, Han SK, Rust T, Popp B (2004) A viable microbial community in a subglacial volcanic crater lake, Iceland. Astrobiology 4:327–344
14. Gich F, Schubert K, Bruns A, Hoffelner H, Overmann J (2005) Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. Appl Environ Microbiol 71:5908–5919
15. Glatz RE, Lepp PW, Ward BB, Francis CA (2006) Planktonic microbial community composition across steep physical/chemical gradients in permanently ice-covered Lake Bonney, Antarctica. Geobiology 4:53–67
16. Green WJ, Gardner TJ, Ferdelman TG, Angle MP, Varner LC, Nixon P (1989) Geochemical process in the Lake Fryxell Basin (Victoria Land, Antarctica). Hydrobiologia 172:129–148
17. Irgens RL, Gosink JJ, Staley JT (1996) *Polaromonas vacuolata* gen nov, sp nov, a psychrophilic, marine, gas vacuolate bacterium from Antarctica. Int J Syst Evol Microbiol 46:822–826
18. Jung DO, Achenbach LA, Karr EA, Takaichi S, Madigan MT (2004) A gas vesiculate planktonic strain of the purple non-sulfur bacterium *Rhodoferax antarcticus* isolated from Lake Fryxell, Dry Valleys, Antarctica. Arch Microbiol 182:236–243
19. Karr EA, Sattley WM, Rice MR, Jung DO, Madigan MT, Achenbach LA (2005) Diversity and distribution of sulfate-reducing bacteria in permanently frozen Lake Fryxell, McMurdo Dry Valleys, Antarctica. Appl Environ Microbiol 71:6353–6359
20. Ludwig W, Strunk O, Klugbauer S, Klugbauer N, Weizenegger M, Neumaier J, Bachleitner M, Schleifer KH (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554–568
21. Lyons WB, Fountain A, Doran P, Priscu JC, Neumann K, Welch KA (2000) Importance of landscape position and legacy: the evolution of the lakes in Taylor Valley, Antarctica. Freshw Biol 43:355–367
22. Madigan MT (1998) Isolation and characterization of psychrophilic purple bacteria from Antarctica. In: Peschek GA, Loffelhardt W, Schmetterer G (eds) The phototrophic prokaryotes. Plenum, New York, pp 699–706
23. Madigan MT, Jung DO, Woese CR, Achenbach LA (2000) *Rhodoferax antarcticus* sp. nov., a moderately psychrophilic purple nonsulfur bacterium isolated from an Antarctic microbial mat. Arch Microbiol 173:269–277
24. Morris RM, Longnecker K, Giovannoni SJ (2006) *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. Environ Microbiol 8:1361–1370
25. Nedashkovskaya OI, Suzuki M, Lysenko AM, Snauwaert C, Vancanneyt M, Swings J, Vysotskii MV, Mikhailov VV (2004) *Cellullophaga pacifica* sp nov. Int J Syst Evol Microbiol 54:609–613
26. Olsen GJ, Matsuda H, Hagstrom R, Overbeek R (1994) fastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput Appl Biosci 10:41–48
27. Page KA, Connon SA, Giovannoni SJ (2004) Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. Appl Environ Microbiol 70:6542–6550
28. Priscu JC, Downes MT, McKay CP (1996) Extreme supersaturation of nitrous oxide in a permanently ice-covered Antarctic lake. Limnol Oceanogr 41:1544–1551
29. Priscu JC (1997) The biogeochemistry of nitrous oxide in permanently ice-covered lakes of the McMurdo Dry Valleys, Antarctica. Glob Chang Biol 3:301–305
30. Rappe MS, Connon SA, Vergin KL, Giovannoni SJ (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418:630–633
31. Sassen R, Roberts HH, Carney R, Milkov AV, DeFreitas DA, Lanoil B, Zhang CL (2004) Free hydrocarbon gas, gas hydrate, and authigenic minerals in chemosynthetic communities of the northern Gulf of Mexico continental slope: relation to microbial processes. Chem Geol 205:195–217
32. Sattley WM, Madigan MT (2006) Isolation, characterization, and ecology of cold-active, chemolithotrophic, sulfur-oxidizing bacteria from perennially ice-covered Lake Fryxell, Antartica. Appl Environ Microbiol 72:5562–5568
33. Shivaji S, Reddy GSN, Suresh K, Gupta P, Chintalapati S, Schumann P, Stackebrandt E, Matsumoto GI (2005) *Psychrobacter vallis* sp nov and *Psychrobacter aquaticus* sp nov, from Antarctica. Int J Syst Evol Microbiol 55:757–762
34. Skidmore M, Anderson SP, Sharp M, Foght J, Lanoil BD (2005) Comparison of microbial community compositions of two subglacial environments reveals a possible role for

microbes in chemical weathering processes. Appl Environ Microbiol 71:6986–6997
35. Spigel RH, Priscu JC (1996) Evolution of temperature and salt structure of Lake Bonney, a chemically stratified Antarctic lake. Hydrobiologia 321(3):177–190
36. Spigel RH, Priscu JC (1998) Physical limnology of the McMurdo Dry Valley lakes. In: Priscu JC (ed) Ecosystem dynamics in a polar desert: the McMurdo Dry Valleys, Antarctica, vol 72. American Geophysical Union, Washington, DC, pp 153–188
37. Takacs CD, Priscu JC (1998) Bacterioplankton dynamics in the McMurdo Dry Valley lakes, Antarctica: Production and biomass loss over four seasons. Microb Ecol 36:239–250
38. Voytek MA, Priscu JC, Ward BB (1999) The distribution and relative abundance of ammonia-oxidizing bacteria in lakes of the McMurdo Dry Valley, Antarctica. Hydrobiologia 401:113–130
39. Wharton Jr RA, Lyons WB, Des Marais D (1993) Stable isotope biogeochemistry of carbon and nitrogen in a perennially ice-covered Antarctic lake. Chem Geol 107:159–172