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ORIGINAL PAPER

Cultivable bacteria from ancient algal mats from the McMurdo Dry Valleys, Antarctica

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Abstract The McMurdo Dry Valleys in Antarctica are a favorable location for preservation of dormant microbes due to their persistent cold and dry climate. In this study, we examined cultivable bacteria in a series of algal mat samples ranging from 8 to 26539 years old. Cultivable bacteria were found in all samples except one (12303 years old), but abundance and diversity of cultivable bacteria decreased with increasing sample age. Only members of the *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* were found in the ancient samples, whereas bacteria in the 8-year-old sample also included *Cyanobacteria*, *Proteobacteria*, and *Deinococcus-Thermus*. Isolates of the Gram-positive spore-forming bacterium *Sporosarcina* were found in 5 of 8 samples. The growth of these isolates at different temperatures was related to the phylogenetic distance among genotypes measured by BOX-PCR. These findings suggest that adaptation to growth at different temperatures had occurred among *Sporosarcina* genotypes in the Dry Valleys, causing the existence of physiologically distinct but closely related genotypes. Additionally, fully psychrophilic isolates (that grew at 15°C, but not 25°C) were found in ancient samples, but not in the modern sample. The preservation of viable bacteria in the Dry Valleys could potentially represent a legacy of bacteria that impacts on current microbial communities of this environment.

Keywords Antarctica · Ancient algal mats · Bacteria · Psychrophiles · Dormancy

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Introduction

The McMurdo Dry Valleys in Antarctica possess environmental conditions which are challenging to microbial life, including persistent cold temperatures, scarcity of water, extreme seasonality, and freeze–thaw cycles (Sun and Friedmann 1999). These same conditions may also promote the persistence of dormant microbes in the Dry Valleys, as cold and dry climates generally favor preservation of biomolecules (Kennedy et al. 1994; Billi and Potts 2002). Viable bacterial cells have been recovered from glacial ice and permafrost several hundreds of thousands of years old (Vishnivetskaya et al. 2000; Christner et al. 2000, 2003). The potential for microbes to survive over these timescales in the Dry Valleys, however, is unclear due to the lack of water, in contrast to previously investigated environments.

Previous research has established that many microorganisms in the Dry Valleys are able to remain viable over several years or decades of dormancy. Viable cyanobacteria and other microbes have been recovered from algal mat communities after more than two decades of desiccation (Hawes et al. 1992; McKnight et al. 2007). Bacteria have also been isolated from decades-old materials originating from human Antarctic expeditions (Nedwell et al. 1994; Ronimus et al. 2006; Hughes and Nobbs 2004). Yet,

Table 1 Sample ^{14}C ages (years before present) and locations of origin

^{14}C age	Location	Age class	Reference[a]
8	Victoria	1	NA
8619	Wright	2	Hall et al. (2001)
8643	Victoria	2	Hall et al. (2002)
11164	Victoria	3	NA
11851	Victoria	3	Hall et al. (2002)
12303	Victoria	3	Hall et al. (2002)
12910	Taylor	3	Hall and Denton (2000)
26539	Wright	4	NA

The age of the modern sample (8) is based on the date of collection

[a] NA indicates samples not previously described

the potential for bacterial cells to tolerate millennia of dormancy in the Dry Valleys has not been examined.

In this study, we examined ancient samples of algal mats originating from glacial lakes that occupied the Dry Valleys during the late Holocene (Doran et al. 1994; Hall et al. 1997, 2001, 2002). Samples represented a chronological sequence of ^{14}C age from 8619 to 26539 years before present [Table 1 (throughout this manuscript, samples are referred to by their ^{14}C age in years before present, ypb)]. ^{14}C ages are thought to approximately indicate the most recent periods of significant primary productivity in these samples (Hall et al. 2001, 2002). A sample of a modern algal mat was included for comparison to ancient samples. We expected that the abundance and diversity of viable cells in ancient samples would decline with increasing sample age. We used BOX-PCR, a high-resolution genotyping method which uses a primer targeting BOX elements conserved in a wide range of bacteria (Koeuth et al. 1995), to examine the relatedness of selected isolates from samples of different ages. In addition, the temperature responses of these isolates was evaluated by screening for growth on solid media at temperatures between 5 and 35°C, and were related to BOX-PCR profile identity.

Materials and methods

Sample collection and characterization

Samples of ancient algal mats were collected aseptically from 5 to 15 cm below the soil surface in Taylor, Wright, and Victoria valleys (Table 1). The modern sample (8 ybp) was collected from the shoreline of Upper Victoria Lake; the age of this sample is based on collection date and not ^{14}C age. Ancient mat samples were stored dried at room temperature following collection in 1993 or 1994, and sample 8 was stored under identical conditions since collection in 1999. For the purpose of statistical analysis, samples were divided into four classes based on age. Age class 1 contained the modern sample (8 ybp). Age class 2 contained samples 8619 and 8643. Age class 3 contained samples 11164, 11185, and 12910, and age class 4 contained sample 26539.

Viable bacteria cultivation and abundance

Cultivation of bacteria from samples was done by a variety of methods to increase the probability of recovering viable bacteria and maximize the diversity of bacteria recovered. For all cultivation attempts, media, solutions, and all other materials were sterilized by autoclaving. Negative controls consisted of pieces of autoclaved glass fiber filters, which were handled and homogenized alongside samples and subsequently inoculated into the respective medium. Negative controls were performed at a ratio of 1 negative control to 3 samples. Cultivation methods used were: (1) heterotrophic plate counts, (2) autotrophic enrichment cultures, and (3) heterotrophic enrichment cultures.

Heterotrophic plate counts were performed, in triplicate, for each sample in a full factorial experiment with medium and incubation temperature as factors. The media used were R2A (Reasoner and Geldreich 1985) or 10% strength R2A, and the incubation temperatures were 5, 15, or 25°C. To facilitate plating, samples were homogenized in 1 mL of 0.85% NaCl by grinding with 5 mm steel beads on a variable speed vortexer (MoBio Laboratories Inc., Carlsbad, CA, USA). Sample homogenates were plated in a tenfold dilution series. For sample 8, a countable number of colonies were obtained from dilutions containing <0.01 mg of material per plate. Countable colonies were obtained from approximately 0.5 mg material for sample 8619, and 1–20 mg material for other ancient samples. Plates were incubated for 60 days or until colonies were observed. Colonies were counted and colony morphology was noted. Plates were re-counted daily until the number of colonies stopped increasing. Representatives of each colony morphology type from each sample were isolated as pure cultures by streak plating and screened by amplified ribosomal DNA restriction analysis (ARDRA) as described below. For statistical analysis of plate count data, CFU data were $\log_{10}$ transformed and a linear mixed model ANOVA was used to test the significance of the fixed factors: temperature, isolation medium, and age class (and interactions among these effects). Sample identity was used as a random factor to account for repeated measurements on the same samples.

Autotrophic enrichment cultures were performed in 100 mL of DYIY liquid medium (Lehman 1976) and incubated at 15°C under illumination from a 40-W broad-spectrum fluorescent lights. Approximately, 10 mg of sample was used to inoculate each culture. Cultures were

performed in duplicate and incubated for 60 days or until they appeared turbid. The medium was spread onto DYIY plates to assay cultures for growth and to isolate individual colonies. Representative isolates from all cultures were subsequently screened by ARDRA.

Heterotrophic enrichment cultures from each sample were carried out in a full factorial experiment with medium and incubation temperature as factors and triplicate replication. Incubations were performed at 5, 15, or 25°C. Heterotrophic enrichment culture media included tryptic soy broth (TSB), 20% strength TSB, or a marine nutrient medium (MN). The marine nutrient medium was based on the artificial seawater (ASW) formulation of Keller et al. (1987) amended with 1 g L^{-1} yeast extract and 5 g L^{-1} peptone. Enrichment cultures were inoculated with small amounts of sample (selected based on plate count data) to maximize the possibility of finding unique bacterial isolates. The mass of the sample used to inoculate heterotrophic enrichments ranged from approximately 1 mg (sample 26539) to 0.01 mg (sample 8). Heterotrophic enrichment cultures were incubated for 60 days or until an observed increase in turbidity. Culture medium was spread onto agar plates to detect growth and isolate individual colonies. Representative isolates from all cultures were subsequently screened by ARDRA.

Culture isolation and ARDRA screening

ARDRA was used as a screening method to assess cultivable bacterial diversity and to select isolates for 16S rRNA gene sequencing. Pure cultures of isolates were obtained from each of the three cultivation approaches by streak plating on solid media corresponding to the original culture medium. A total of 262 bacterial isolates were obtained and screened by ARDRA: 120 from heterotrophic enrichment cultures, 12 from autotrophic enrichment cultures, and 130 from heterotrophic plate counts.

16S rRNA gene fragments were PCR-amplified using primers Eub338F-0-III (Blackwood et al. 2005) and 1391R (Barns et al. 1994). Templates for PCR were either single colonies or DNA extracts (for those isolates on which direct PCR of colonies was not successful) following a procedure modified from Surzycki (2000). PCR conditions were: 0.2 μM each primer, 0.2 mM each dNTP, 0.025 U μL^{-1} Gene Choice Taq DNA polymerase (Cat. No. T-28), 1× Gene Choice ammonium buffer [1.5 mM $MgCl_2$, 75 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, pH 8.5], 0.5 mM $MgCl_2$ (total 2.0 mM $MgCl_2$), and 0.1 μg μL^{-1} BSA. Thermal cycling was performed as follows: an initial denaturation step of 3 min at 95°C followed by 25–30 cycles of 30 s at 95°C, 30 s at 57°C, and 90 s at 72°C, and a final elongation step of 5 min at 72°C. The PCR product was digested by *Hae*III or *Hin*P1I and *Msp*I in combination (New England Biolabs, Ipswich, MA, USA). Digests used 3 U of each enzyme and were incubated overnight at 37°C. Digests were analyzed by electrophoresis in a 3% NuSieve agarose gel at 2.5 V cm^{-1} for 150 min followed by staining with ethidium bromide (0.5 μg mL^{-1}). Stained gels were photographed using a GelDoc imaging system (BioRad, Hercules, CA, USA), and band identification and pattern matching were performed using GelCompare II (v. 4.6; Applied Maths, Austin, TX, USA).

BOX-PCR screening

A single ARDRA type (ARDRA type 1) was observed in 5 of the 8 samples. To provide sufficient numbers of isolates for further study, additional colonies of ARDRA type 1 were isolated from heterotrophic count plates. The selection of colonies was balanced among medium and temperature treatments. BOX-PCR (Koeuth et al. 1995) was applied to investigate the genetic relatedness of ARDRA type 1 isolates from plates. Isolates from enrichment cultures were not genotyped. Genomic DNA for BOX-PCR was extracted from cultures grown on R2A slants using a procedure modified from Surzycki (2000). DNA quantity and purity were measured by absorbance at 260 and 280 nm in a Synergy 2 plate reader (Biotek Instruments, Winooski, VT, USA). Approximately, 100 ng of DNA was used as template in a 25-μL reaction. PCR reaction conditions were: 2.0 μM BOX A1R primer (Koeuth et al. 1995), 0.5 mM each dNTP, 1× Gene Choice ammonium buffer [1.5 mM $MgCl_2$, 75 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, pH 8.5], 3.0 mM $MgCl_2$ (total 4.5 mM $MgCl_2$), 0.05 U μL^{-1} Gene Choice Taq DNA polymerase (Cat. No. T-28), and 0.5 μg μL^{-1} BSA. Negative controls, in which water was substituted for template, were included with all reaction sets. Reactions were cycled as follows: an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 1 min at 94°C, 45 s at 46°C, and 8 min at 72°C, and a final extension of 12 min at 72°C. BOX-PCR profiles were acquired by electrophoresis in 1.5% NuSieve agarose gel for 6 h at 2.5 V cm^{-1}. Gels were stained with ethidium bromide (0.5 μg mL^{-1}) and photographed on a GelDoc imaging system (BioRad, Hercules, CA, USA).

BOX-PCR profiles were analyzed in GelCompare II (v. 4.6; Applied Maths, Austin, TX, USA). Bands were identified, band intensities were scaled to the total intensity of each profile, and pairwise Euclidean distances among profiles were computed. Analytical variation in BOX-PCR profiles was assessed by generating duplicate profiles from 16 isolates (Louws et al. 1994). Duplicate profiles were derived from separate cultures and DNA extractions. Duplicate profiles had an average Euclidean distance of

0.12, and distances were not normally distributed. A maximum distance among duplicate profiles of 0.15 was subsequently used as a cutoff value for genotype identity. Rarefaction analysis to completeness of sampling ARDRA type 1 genotypes in each sample was performed using Estimate S (v. 7.5.2; Colwell 2005). A *G* test was used to determine whether genotypes were randomly distributed among the isolation temperatures and media treatments used for heterotrophic plate counts.

16S rRNA gene sequencing

To determine the phylogenetic affiliation of isolates, partial 16S rRNA gene sequences were obtained from representatives of each ARDRA type and each BOX-PCR genotype. PCR products for sequencing were obtained as described above using the Eub338F-0-III and 1391R primers. Sequencing was performed by the Genome Sequencing Center at Washington University of Saint Louis using the Eub338F-0-III primer. A total of 60 sequences were acquired (40 from BOX-PCR genotypes and 20 from other ARDRA types). Sequences were deposited in Genbank under accession numbers HQ822284 through HQ822348). The taxonomic affiliation of sequences was determined by nucleotide BLAST search (Zhang et al. 2000). To examine the relatedness of BOX-PCR genotypes, sequences from individual genotypes were aligned using MUSCLE (Edgar 2004), and pairwise Jukes–Cantor corrected distances were calculated in MEGA (v. 4.0; Tamura et al. 2007).

ARDRA type 1 growth temperature screening

Relative growth rates at 5, 15, 25, and 35°C were determined for at least one representative of each BOX-PCR genotype within ARDRA type 1. Isolates were grown in Tryptic soy broth (TSB) at 15°C until exponential growth phase was reached. Cultures were then inoculated onto Tryptic soy agar (TSA) plates which were incubated at the respective temperatures. The plates were inspected daily after inoculation and time to appearance of the colonies was recorded to the nearest 24 h. The effect of temperature on time to appearance of the colonies was evaluated using a mixed model ANOVA with genotype designated a random factor. Isolates were classified into psychrophiles and psychrotrophs according to definitions adapted from Morita (1975), and psychrophiles were further divided into somewhat psychrophilic, displaying some growth at 25°C, and fully psychrophilic, displaying no growth at 25°C. Unifrac analysis (Lozupone and Knight 2005) was used to test whether isolate response to growth temperature was related to BOX-PCR profile data. In this case, phylogenetic information was represented by a neighbor-joining tree of BOX-PCR profile Euclidean distances (constructed using the Neighbor program in Phylip v 3.68; Felsenstein 2005), and temperature response classes were used in lieu of environment data. Unifrac tested the null hypothesis that distances among BOX-PCR profiles were randomly related to temperature response classes. We expected that closely related genotypes are similar in temperature response, which would be indicated by rejection of the null hypothesis.

Results

Heterotrophic plate counts

Heterotrophic plate counts were performed to determine whether cultivable bacteria were present in ancient samples and whether abundance of cultivable bacteria was related to sample age. Cultivable bacteria were found in all samples except 12303 (Table 2). CFU abundance was highest in sample 8 and varied by more than 4 orders of magnitude among samples (Fig. 1). Medium and age class had

Table 2 Cultivable bacteria recovered from samples. CFU per mg sample are from heterotrophic plate counts on R2A medium incubated at 15°C

Sample	CFU mg^{-1}	ARDRA types (heterotrophic plate counts)	ARDRA types (autotrophic enrichment)	ARDRA types (heterotrophic enrichment)
8	$2.09 \pm 0.32 \times 10^4$	1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 15	8	1, 3
8619	$3.72 \pm 0.25 \times 10^3$	1, 2	1	1
8643	110 ± 20	1	1	1
11164	0.66 ± 0.13	1	–	1
11185	2.34 ± 1.4	10, 11	–	10
12303	–	–	–	–
12910	126 ± 17	1, 12	12	–
26539	1.0 ± 0.02	13	–	–

Numbers represent ARDRA types from cultures. (–) denotes samples from which cultivable bacteria were not detected

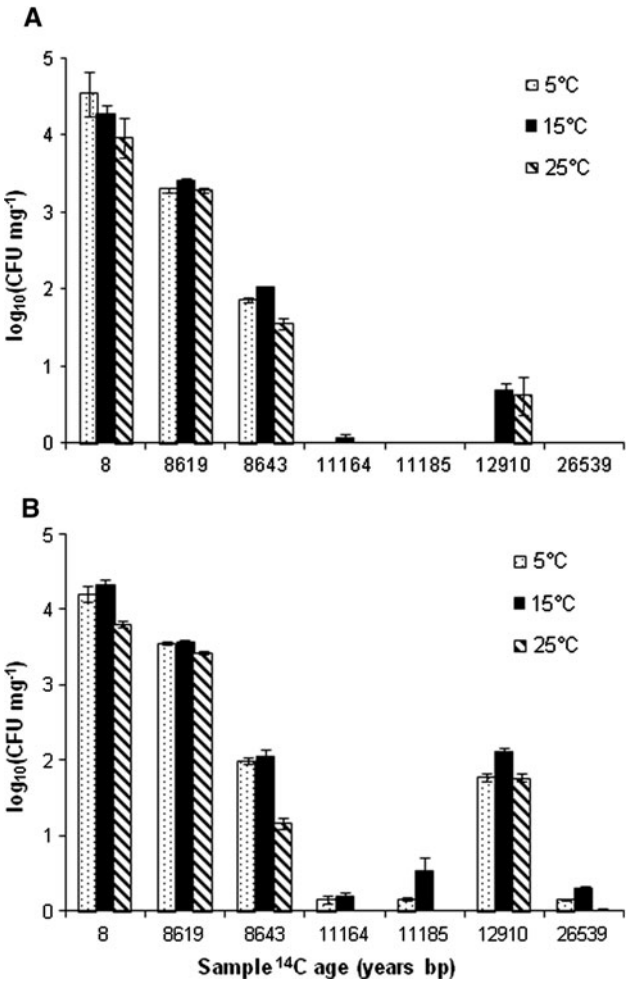

Fig. 1 CFU abundance on 10% R2A plates (**a**) and R2A plates (**b**). *Error bars* represent the standard error of triplicate plates

significant effects on CFU abundance ($p < 0.01$). A significant interaction between temperature and age class was also found ($p = 0.011$). CFU abundance on 10% R2A was, on average, 47% lower than CFU abundance on full strength R2A. For samples 11164, 11185, and 26539, cultivable bacteria were not found at 25°C.

Autotrophic enrichment cultures

Autotrophic enrichment cultures in liquid medium were performed to determine whether cultivable autotrophs were present in ancient samples. Cyanobacteria were isolated from sample 8 and identified to the genus *Microcystis* based on morphology. Cyanobacteria were not isolated from the ancient samples after incubation for 8 weeks. Cultivable bacteria matching ARDRA types from heterotrophic plate counts were found in enrichments from samples 8619, 8643, and 12910 (Table 2).

Heterotrophic enrichment cultures

Cultivable bacteria were found in heterotrophic enrichment cultures of samples 8, 8619, 8643, 11164, and 12910 (Table 2). Cultivable bacteria were found in all types of media and at all temperatures, except for samples 11164 and 12910, which were positive for growth at 5 and 15°C, but not 25°C.

Diversity of cultivable bacteria

ARDRA profiles from 262 bacterial isolates were resolved into a total of 14 types (Tables 2, 3). Bacterial ARDRA types found in autotrophic and heterotrophic enrichment cultures were also found in heterotrophic plate counts. A greater number of ARDRA types were cultivated from sample 8 (11 types) than from individual ancient samples (1–2 types; Table 2). Cultivable bacteria in sample 8 were dominated by ARDRA type 9 (closely related to *Micromonospora*; Table 3), which represented more than 90% of CFUs.

16S rRNA gene sequences of representative isolates of each ARDRA type were obtained to identify cultures (Table 3). Length of sequences obtained ranged from 200 base pairs to more than 700 base pairs. Cultivable bacteria found in sample 8 included members of the *Firmicutes, Actinobacteria*, *Proteobacteria*, *Cyanobacteria*, and *Deinococcus-Thermus*. ARDRA type 8 was a *Cyanobacteria* recovered only from sample 8 and classified as *Microcystis* based on morphological analysis. Cultivable bacteria from ancient samples were restricted to the *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*.

BOX-PCR genotyping

ARDRA type 1, most closely related to the genus *Sporosarcina*, was recovered from 5 samples, and 108 isolates of this type were examined by BOX-PCR. Isolates from heterotrophic plates were genotyped by BOX-PCR and divided into 28 genotypes (Table 4). Rarefaction analysis using Chao's richness estimator ($Chao_1$) indicated that genotypes were thoroughly sampled for samples 8619, 8643, and 11164 (Fig. 2). Genotypes were generally not common among algal mat samples; only two genotypes occurred in more than one sample. However, within samples, genotypes were common to temperature and medium treatments, with the exception of some psychrophilic genotypes which did not occur at 25°C. Because only a single ARDRA type 1 isolate was found from sample 12910, this isolate was not included in subsequent statistical analyses.

According to the G-test, genotypes were distributed non-randomly with respect to both temperature ($p = 0.011$, 28 degrees of freedom) and medium ($p = 0.018$, 56 degrees of

Table 3 Phylogenetic affiliation of ARDRA types as determined via BLAST

ARDRA type	Nearest cultivated relative	Max. BLAST identity (%)	GenBank accession
1	*Sporosarcina* sp. MB6	98–100	AB490786
1	*Planococcus psychrotoleratus*	100	AY771711
2	*Micrococcus luteus*	98–100	GQ369519
3	*Tetrasphaera* sp. Ellin176	99	AF409018
4	*Roseomonas frigidaquae*	99	EU290160
5	*Frigoribacterium* sp. D21	100	DQ652546
6	*Polaromonas* sp. 3010	99	EF423340
7	*Deinococcus aquatilis*	97	AM940971
9	*Micromonospora chokoriensis*	99–100	GQ163478
10	*Paenibacillus* sp. KAR72	98	EF451701
11	*Paenibacillus contaminans*	96	EF626690
12	*Pedobacter* sp. B4a-b5	95–98	FJ897516
13	*Arthrobacter* sp. SH-61B	100	FN377733
14	*Janibacter anophelis*	100	GQ280042
15	*Massilia* sp. M1	98	GQ200828

When ranges of BLAST identity are given, these represent multiple sequences for an ARDRA type

Table 4 Abundance of BOX-PCR genotypes from ARDRA type 1 in different temperature response classes

Sample	Number of isolates	Number of genotypes	Fully psychrophilic	Somewhat psychrophilic	Psychrotrophic
8	18	13	0	7	6
8619	30	9	2	4	3
8643	48	4	1	2	1
11164	12	4	2	1	1
Total	108	28	5	14	11

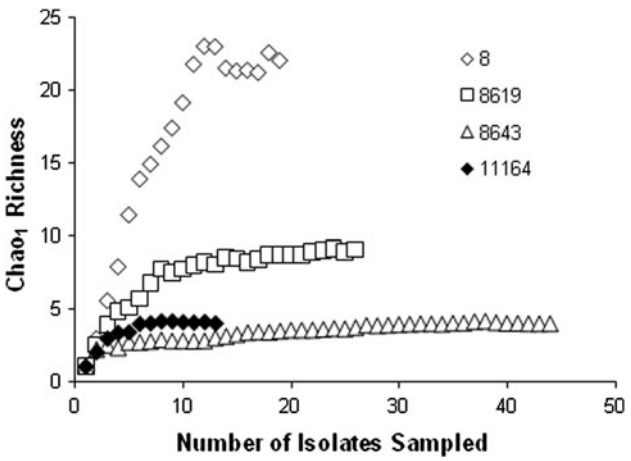

Fig. 2 Rarefaction analysis of BOX-PCR genotypes (ARDRA type 1) from individual samples. Analysis was performed in Estimate S (Colwell 2005) using 50 iterations at each sample size

freedom), suggesting the culture conditions selected for the growth of certain genotypes. For example, in sample 8643, genotype 13 occurred predominately on plates incubated below 25°C and genotype 14 occurred predominately on plates incubated at 25°C.

Partial 16S rRNA gene sequences were acquired from 40 ARDRA type 1 isolates representative of each BOX-PCR genotype. Phylogenetic sequence analysis revealed that the members of ARDRA type 1 were closely related, with an average pairwise Jukes–Cantor corrected distance of 0.81% (standard deviation of 1.4%). Isolate 16S rRNA gene sequences were grouped into 3 distinct clusters (Fig. 3). The majority of isolates were identified as members of the genus *Sporosarcina* by both BLAST (98–100% identity to *Sporosarcina*) and phylogenetic analysis (Fig. 3). One isolate from sample 11164 was related to the *Planococcus* species (100% maximum identity).

Temperature response screening of genotypes

To examine temperature response differences among genotypes, 36 isolates of ARDRA type 1, including at least one member of each genotype, were screened for growth at temperatures ranging from 5 to 35°C. All genotypes grew at 5 and 15°C, and all but 5 genotypes grew at 25°C. No

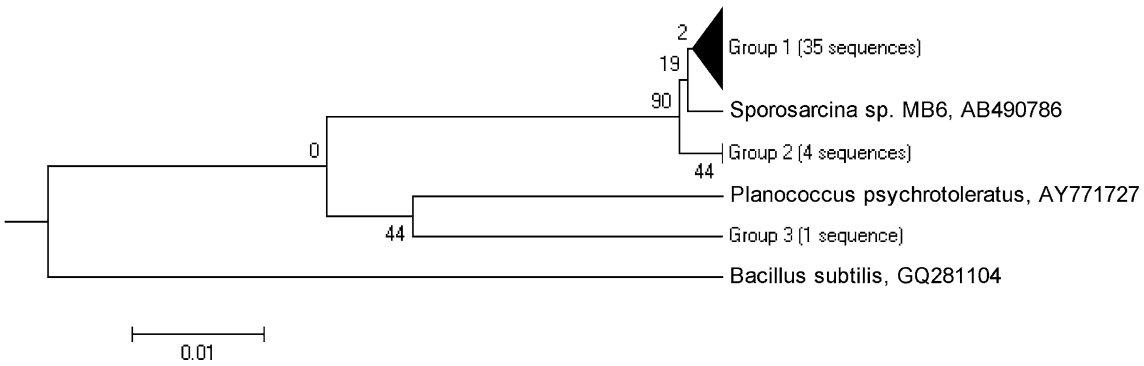

Fig. 3 Neighbor-joining tree of 16S rRNA gene sequences from ARDRA type I isolates. For sequence groups with 100% identity, a single representative sequence was chosen. The tree was tested with 500 bootstrap replicates. Additional sequences from members of the *Firmicutes* were included for comparison

genotypes grew at 35°C. Temperature significantly affected time to colony appearance (mixed model ANOVA, $p < 0.001$). Tukey's HSD test was used to compare differences in time to colony appearance among temperatures; time to appearance was significantly longer at 5°C than at 15 or 25°C; time to appearance at 15 and 25°C were not significantly different.

Genotypes were grouped into 3 categories based on screening results (Table 4): (1) genotypes that did not show growth at 25°C were considered fully psychrophilic, (2) genotypes that showed a shorter time to appearance of colonies at 15°C than 25°C, but that still grew at 25°C, were considered somewhat psychrophilic, and (3) genotypes that displayed a shorter time to appearance of colonies at 25°C than 15°C, or equal time to appearance of colonies at 15 and 25°C, were considered psychrotrophic. Psychrotrophic isolates were more common in age class 1, whereas fully psychrophilic isolates were only found from age classes 2 and 3. The distribution of temperature response groups was significantly different from an even distribution among age classes (G test, $p = 0.024$, 6 degrees of freedom).

To assess whether temperature response was related to genotype phylogeny as determined from BOX-PCR, Unifrac was used to test the null hypothesis that temperature response classes were distributed randomly over a neighbor-joining tree of BOX-PCR profiles. Temperature response classes were non-randomly distributed (Unifrac test of total significance, $p < 0.001$), indicating that temperature response was associated with genetic structure of isolates as determined by BOX-PCR typing.

Discussion

As expected, cultivable bacteria were more abundant and more taxonomically diverse in sample 8 than in ancient samples. Bacteria from ancient samples primarily belonged to the *Firmicutes*, but members of the *Actinobacteria* and *Bacteroidetes* were also found. The modern sample contained representatives of *Cyanobacteria*, *Proteobacteria*, and *Deinococcus-Thermus* in addition to *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. These results are broadly similar to findings from bacterial 16S rRNA gene clone libraries from these samples (Antibus et al. 2011). Several of the genera cultivated in this study have been previously cultivated from modern Antarctic algal mats, namely *Micrococcus* (Brambilla et al. 2001; Van Trappen et al. 2002), *Sporosarcina* (Reddy et al. 2003), *Arthrobacter* (Van Trappen et al. 2002), *Planococcus* (Reddy et al. 2002), and *Frigoribacterium* (Brambilla et al. 2001).

Cultivable bacteria belonging to endospore-forming genera within the *Firmicutes* (*Planococcus*, *Sporosarcina*, and *Paenibacillus*) were readily isolated from ancient samples. The recovery of *Firmicutes* from ancient samples was not unexpected, as *Firmicutes* endospores are extremely resilient to adverse environmental conditions (Gould 2006) and *Firmicutes* have been identified in a wide range of ancient materials (Renberg and Nilsson 1992; Kennedy et al. 1994; Gorbushina et al. 2007; Rollo et al. 2007, Yung et al. 2007).

In contrast, the recovery of *Actinobacteria* and *Bacteroidetes* from ancient samples was less expected, as these groups are considered less capable of long-term dormancy than *Firmicutes*. *Actinobacteria* have been found in ancient permafrost and ice (Vishnivetskaya et al. 2000; Willerslev et al. 2004; Johnson et al. 2007); however, the cause of their persistence is unclear. Johnson et al. (2007) suggest that *Actinobacteria* are metabolically active within permafrost, while Suzina et al. (2004, 2006) suggest that non-spore resting cells are important for persistence. It is possible that Antarctic *Actinobacteria* possess characteristics which enable them to withstand long-term dormancy or that the ancient samples were colonized over the millennia by bacteria from surrounding soils.

Cyanobacteria, identified as *Microcystis*, were cultivated from the modern sample, but cultivable *Cyanobacteria* were not found in ancient samples. *Microcystis stagnalis* has been cultured from lakes and algal mats in the Dry Valleys (Spaulding et al. 1994; Vincent 2000b). Many Antarctic *Cyanobacteria* are resilient to periods of dormancy lasting several years or decades (Hawes et al. 1992; McKnight et al. 2007), but survival of *Cyanobacteria* over longer periods of dormancy has not been well studied due to a lack of suitable environments for study (Billi and Potts 2002). Our findings may be interpreted to mean that *Cyanobacteria* are less able to remain viable over long periods of dormancy in the Antarctic than heterotrophic bacteria. Alternatively, heterotrophic bacteria in samples may be consuming ancient carbon derived from *Cyanobacteria*.

BOX-PCR genotyping was applied to ARDRA type I isolates to assess genotypic diversity of closely related bacteria recovered from samples of different ages. Because adaptation to low temperatures is thought to have been an important factor in the evolution of some Antarctic microbes (Franzmann and Dobson 1993; reviewed in Vincent 2000a; reviewed in Deming 2002), we examined the response of isolate genotypes to growth at temperatures between 5 and 35°C. The majority of genotypes found in this study (82%) are psychrotrophic based on the definition of Morita (1975), while 18% are psychrophilic. This finding was expected, as novel bacteria from the Antarctic are often psychrotrophs rather than psychrophiles (e.g., Spring et al. 2003; Van Trappen et al. 2004; Shivaji et al. 2004; Prabahar et al. 2004; Yi et al. 2005; Hong et al. 2008). However, psychrophilic isolates were recovered from samples thousands of years old. Therefore, it is possible that psychrophilic traits also confer enhanced resistance during extended periods of dormancy at very low temperatures.

As expected, temperature response classes were non-randomly distributed over a phylogenetic tree of isolate BOX-PCR profiles, indicating that variation in range of growth temperatures reflects the genetic relatedness of genotypes. Different growth temperature ranges displayed by genotypes may represent life-history traits which are advantageous under varying conditions, as suggested by Vincent (2000a) (i.e., strains capable of relatively rapid growth in warm temperatures versus strains capable of growth in cold temperatures). However, physiological traits other than those related to temperature may contribute to differences among genotypes. We found evidence that genotypes differed in their response to growth medium (genotype occurrence was significantly different on 10% R2A and R2A plates) suggesting that some genotypes required a richer medium for growth. In addition, interaction may exist between physiological traits: temperature and nutrient limitation have been identified as potential co-limiting factors for bacterial growth in the Antarctic (Wiebe et al. 1992; Nedwell 1999).

Conclusions

Cultivable bacteria were isolated from a chronological series of algal mat samples, suggesting that bacteria have the potential to remain viable for millennia in the Dry Valleys. Although we cannot fully exclude the possibility of colonization by soil bacteria, ancient mat samples displayed low abundance and diversity of cultivable bacteria, which was expected due to loss of viability over time. Some isolates in this ancient, extreme environment, such as those in ARDRA type 1 related to *Sporosarcina*, have distinct traits that may explain their persistence, such as the ability to form endospores and grow more readily at low temperatures. Although it is possible that these ancient communities may include organisms that have colonized the material after deposition, these bacteria have the potential to resume metabolism and growth. Given the right environmental conditions, this source of microbial inocula could result in selective "recycling" of bacteria from ancient materials, with implications for the composition of modern bacterial communities.

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