Prospecting for ice association: characterization of freeze–thaw selected enrichment cultures from latitudinally distant soils

Sandra L. Wilson, Paul Grogan, and Virginia K. Walker

Abstract: Freeze–thaw stress has previously been shown to alter soil community structure and function. We sought to further investigate this stress on enriched microbial consortia with the aim of identifying microbes with ice-associating adaptations that facilitate survival. Enrichments were established to obtain culturable psychrotolerant microbes from soil samples from the latitudinal extremes of the Canadian Shield plateau. The resulting consortia were subjected to consecutive freeze–thaw cycles, and survivors were putatively identified by their 16S rRNA gene sequences. Even though the northerly site was exposed to longer, colder winters and large spring-time temperature fluctuations, the selective regime similarly affected both enriched consortia. Quantitative PCR and metagenomic sequencing were used to determine the frequency of a subset of the resistant microbes in the original enrichments. The metagenomes showed 22 initial genera, only 6 survived and these were not dominant prior to selection. When survivors were assayed for ice recrystallization inhibition and ice nucleation activities, over 60% had at least one of these properties. These phenotypes were not more prevalent in the northern enrichment, indicating that regarding these adaptations, the enrichment strategy yielded seemingly functionally similar consortia from each site.

Key words: antifreeze activity, freeze–thaw resistance, ice nucleation, ice recrystallization, soil bacteria.

Résumé : Il a été démontré que le stress causé par la congélation–décongélation affecte la structure et la fonction de la communauté d'un sol donné. Nous désirions étudier plus en profondeur ce type de stress sur des consortiums microbiens enrichis dans le but d'identifier des microbes possédant des traits d'adaptation à la glace qui faciliteraient leur survie. Des enrichissements ont été réalisés afin d'obtenir des microbes psychrotolérants cultivables à partir d'échantillons de sol provenant de latitudes extrêmes du plateau du Bouclier canadien. Les consortiums résultants ont été soumis à ces cycles consécutifs de congélation–décongélation et les survivants ont été identifiés putativement à partir des séquences de leur gène d'ARNr 16S. Même si le site le plus nordique était exposé à des hivers plus froids et plus longs ainsi qu'à d'importantes fluctuations de températures printanières, le protocole de sélection affectait de façon similaire les deux consortiums enrichis. La Q-PCR et le séquençage métagénomique ont été utilisés pour déterminer la fréquence d'une sous-catégorie de microbes au sein des populations enrichies originales. Les métagénomes ont révélé la présence de 22 genres initiaux dont 6 seulement survivaient, et ceux-ci n'étaient pas dominants avant la sélection. Lorsque les survivants ont été évalués sur le plan de l'inhibition de la recristallisation de la glace et de la nucléation de la glace, plus de 60 % possédaient au moins une de ces propriétés. Ces phénotypes n'étaient pas plus abondants dans l'enrichissement nordique, indiquant que, en ce qui concerne ces adaptations, la stratégie d'enrichissement a généré des consortiums apparemment similaires d'un point de vue fonctionnel sur chaque site.

Mots-clés : activité antigel, résistance à la congélation–décongélation, nucléation de la glace, recristallisation de la glace, bactéries du sol.

[Traduit par la Rédaction]

Introduction

Becking's well-known principle of "everything is everywhere [but] the environment selects" (Baas Becking 1934; de Wit and Bouvier 2006) is viewed as rather deterministic for today's enthusiasm for stochastic processes in environmental microbiology. Perhaps in keeping with the modern view, the literature relating to psychrotolerant or psychrophilic microbes demonstrates that for the most part, these microbes are sought, and found, in extremely cold locations such as glaciers and the Arctic or Antarctic. It is from these collections that we know that such microbes have evolved a number of strategies to counter the challenges posed by

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freeze–thaw stress. These strategies can include the production of cold shock or cold adaptive proteins, of proteins with low temperature alterations, of cryoprotectants, including sugars such as glycerol and trehalose, and alterations in membrane fatty acid composition to help maintain membrane fluidity (Bej and Mojib 2010; Casanueva et al. 2010; Chattopadhyay and Jagannadham 2001; Gómez Zavaglia et al. 2000; Jones et al. 1987; Metpally and Reddy 2009). Despite these adaptations, freeze–thaw stress remains a challenge to microbes and microbial communities (e.g., Yergeau and Kowalchuk 2008), partially because of ice formation (Mazur 1963). Thus microbes that avoid freezing and the damage due to ice crystal growth by decreasing extracellular ice nucleation temperatures and (or) regulating the temperature of freezing and the shape and growth of external ice crystals (e.g., D'Amico et al. 2006; Xu et al. 1998) should show enhanced survival at freezing temperatures.

Microbial antifreeze proteins (AFPs) and ice nucleation proteins (INPs) directly associate with ice and presumably reduce its damaging effects on the organism (e.g., Deininger et al. 1988; Garnham et al. 2008; Gilbert et al. 2004; Raymond et al. 2008; Xiao et al. 2010). AFPs adsorb to ice crystals thereby decreasing the freezing temperature, modifying the ice crystal phenotype, and preventing ice recrystallization (Knight and Duman 1986; Raymond and DeVries 1977). In contrast, INPs precipitate extracellular ice formation by acting as a template for ice growth at high subzero temperatures, thus decreasing the likelihood of intracellular ice formation (Zachariassen and Hammel 1976). Both AFPs and INPs have industrial applications, especially within the energy, food, medical, and recreational industries, and therefore, the isolation of microbes with these activities is of considerable interest.

It is not known if low temperature adaptations, including cryoprotectants, are disadvantageous under nonselective conditions in temperate climates, and thus, if it is necessary to prospect for microbes with ice-associating adaptations in extremely cold habitats. However, given the ubiquity of microbes and Becking's principle, we hypothesized that freeze-hardy microbes could be isolated from soils derived from more accessible and less extreme sites. To identify such potentially "rare" microbes with various low-temperature-resistant phenotypes we reasoned that we would have to subject enrichment cultures from such sites to multiple freeze–thaw cycles (Walker et al. 2006). Further, our objectives were to compare the stringency of this selective regime for the recovery of resistant microbes in two latitudinally distant consortia, one remote (and more northerly) and the other local, and to assess the recovered microbes for cold adaptations that would be relevant to industry.

Materials and methods

Soil samples and culture conditions

Soil samples were obtained in the late summer from the top 1–2 cm of organic soil from two locations in the Canadian Shield, a plateau where Precambrian granite and gneiss outcrops have been exposed by glaciers and there is a relatively shallow active layer. Soil from the more southerly Gould Lake watershed, Ontario (44°26′N, 76°35′W; pH ~6.5) overlays bedrock, with the northern Daring Lake watershed, Northwest Territories (64°52′N, 111°35′W; pH ~6.5) underlain by permafrost and bedrock. Both soils are subjected to a continental climate, but with the latter experiencing a significantly longer and colder winter than the southerly site, with ~82 frost-free days, and mean February temperatures of approximately –27 °C (compared with ~156 frost-free days and mean February temperatures of –6 °C for the Gould Lake region). Soil samples, with large roots removed, were collected in triplicate sterile tubes and composited prior to enrichment. For logistical reasons, the samples were transported and stored at 4 °C (Gould Lake) or –20 °C (Daring Lake) until used. Soil pH was determined by the liquid portion resulting from a slurry of 1 g soil in 5 mL water, which was vigorously mixed and allowed to sediment (Kumar et al. 2011).

Soil enrichment cultures were obtained by culturing the soil composites (1 g) in ≥3 mL of 10% tryptic soy broth (TSB) (3 g tryptic soy broth (Bacto, Dickinson and Company, Sparks, Maryland, USA), 0.1 g KNO_3, 0.1 g $(NH_4)_2SO_4$, and 0.1 g K_2HPO_4 in 1 L of deionized water), while being shaken at 100 r/min (Gyrotory shaker G2, New Brunswick Scientific Co. Inc., Edison, New Jersey, USA) for ~48 h at 22 °C. Isolates subsequently derived from the enrichment cultures and control bacteria (*Escherichia coli* TG-2 and *Chryseobacterium* sp. strain C14) were cultured in 10% TSB as above. Cultures were transferred to 4 °C (overnight prior to freeze–thaw treatment or for ~48 h prior to assay; Walker et al. 2006; Wilson et al. 2006) to facilitate the production of any cold-inducible products. Transformed *E. coli* (see below) was cultured in Luria broth (LB; 10 g NaCl, 10 g bacto-tryptone, and 5 g yeast extract in 1 L deionized water, pH 7.0) overnight at 37 °C, with agitation.

Freeze–thaw selection

Aliquots (2 mL) of the enrichment cultures (in 10% TSB, in triplicate), without added cryoprotectants, were treated with multiple freeze–thaw cycles using an automated cryocycler, wherein samples were frozen (–18 °C) and thawed (5 °C) on an hourly basis, with a single cycle consisting of 1 h at each temperature. In practice, the samples were at or below 0 °C for ~95 min each cycle (Walker et al. 2006). Following the first 48 cycles, the cultures were used to inoculate fresh media and were subjected to a second round of 48 freeze–thaw cycles. Freeze–thaw tolerance was monitored periodically during the selection by removing samples and determining viable cell counts (colony-forming units (CFU)/mL; i.e., at 22 °C on 10% TSB agar plates). *Chryseobacterium* sp. strain C14, with known freeze–thaw resistance (Walker et al. 2006), and *E. coli* TG-2, with no known ice-association properties (Wilson et al. 2006), were used as the positive and negative controls, respectively. Silver iodide crystals were added to the controls and freeze–thaw resistant isolates (see below) to ensure nucleation of samples (Walker et al. 2006).

Culturable bacteria that survived both sets of 48 freeze–thaw cycles were deemed to be freeze–thaw resistant and were isolated as monocultures (at 22 °C on 10% TSB agar plates). The 16S rRNA gene from morphologically distinct (with redundancy) isolated monocultures was PCR amplified and sequenced with universal bacterial 16S rRNA gene primers 8F and r1406 as below, or with 530F (5′-

GTGCCAGCMGCCGCGG-3′) and R1492 (5′-TACGGYTACCTTGTTACGACT-3′; http://openwetware.org/wiki/Bacterial_species_identification#Universal_Bacterial_Primers; Lane 1991). Isolates with similar sequences were compared using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Thompson et al. 1994). Those with a sequence similarity of ≥98% were deemed to be the same and were recorded once per genus. This final set of isolates was subsequently subjected to an additional set of 48 freeze–thaw cycles.

Clone library construction and analysis

Clone libraries were constructed to estimate the consortia richness of the enrichment cultures from the two locations prior to freeze–thaw selection. The libraries were assembled in duplicate by extracting DNA from the free-living community within the liquid portion of the soil enrichments (SoilMaster DNA extraction kit; Epicentre, Madison, Wisconsin, USA). The 16S rRNA gene sequence was PCR amplified from the extracted DNA with "universal" bacterial 16S rRNA gene primers (8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and r1406 (5′-ACGGGCGGTGTGTAC-3′) SigmaGenosys, Oakville, Ontario, Canada (Hicks et al. 1992; Lane et al. 1985)), *Taq* polymerase (1 unit, Fermentas, Burlington, Ontario, Canada), and cycling conditions Telang et al. 1997 or the T7 Select System (Novagen, Madison, Wisconsin, USA). The expected 1.4 kb amplified 16S rRNA gene sequence product was confirmed by agarose gel electrophoresis. The amplified and purified (Qiagen, Mississauga, Ontario, Canada) products were cloned into pCR2.1 and subsequently transformed into *E. coli* DH5-α Top 10′ cells (TOPO cloning, Invitrogen, Carlsbad, California, USA). The inserted 16S rRNA genes of randomly selected clones were then PCR amplified either as above from the isolated vector (Miniprep, Qiagen) or directly from the clone with M13 forward (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers (TOPO cloning, Invitrogen).

Libraries were screened using restriction fragment length polymorphism (RFLP) analysis, which consisted of two single restriction digests, *Hae*III (5′-GG/CC-3′) or *Hha*I (5′-G/CGC-3′) per clone (as per recommendations, Fermentas). After digestion (37 °C for 1 h), the DNA was visualized on 1.0%–1.5% agarose gels and the cloned inserts categorized according to their RFLP fingerprint. Clones with unique RFLP patterns were sequenced on both strands using the 8F and r1406 or the M13 forward and reverse primers. The representative clones to be sequenced were randomly picked, with some redundancy. Sequence quality was assessed with CodonCode Aligner (http://www.codoncode.com/aligner/trial.htm). The inverse complement of the reverse sequence was obtained using "Manipulate and Display a DNA sequence" by Molecular Toolkit (http://arbl.cvmbs.colostate.edu/molkit/manip). Verified sequences were entered into BLASTn from NCBI (http://www.ncbi.nlm.nih.gov/blast; Altschul et al. 1997), or the Ribosomal Database Project II (http://rdp.cme.msu.edu; Cole et al. 2005, 2009), and the putative identity of the microbe from which the insert was derived was determined based on the closest phylogenetic relative with ≥97% identity.

Semiquantitative real-time PCR

Semiquantitative PCR (Q-PCR) (modified from Shelburne et al. 2000) was performed (QuantiTect SYBR Green PCR Kit, Qiagen; Cepheid Smart Cycler Version 2.0d) to estimate the relative abundance of genera corresponding to certain isolates in the original enrichment cultures. Cycling conditions were as follows: an initial denaturation (15 min at 95.0 °C), 45 cycles (15 s at 95.0 °C, 30 s at 57.0 °C, 30 s at 72.0 °C, 15 s at 72.0 °C with optics), and a melt curve (60.0–95.0 °C at 0.2 °C/s with optics). Universal, *Bacillus*, *Paenibacillus*, and *Pseudomonas* specific primers were used at different concentrations and under different conditions, with the reproducibly optimal concentration for each primer pair as stated in Table 1. Primers with satisfactory specificity to other isolated genera could not be generated even after numerous attempts. Standard curves (cycle threshold (C_T) versus DNA concentration) were determined in duplicate using the 16S rRNA gene sequence of the isolates. For the experimental reactions (in triplicate), template DNA was obtained by dilutions of the original enriched consortia used to construct the clone libraries. In all cases, each experimental condition was duplicated within each Q-PCR reaction, and no template controls were included. The average C_T was calculated, as was the proportion of the total 16S rRNA genes present, which in turn was determined by averaging the C_Ts from both pairs of universal primers.

Metagenomic analysis

Metagenomic analysis was performed on genomic DNA extracted (duplicate extractions, pooled) from the enrichment cultures derived from the two soil-derived consortia prior to freeze–thaw cycling (Qiagen DNeasy kit, Gram-positive bacteria protocol). Library preparation (DNA shearing, adapter ligation), sequence generation, and initial sequence filtering were done by Genome Quebec (Montréal, Quebec, Canada) using Roche 454 sequencing technology (Roche GS-FLX sequencing platform). Linker sequences, terminal N nucleotides, reads shorter than 50 bp, and reads otherwise considered poor quality were omitted (GSAssembler Suite, Roche). The remaining sequences were compared with those in the Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) using the BLASTn program from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1997). Subsequently, "sfffile" (GSAssembler Suite) was used to extract 16S rRNA gene sequence reads, which were then compared with a database of bacterial 16S rDNA sequences from NCBI (September 2010). The "Fetch Taxonomic Representation" and "Summarize Taxonomy" functions of Galaxy Genomics (http://galaxy.psu.edu) were used to determine the putative taxonomic identity corresponding to the top BLAST "hit" per sequence. This then allowed for various taxonomic level groupings of the sequences.

Characterization of the freeze–thaw resistant isolates

Ice recrystallization inhibition (IRI) and ice-shaping assays were performed as previously described with whole-cell cultures (Wilson et al. 2006; Wilson and Walker 2010). Samples in which there was no visible growth of ice crystals over the ≥16 h incubation period at –6 °C were deemed to exhibit IRI activity (Tomczak et al. 2003). Ice-shaping activity was assessed using a nanolitre osmometer (Clifton Technical Physics, Hartford, New York, USA) and a Leitz Dialux 22 microscope. Ice-shaping was indicated by ice crystal morphologies that deviated from thin disks (Chakrabartty and

Table 1. Q-PCR primer sequences and threshold cycles (C_T).

rRNA gene[a]	Forward primer	Reverse primer	Primer (μmol/L)	Percentage of universal[b] (C_T)[c]	
				Gould Lake	Daring Lake
Universal (89 bp)	CCATGAAGTCGGAATCGCTAG (Shelburne et al. 2000)	GCTTGACGGGCGGTGT (Shelburne et al. 2000)	0.4	100.00±0.00 (17.43±1.10)	100.00±0.00 (14.66±0.65)
Universal (466 bp)	TCCTACGGGAGGCAGCAGT (Nadkarni et al. 2002)	GGACTACCAGGGTATCTA… (Nadkarni et al. 2002)	0.3	100.00±0.00 (14.78±0.28)	100.00±0.00 (12.76±0.54)
Bacillus	CTAACCAGAAAGCCACGGC (Chiang et al. 2006)[d]	CCCAGTTTCCAATGACCCT (Merrill et al. 2003)[e]	0.1	20.47±1.91 (19.19±0.33)	1.09±0.33 (21.38±1.03)
Paenibacillus	GCTCGGAGAGTGACGGTACCTGAGA (Shida et al. 1997)	CTACCAGGGTATCTAATCC (Steven et al. 2007)	0.2	0.48±0.21 (31.68±0.93)	0.05±0.03 (33.24±1.66)
Pseudomonas	GACGGGTGAGTAATGCCTA (Spilker et al. 2004)	CACTGGTGTTCCTTCCTATA (Spilker et al. 2004)	0.3	4.72±1.84 (18.79±0.72)	20.96±1.14 (24.41±0.22)

[a]16S rRNA genes were amplified using either "universal" or genus-specific primers.
[b]Means and standard deviations of the amplified product as a percent of the amplified product obtained with the universal primers.
[c]Amplified product was estimated using the C_T determined by the level of intercalated fluorescent dye. Means and standard deviations are shown.
[d]Inverse complement of primer No. 7.
[e]Inverse complement of primer Bac629F.

Hew 1991). Ice nucleation activity (INA) assays were conducted by loading 1 μL samples of cultures (approximately 10^8 CFU/mL) onto a polarizing film on a covered ethylene glycol bath. The bath temperature was decreased by approximately 0.2 °C/min from –1 to –20 °C. As a semiquantitative estimate, the temperature at which ≥90% of the droplets froze (T_{90}) was deemed to be the ice nucleation temperature and isolates were classified according to INA type (Yankofsky et al. 1981), with a lower limit of activity at –9 °C; quantified results of a subset of nucleation profiles are shown as log nuclei per millilitre versus temperature (modified from Maki et al. 1974; Vali 1971).

To ascertain if the cryocycler selected for microbes demonstrating AFP and (or) INP activity, we randomly picked colonies ($n = 8$) from each of the unselected enriched consortia derived from the collection sites. These colonies were assayed for IRI, ice-shaping, and INA activity. For all assays, *Chryseobacterium* sp. strain C14, fish Type III AFP (10 mg/mL; A/F Protein, Inc., Waltham, Massachusetts, USA), or purified recombinant AFPs prepared as described (Gordienko et al. 2010), served as positive controls for the IRI and ice-shaping assays. Commercial INPs (*Pseudomonas syringae* preparation; Wards Natural Science Establishment, Rochester, New York USA) were used as positive controls for the INA assays. In all cases, *E. coli* TG-2 and 10% TSB were used as the negative controls. All ice-associating assays were repeated 3–5 times.

Statistical analysis

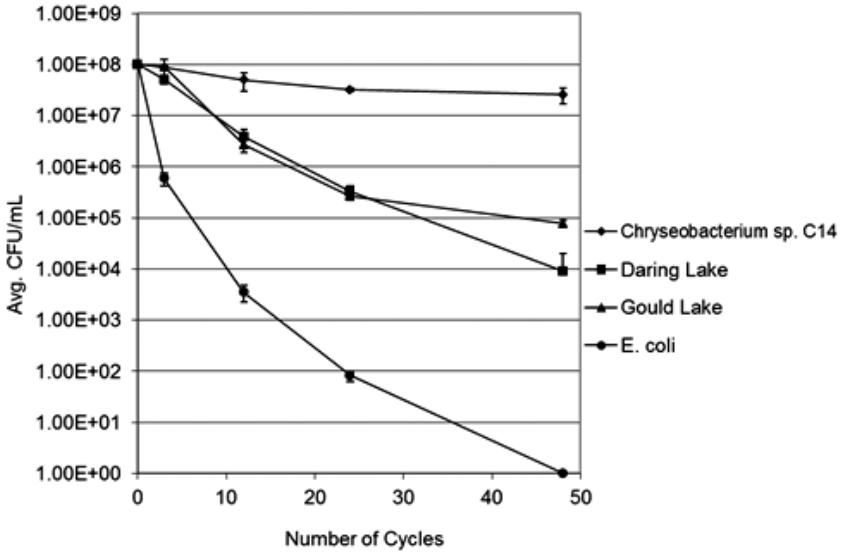
The efficacy (coverage) of the clone libraries was determined on the basis of the total cultured richness captured by the random sampling of clones, equal to $(1 - (n/N))$, where n is the number of clone types encountered once, and N is the total number of clones (Good 1953). The Shannon–Weaver Index (Shannon and Weaver 1949) was used to assess the diversity, or taxon evenness and richness, of the initial enrichments, based on the clone libraries, as well as the diversity retained after freeze–thaw selection, based on observed morphologies and sequencing results. Finally, Student's t tests were used to identify significant differences ($p \leq 0.05$) between and within the freeze–thaw selection curves, and for the diversity reduction.

Results

Freeze–thaw selection

When enrichment cultures from the 2 sites were subjected to freeze–thaw treatment, there was little loss in culture viability over the first 3 freeze–thaw cycles (Fig. 1). However, there was a 50-fold reduction after 12 cycles, and viability was reduced almost 3 orders of magnitude after 24 cycles. At the end of 48 cycles, the viable cell number was reduced more than 1000-fold for both consortia, but with a significantly higher recovery of colonies in the Gould Lake samples (t test; $p = 0.04$; first set of 48 cycles). Repeating the freeze–thaw regime did not result in a significant difference in the second set of freeze–thaw treatments compared with the first, for soil enrichments from either location. When surviving isolates were identified according to 16S rRNA gene sequences and subsequently subjected to 48 freeze–thaw cycles individually, *Pseudomonas* sp. strain DL13 viability decreased

Fig. 1. Average colony-forming units (CFU) per millilitre for the Daring Lake (NT) and Gould Lake (ON) enriched consortia over 48 freeze–thaw cycles. *Chryseobacterium* sp. strain C14 and *Escherichia coli* TG-2 were used as relatively freeze–thaw resistant and susceptible controls, respectively. Each culture was tested in triplicate; in several cases, error bars on the data points are too small to be depicted. The first set of 48 freeze–thaw cycles is shown, as there was no difference between the first and second sets (*t* test; $p = 0.19–0.59$ and 0.11–0.52 over 3–48 cycles, with $p = 0.26$ and 0.49 at 48 cycles, respectively, for the Gould and Daring lakes curves).



10^5-fold, *Paenibacillus* sp. strain Y1B viability decreased 10^7-fold, and the viability of the remaining 6 isolates decreased ~10^2-fold. These individual survivalship curves lay between the positive control isolate, *Chryseobacterium* sp. strain C14, which lost little viability (as determined by CFU/mL), and the negative control, *E. coli* TG-2, which lost all viability over the duration of the treatment cycles (Fig. 1).

Assessments of diversity, before and after freeze–thaw selection

To determine if the number of genera decreased over the course of freeze–thaw selection, in addition to cell abundance, diversity was assessed by construction and analysis of clone libraries for both sites. Table 2 summarizes the enriched consortia richness before selection, with redundancy removed (i.e., each clone type is represented once). It also shows a complete list of the putative identities (based on NCBI BLASTn or Ribosomal Database Project II searches) of the isolates recovered post freeze–thaw treatment (see above). To ensure that the constructed clone libraries were themselves efficacious, RFLP analysis was used to determine the coverage of the clone libraries. The clone libraries for the cultured Daring and Gould lakes consortia accounted for 0.63 and 0.43, respectively, of the total cultured richness captured by the random sampling of clones containing the 16S rRNA gene inserts (coverage; Table 3). Thus, overall, after 2 cycle sets of freeze–thaw selection, the diversity of identified species in each consortium significantly decreased by ~10-fold (Table 2; Shannon–Weaver index, *H* values). With 20 and 17 species detected within the clone libraries for Daring and Gould lakes prior to freeze–thaw selection, respectively, and with 4 species per lake post freeze–thaw selection, taxon richness decreased an estimated 5-fold.

Semiquantitative real-time PCR and metagenomic analysis

Although diversity, richness, and viability clearly decreased after treatments of enrichment cultures from both sites (Fig. 1; Table 2), these analyses do not address the possibility that the most predominant isolates might simply increase in abundance in response to freeze–thaw selection. To address this hypothesis, real-time Q-PCR using universal and genus-specific primers was used to further investigate the relative abundance of a subset of the identified freeze–thaw resistant genera in the original soil cultures. *Paenibacillus* accounted for 0.1% and 0.5% of the initial Daring and Gould lakes consortia, respectively (Table 1). As well, *Bacillus* represented 1% and 21% and *Pseudomonas* 21% and 5% of the unselected enrichment cultures from the Daring and Gould lakes samples. Combined, these genera represent about ¼ of the total 16S rRNA genes in the initial enrichment cultures. Multiple attempts to estimate the proportion of the initial cultures represented by *Chryseobacterium*, using a variety of primers (both published and designed) as well as reaction conditions (annealing temperatures and $MgCl_2$ concentrations), did not yield quantifiably reliable amplified products, and subsequent sequencing did not consistently include *Chryseobacterium*. This result suggests that *Chryseobacterium* sequences were present in the initial cultures at a very low abundance. To support conclusions obtained from these necessarily limited data sets, the microbial richness within metagenomes was determined.

We performed 454 metagenomic sequencing on genomic DNA extracted from the enrichment cultures prior to freeze–thaw selection. The two metagenomes were sequenced in a partial plate run. Of the 78 286 reads generated for the Daring Lake library, 77 487 passed quality control. Similarly, of

Table 2. Richness of the northerly (Daring Lake) and southerly (Gould Lake) soil enrichment cultures before and after freeze–thaw selection.

Before selection[a]				After selection[b]		
Putative identity and sample ID[c]	Acc. No.[d]	*N* (% match)[e]	No. of RFLP patterns[f]	Putative identity and sample ID[c]	Acc. No.[d]	*N* (% match)[e]
Daring Lake						
Actinobacterium sp. strain D3C	AY661610	1169 (97)	NA	*Chryseobacterium piscium* DL11[g]	DQ862541.1	1224 (97)
Arthrobacter sp. strain D3E	AB167248.7	1164 (98)	NA	*Paenibacillus* sp. strain YIB	AJ495806.1	1238 (98)
Bacillus sp. strain D5B	AY748912.1	1236 (100)	NA	*Pseudomonas borealis* YIC	AJ012712.1	1257 (100)
Unidentified Y12	AJ575723.1	1309 (95)	1	*Pseudomonas* sp. strain DL13[g,h]	DQ011923.1	1248 (100)
Unidentified Y3	AF532770.1	1306 (89)	1			
Burkholderia fungorum Y7	AJ544690.1	1293 (98)	2			
Burkholderia sp. strain DL17	DQ118949.1	1388 (98)	2			
Burkholderia sp. strain Y2	AY178076.1	1305 (98)	1			
Burkholderia sp. strain Y8	DQ118949.1	1241 (98)	1			
Burkholderia sp. strain Y9	AF215704.1	1243 (98)	1			
Enterobacteriaceae DL9	NA	1228 (100)	2			
Escherichia coli DL3	AB305017.1	1146 (99)	7			
Unidentified Y1	EF111071.1	1320 (96)	1			
Luteibacter rhizovicina D7D	AJ580498.1	1247 (99)	NA			
Paenibacillus sp. strain DL4	DQ339607.1	1261 (99)	1			
Photorhabdus sp. strain DL16	AM084246	1189 (100)	2			
Propionibacterium sp. strain Y4	AM410900.1	1239 (100)	1			
Pseudomonas borealis DL7	AJ012712.1	1219 (99)	2			
Pseudomonas sp. strain D5A	DQ011926.1	1179 (99)	NA			
Unidentified Y6	AF409002.1	1247 (96)	1			
Diversity (*H*)			**2.53**			**0.21**
Gould Lake						
Acinetobacter sp. strain GL5	EF103570.1	1113 (99)	1	*Bacillus* sp. strain G1a1[g,h]	AY965249.1	1156 (99)
Arthrobacter sp. strain G3B	AJ785759.1	1135 (99)	NA	*Buttiauxella* sp. strain G2b1[g]	DQ223872.1	1151 (99)
Bacillus pocheonensis GL3	AB245377.1	1235 (98)	1	*Enterobacteriaceae* G3b1[g]	NA	1266 (NA)
Bacillus sp. strain K2	AJ920000.1	1281 (99)	5	*Pantoea agglomerans* GLY	AF157694	854 (99)
Bacillus sp. strain K6	DQ985273.1	1285 (99)	1			
Chryseobacterium indoltheticum K8	ATCC27950	1307 (97)	1			
Chryseobacterium sp. strain GL8	DQ673675.1	1220 (99)	1			
Clostridium sp. strain GL7	DQ479415.1	1207 (99)	1			
Enterobacter ludwigii K4	AJ853891	1341 (98)	1			
Erwinia persicina G3E	AJ937837.1	1229 (99)	NA			
Flavobacterium sp. strain GL10	EU707556.1	1213 (99)	1			
Oxalobacteraceae strain GL4	AY429715.1	1233 (97)	1			
Paenibacillus sp. strain GL6	AM162313.1	1121 (99)	1			
Propionibacterium acnes K11	AB108483.1	1322 (99)	1			
Pseudomonas sp. strain G3F	AY263482.1	1175 (99)	NA			
Massilia plicata G3H	AY966000.1	1178 (99)	NA			
Unidentified K1	DQ819169.1	1266 (93)	1			
Diversity (*H*)			**2.71**			**0.25**

[a]Richness prior to selection was based on the 16S rDNA clone libraries. Diversity (*H*) is indicated, as calculated with the Shannon–Weaver index (Shannon and Weaver 1949).

[b]Richness following selection was based on 16S rDNA sequencing of morphologically distinct isolates. Diversity (*H*) is indicated as calculated with the Shannon–Weaver index (Shannon and Weaver 1949).

[c]Putative identity of the isolate, based on the database search results, followed by the sample identifier assigned in the laboratory.

[d]Accession number corresponding to the closest match in the database.

[e]*N* is the number of nucleotides entered into the databases; the percent identity between the isolate and its closest match is indicated in parentheses.

[f]Number of times the RFLP banding pattern corresponding to this isolate occurred in the clone libraries, used for statistical analysis of the clone library (Shannon–Weaver index and Coverage). NA, not available.

[g]Demonstrated ice recrystallization inhibition activity.

[h]Demonstrated Type 1 ice nucleation activity.

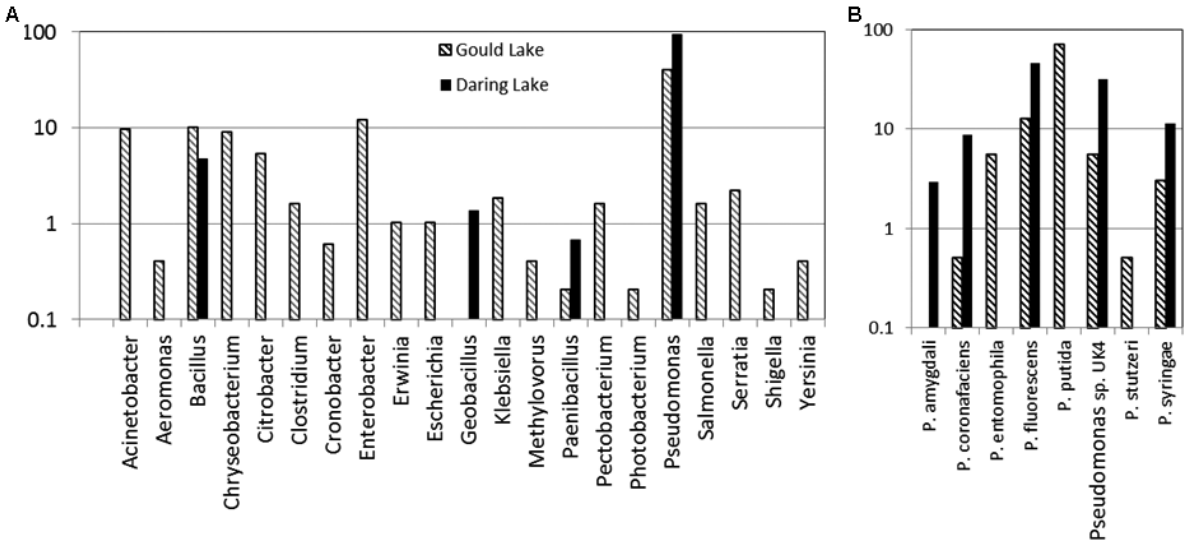
the 87 967 reads in the Gould Lake library, 87 168 reads were maintained. Unassembled quality controlled reads were mined for 16S rRNA gene sequences. Of these, an average of 390 reads contained sequences of interest; 293 and 487 for the Daring and Gould lakes libraries, respectively. The genus level 16S rRNA gene profile (Fig. 2A) showed greater richness in the Gould Lake (20 genera) enrichment than in the Daring Lake (4 genera) enrichment, which may have in part

Table 3. Coverage represented by the clone libraries.

Sample	No. of clones analyzed	No. of RFLP fingerprint groups	No. of unique RFLP patterns	Coverage[a]
Daring Lake	27	16	10	0.63
Gould Lake	28	19	16	0.43

[a]Coverage was calculated as follows: $C = 1 - (n/N)$, where C is coverage, n is no. of clone types encountered once, and N is total no. of clones (Good 1953).

Fig. 2. A summary of the phylogenetic diversity obtained from the 16S rDNA fragment containing reads (percentage of total) within the enriched metagenomes from Daring and Gould lakes. The genus-level results are shown (A), as is the species composition of the most abundant genus, *Pseudomonas* (B).



been influenced by the enrichment culture conditions. Nevertheless, the pyrosequencing data showed that the predominant genus in both sampled sites was *Pseudomonas*, which was in turn composed of a number of species (Fig. 2B). The Gould Lake enrichment was represented by 7 identified *Pseudomonas* species, 5 of which were also represented in the Daring Lake enrichment. The genera-level richness, captured by pyrosequencing, overlapped with that from the clone libraries (Table 2), although these were not identical. Markedly, however, the predominant genera identified by 454 pyrosequencing were not identical to those isolates recovered after freeze–thaw treatment (Table 2; Fig. 2). It should be noted that no sequences corresponding to *Chryseobacterium* were found in the Daring Lake library or in the metagenomic analysis (Table 2; Fig. 2). Taken together, both the Q-PCR and metagenomic analysis results indicate that the surviving microbes were somewhat phylogenetically diverse and that not all were represented in the enrichments prior to freeze–thaw selection, indicating their initial low abundance.

Characterization of freeze–thaw selected isolates

Two freeze–thaw resistant isolates derived from Daring Lake (*Chryseobacterium piscium* DL11 and *Pseudomonas* sp. strain DL13) and 3 isolates from Gould Lake (*Bacillus* sp. strain G1a1, *Buttiauxella* sp. strain G2b1, and *Enterobacteriaceae* strain G3b1) showed IRI, similar to that seen for Type III AFP or *Chryseobacterium* sp. strain C14 (Fig. 3, panel I). Some of the isolates showed large ice crystals, which did not appear to recrystallize further, and thus, they were classified as having ice-associating properties, and putative IRI activity. *Buttiauxella* sp. strain G2b1 demonstrated ice-shaping activity (Fig. 3 panel II A). Two isolates, *Pseudomonas* sp. strain DL13 and *Bacillus* sp. strain G1a1 had type 1 INA, the strongest type of nucleator (see Yankofsky et al. 1981 for INP activity classification type), similar to that observed in commercial *P. syringae* preparations (Fig. 4). In all, 5 of the 8 isolates recovered following freeze–thaw selection demonstrated one or more of the as-

Fig. 3. Representative ice-association activity assays. (I) Ice recrystallization inhibition assay of a freeze–thaw survivor, *Pseudomonas* sp. strain DL13. Digital images were captured prior to (I A) and following (I B) 16 h of incubation at –6 °C; capillary tubes are 1 mm in diameter. (II) Ice-shaping assay, where panel II A is a representative crystal morphology in the presence of *Buttiauxella* sp. strain G2b1, and panel II B shows *Chryseobacterium piscium* DL11, which also represents the remainder of the isolates with the same phenotype.

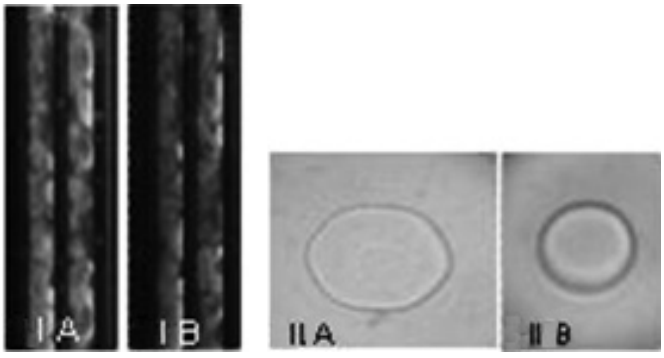
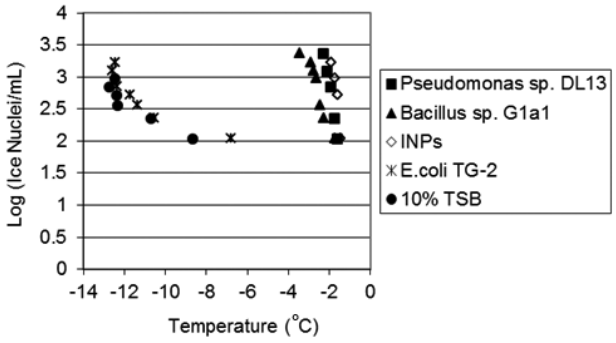


Fig. 4. Representative ice nucleation activity of freeze–thaw resistant isolates as indicated by the logarithm of ice nuclei per millilitre. Samples include *Pseudomonas* sp. strain DL13, *Bacillus* sp. strain G1a1, *Pseudomonas syringae* preparation (ice nucleation proteins; INPs), *Escherichia coli* TG-2, and 10% TSB (media control).



sayed ice-association activities (Table 2). Comparatively, of 16 microbes randomly picked from the Daring and Gould lakes derived enrichments before selection, none showed any such activities.

Discussion

Prospecting for microbes with ice-association characteristics is often undertaken in extreme environments. In contrast, we made collections prior to the first frost at 2 relatively exposed sites in the Canadian Shield plateau, characterized by a continental climate. These sites are located at the northerly and southerly extremes of this vast topography, with Daring Lake winters significantly longer, ~21 °C colder, and showing large temperature fluctuations particularly in the spring (Nobrega and Grogan 2007). Therefore, one might anticipate that enrichment cultures from Daring Lake would be more freeze-resistant than those derived from the more southern location. Indeed, we initially hypothesized that the Daring Lake enrichments would be a richer source of isolates with freeze–thaw adaptations and ice-association activities. However, this was not the case.

The clone libraries, prior to selection, represented about half (63% and 43% from the Daring and Gould Lake collections, respectively) of the total cultured richness (Good 1953; Table 3). RFLP analysis and subsequent sequencing showed that the enrichment preparations from both soils had half a dozen genera in common (Table 2). In response to freeze–thaw stress, viable cell numbers of both enrichments decreased in step until there was a 3–4 logarithm decline in viability after 48 freeze–thaw cycles (Fig. 1), coupled with a 10-fold decrease in diversity (Table 2). In contrast to less stringent procedures (Männistö et al. 2009), such severe selection allows only the most hardy, culturable isolates to survive (Walker et al. 2006), and in both consortia, freeze–thaw resistant bacteria were isolated. These 8 isolates represented 6 different genera (Table 2); *C. piscium*, *Paenibacillus* sp., and 2 *Pseudomonas* spp. were isolated from the Daring Lake consortia, while *Bacillus* sp., *Buttiauxella* sp., *Pantoea agglomerans* (synonym *Erwinia herbicola*), and an *Enterobacteriaceae* strain were isolated from the Gould Lake consortia. When assayed for freeze–thaw resistance singly, *C. piscium* DL11, *Pseudomonas borealis* Y1C, *Bacillus* sp. strain G1a1, *Buttiauxella* sp. strain G2b1, *Enterobacteriaceae* strain G3b1 and *P. agglomerans* GLY were more freeze–thaw resistant than the enriched consortia from which they were recovered, while *Pseudomonas* sp. strain DL13 was slightly more susceptible. *Paenibacillus* sp. strain Y1B appeared highly susceptible as a monoculture and lost nearly all viability. Thus, although the Daring Lake collection site was 20° latitude farther north, resistant isolates were not more numerous in this enrichment than in the more southern enrichment.

All of the identified genera from the northern and southern enrichments have previously been isolated from low-temperature environments such as glaciers (Christner et al. 2000; Miteva et al. 2004), cryoconite sediments (Christner et al. 2003), accretion ice (Christner et al. 2001), and polar regions (Gilbert et al. 2004; Lee et al. 2004; Nelson and Parkinson 1978). Microbes active within these environments are presumably cold-adapted. These properties are likely also responsible for the recovery of microbes after our freeze–thaw treatments, reflecting the stringency of this selective regime. Based on the characterization of the bacteria, clone sequencing and the RFLP data (Table 2), the recovered resistant isolates apparently represent only a small portion of the original enrichment. Q-PCR estimates indicate that the recovered species were present in the original cultures in a much lower proportion at both the Daring Lake and Gould Lake sites; enriched cultures were represented by the genera *Bacillus* (1.1% and 20.5%), *Paenibacillus* (0.1% and 0.5%), and *Pseudomonas* (21.0% and 4.7%), respectively (Table 1). Taken together, these analyses convincingly demonstrate that freeze–thaw treatments in the cryocycler do not simply select for the most abundant microbes. Although the selection was culture-based to facilitate the future recovery of useful products, the rigorous selection of a few bacteria at low abundance in the original enrichment and without apparent bias towards a particular phylogenetic grouping was successfully achieved.

It is not surprising, given the overlap of bacteria isolated from the 2 sites and from cold environments, that the genera of the bacteria recovered here, including *Bacillus* (Nejad et al. 2004), *Chryseobacterium* (Walker et al. 2006), *Pseudomonas* (Xu et al. 1998), and *Erwinia* (Koda et al. 2000), have previously been associated with low-temperature adaptations, including INP and (or) AFP activity. In total, 63% of the surviving isolates, originating from both sites and certainly not dominated by the higher latitude consortia, had ice-associating properties, which presumably would decrease or prevent ice-induced injury (Table 2; Figs. 3 and 4). *Pseudomonas* sp. strain DL13 and *Bacillus* sp. strain G1a1 demonstrated both IRI and type 1 INA activities. *Buttiauxella* sp. strain G2b1 had ice-shaping activity and *C. piscium* DL11, *Buttiauxella* sp. strain G2b1, and *Enterobacteriaceae* strain G3b1 all showed IRI. Of the surviving isolates, only *P. borealis* YIC, *Paenibacillus* sp. strain YIB, and *P. agglomerans* GLY did not demonstrate these activities, suggesting that while these activities are beneficial, it is not necessary that all species have such phenotypes to resist freeze–thaw stress. Alternative adaptations such as sporulation (e.g., *Paenibacillus*), cryoprotectants, or cold shock proteins are also important. Nevertheless, the fact that the majority of the recovered isolates showed ice-association activity is even more striking considering that none of the 16 isolates derived from the unselected Daring and Gould lakes enrichments showed any such

activity, demonstrating that these ice-associating activities may not be common at the collection sites, or indeed generally.

Given that one of the goals of these experiments was to prospect for microbes with ice-associating properties for subsequent protein isolation and characterization, a culture-dependent approach was crucial. By subjecting enrichments to freeze–thaw selection we could ensure that isolates could be cultivated and, furthermore, that survival was not due to unculturable consortia members. The metagenome of the pre-selected enrichment cultures (Fig. 2) was generated from sequences unbiased by PCR amplification and, therefore, not only underscores the strong selective pressure of freeze–thaw cycling (Fig. 1), but also should have utility in downstream protein-isolation initiatives. Our results are consistent with previous observations showing that freeze–thaw selection of soil-derived consortia allow the isolation of a few microbes with IRI activities (Walker et al. 2006), but here we also showed isolates with INA activities.

Although the original samples were obtained from different sites, with different environmental (and storage) conditions, which might be expected to lead to differing enrichment culture diversity, similar trends were observed. In hindsight, perhaps this is not surprising considering that although large air temperature fluctuations are common in higher latitude locations especially during spring, soil temperatures tend to be much less dynamic due to thermal inertia associated with the specific heat capacity of soils (e.g., Buckeridge et al. 2010). In addition, pH has a strong influence on soil diversity (e.g., Chu et al. 2010), and both Shield plateau soil types had the same pH of 6.5. The cryocycler protocol mediated the decrease in culturable diversity and enabled the recovery of freeze–thaw resistant microbes from both collections, including the southern, more temperate climate location. These studies demonstrate that even bacteria found in relatively low abundance in an enrichment culture can be isolated given sufficient selective pressure, and many of those recovered had similar ice-associating characteristics. Such stringent selection conditions should prove useful in the isolation and identification of microbes for the production of industrially relevant products, including proteins with ice-associating activity for the food industry, and for energy applications such as ice recrystallization and gas hydrate inhibition, along with the cost-effective savings associated with the implementation of the "everything is everywhere" concept.

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References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**(17): 3389–3402. doi:10.1093/nar/25.17.3389. PMID:9254694.

Baas Becking, L.G.M. 1934. Geobiologie of inleiding tot de milieukunde. Van Stockum, W.P., and Zoon, The Hague, Netherlands; as cited by R. de Wit and T. Bouvier, T. 2006.

Bej, A.K., and Mojib, N. 2010. Cold adaptation in Antarctic biodegradative microorganisms. *In* The ecology, biodiversity and bioremediation potential of microorganisms in extremely cold environments. *Edited by* A.K. Bej, J. Aislabie, and R.M. Atlas. CRC Press, Boca Raton, Fla., USA. pp. 157–177.

Buckeridge, K.M., Cen, Y.-P., Layzell, D.B., and Grogan, P. 2010. Soil biogeochemistry during the early spring in low Arctic mesic tundra and the impacts of deepened snow and enhanced nitrogen availability. Biogeochemistry, **99**(1–3): 127–141. doi:10.1007/s10533-009-9396-7.

Casanueva, A., Tuffin, M., Cary, C., and Cowan, D.A. 2010. Molecular adaptations to psychrophily: the impact of 'omic' technologies. Trends Microbiol. **18**(8): 374–381. doi:10.1016/j.tim.2010.05.002. PMID:20646925.

Chakrabartty, A., and Hew, C.L. 1991. The effect of enhanced α-helicity on the activity of a winter flounder antifreeze polypeptide. Eur. J. Biochem. **202**(3): 1057–1063. doi:10.1111/j.1432-1033.1991.tb16470.x. PMID:1765066.

Chattopadhyay, M.K., and Jagannadham, M.V. 2001. Maintenance of membrane fluidity in Antarctic bacteria. Polar Biol. **24**(5): 386–388. doi:10.1007/s003000100232.

Chiang, Y.-C., Yang, C.-Y., Li, C., Ho, Y.-C., Lin, C.-K., and Tsen, H.-Y. 2006. Identification of *Bacillus* spp., *Escherichia coli, Salmonella* spp., *Staphylococcus* spp., and *Vibrio* spp. with 16S ribosomal DNA-based oligonucleotide array hybridization. Int. J. Food Microbiol. **107**(2): 131–137. doi:10.1016/j.ijfoodmicro.2005.04.028. PMID:16386323.

Christner, B.C., Mosley-Thompson, E., Thompson, L.G., Zagorodnov, V., Sandman, K., and Reeve, J.N. 2000. Recovery and identification of viable bacteria immured in glacial ice. Icarus, **144**(2): 479–485. doi:10.1006/icar.1999.6288.

Christner, B.C., Mosley-Thompson, E., Thompson, L.G., and Reeve, J.N. 2001. Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. Environ. Microbiol. **3**(9): 570–577. doi:10.1046/j.1462-2920.2001.00226.x. PMID:11683867.

Christner, B.C., Kvitko, B.H., II, and Reeve, J.N. 2003. Molecular identification of bacteria and eukarya inhabiting an Antarctic cryoconite hole. Extremophiles, **7**(3): 177–183. PMID:12768448.

Chu, H., Fierer, N., Lauber, C.L., Caporaso, J.G., Knight, R., and Grogan, P. 2010. Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. Environ. Microbiol. **12**(11): 2998–3006. doi:10.1111/j.1462-2920.2010.02277.x. PMID:20561020.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., et al. 2005. The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33**(Database issue): D294–D296. doi:10.1093/nar/gki038. PMID:15608200.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., et

al. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. **37**(Database issue Database): D141–D145. doi:10.1093/nar/gkn879. PMID: 19004872.

D'Amico, S., Collins, T., Marx, J.-C., Feller, G., and Gerday, C. 2006. Psychrophilic microorganisms: challenges for life. EMBO Rep. **7**(4): 385–389. doi:10.1038/sj.embor.7400662. PMID:16585939.

Deininger, C.A., Mueller, G.M., and Wolber, P.K. 1988. Immunological characterization of ice nucleation proteins from *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia herbicola.* J. Bacteriol. **170**(2): 669–675. PMID:3123461.

de Wit, R., and Bouvier, T. 2006. 'Everything is everywhere, but, the environment selects'; What did Baas Becking and Beijerinck really say? Environ. Microbiol. **8**(4): 755–758. doi:10.1111/j.1462-2920.2006.01017.x. PMID:16584487.

Garnham, C.P., Gilbert, J.A., Hartman, C.P., Campbell, R.L., Laybourn-Parry, J., and Davies, P.L. 2008. A Ca^{2+}-dependent bacterial antifreeze protein domain has a novel β-helical ice-binding fold. Biochem. J. **411**(1): 171–180. doi:10.1042/BJ20071372. PMID:18095937.

Gilbert, J.A., Hill, P.J., Dodd, C.E.R., and Laybourn-Parry, J. 2004. Demonstration of antifreeze protein activity in Antarctic lake bacteria. Microbiology, **150**(1): 171–180. doi:10.1099/mic.0.26610-0. PMID:14702410.

Gómez Zavaglia, A., Disalvo, E.A., and De Antoni, G.L. 2000. Fatty acid composition and freeze–thaw resistance in lactobacilli. J. Dairy Res. **67**(2): 241–247. doi:10.1017/S0022029900004179. PMID:10840678.

Good, I.J. 1953. The population frequencies of species and the estimation of population parameters. Biometrika, **40**: 237–264.

Gordienko, R., Ohno, H., Singh, V.K., Jia, Z., Ripmeester, J.A., and Walker, V.K. 2010. Towards a green hydrate inhibitor: imaging antifreeze proteins on clathrates. PLoS ONE, **5**(2): e8953. doi:10.1371/journal.pone.0008953. PMID:20161789.

Hicks, R.E., Amann, R.I., and Stahl, D.A. 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58**(7): 2158–2163. PMID:1379029.

Jones, P.G., VanBogelen, R.A., and Neidhardt, F.C. 1987. Induction of proteins in response to low temperature in *Escherichia coli.* J. Bacteriol. **169**(5): 2092–2095. PMID:3553157.

Knight, C.A., and Duman, J.G. 1986. Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. Cryobiology, **23**(3): 256–262. doi:10.1016/0011-2240(86)90051-9.

Koda, N., Aoki, M., Kawahara, H., Yamade, K., and Obata, H. 2000. Characterization and properties of intracellular proteins after cold acclimation of the ice-nucleating bacterium *Pantoea agglomerans* (*Erwnia herbicola*) IFO12686. Cryobiology, **41**(3): 195–203. doi:10.1006/cryo.2000.2280. PMID:11161552.

Kumar, N., Shah, V., and Walker, V.K. 2011. Perturbation of an Arctic soil microbial community by metal nanoparticles. J. Hazard. Mater. **190**(1–3): 816–822. doi:10.1016/j.jhazmat.2011.04.005. PMID:21546158.

Lane, D.J. 1991. 16S/23S rRNA sequencing. *In* Nucleic acid techniques in bacterial systematics. *Edited by* E. Stackebrandt and M. Goodfellow. John Wiley and Sons, New York, USA. pp. 115–175.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. 1985. Rapid determination of 16S ribosomal RNA sequences from phylogenetic analyses. Proc. Natl. Acad. Sci. U.S.A. **82**(20): 6955–6959. doi:10.1073/pnas.82.20.6955. PMID:2413450.

Lee, Y.K., Kim, H.W., Cho, K.H., Kang, S.-H., Lee, H.K., and Kim, Y. 2004. Phylogenetic analysis of culturable Arctic bacteria. Ocean Polar Res. **26**(1): 51–58. doi:10.4217/OPR.2004.26.1.051.

Maki, L.R., Galyan, E.L., Chang-Chien, M.-M., and Caldwell, D.R. 1974. Ice nucleation induced by *Pseudomonas syringae.* Appl. Microbiol. **28**(3): 456–459. PMID:4371331.

Männistö, M.K., Tiirola, M., and Häggblom, M.M. 2009. Effect of freeze–thaw cycles on bacterial communities of Arctic tundra soil. Microb. Ecol. **58**(3): 621–631. doi:10.1007/s00248-009-9516-x. PMID:19367430.

Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. **47**(2): 347–369. doi:10.1085/jgp.47.2.347. PMID:14085017.

Merrill, L., Richardson, J., Kuske, C.R., and Dunbar, J. 2003. Fluorescent heteroduplex assay for monitoring *Bacillus anthracis* and close relatives in environmental samples. Appl. Environ. Microbiol. **69**(6): 3317–3326. doi:10.1128/AEM.69.6.3317-3326.2003. PMID:12788732.

Metpally, R.P.R., and Reddy, B.V.B. 2009. Comparative proteome analysis of psychrophilic versus mesophilic bacterial species: insights into the molecular basis of cold adaptation of proteins. BMC Genomics, **10**: 11. PMID:19133128. doi:10.1186/1471-2164-10-11.

Miteva, V.I., Sheridan, P.P., and Brenchley, J.E. 2004. Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. Appl. Environ. Microbiol. **70**(1): 202–213. doi:10.1128/AEM.70.1.202-213.2004. PMID:14711643.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology, **148**(1): 257–266. PMID:11782518.

Nejad, P., Ramstedt, M., and Granhall, U. 2004. Pathogenic ice-nucleation active bacteria in willows for short rotation forestry. For. Pathol. **34**(6): 369–381. doi:10.1111/j.1439-0329.2004.00378.x.

Nelson, L.M., and Parkinson, D. 1978. Effect of freezing and thawing on survival of three bacterial isolates from an Arctic soil. Can. J. Microbiol. **24**(12): 1468–1474. doi:10.1139/m78-236. PMID: 747810.

Nobrega, S., and Grogan, P. 2007. Deeper snow enhances winter respiration from both plant-associated and bulk soil carbon pools in birch hummock tundra. Ecosystems, **10**(3): 419–431. doi:10.1007/s10021-007-9033-z.

Raymond, J.A., and DeVries, A.L. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc. Natl. Acad. Sci. U.S.A. **74**(6): 2589–2593. doi:10.1073/pnas.74.6.2589. PMID:267952.

Raymond, J.A., Christner, B.C., and Schuster, S.C. 2008. A bacterial ice-binding protein from the Vostok ice core. Extremophiles, **12**(5): 713–717. doi:10.1007/s00792-008-0178-2. PMID:18622572.

Shannon, C.E., and Weaver, W. 1949. The mathematical theory of communication. University of Illinois Press, Urbana, Ill., USA.

Shelburne, C.E., Prabhu, A., Gleason, R.M., Mullally, B.H., and Coulter, W.A. 2000. Quantitation of *Bacteroides forsythus* in subgingival plaque: comparison of immunoassay and quantitative polymerase chain reaction. J. Microbiol. Methods, **39**(2): 97–107. doi:10.1016/S0167-7012(99)00106-2. PMID:10576699.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L.K., and Komagata, K. 1997. Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus.* Int. J. Syst. Bacteriol. **47**(2): 289–298. doi:10.1099/00207713-47-2-289. PMID:9103612.

Spilker, T., Coenye, T., Vandamme, P., and LiPuma, J.J. 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from

other *Pseudomonas* species recovered from cystic fibrosis patients. J. Clin. Microbiol. **42**(5): 2074–2079. doi:10.1128/JCM.42.5.2074-2079.2004. PMID:15131172.

Steven, B., Briggs, G., McKay, C.P., Pollard, W.H., Greer, C.W., and Whyte, L.G. 2007. Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. FEMS Microbiol. Ecol. **59**(2): 513–523. doi:10.1111/j.1574-6941.2006.00247.x. PMID:17313587.

Telang, A.J., Ebert, S., Foght, J.M., Westlake, D.W.S., Jenneman, G.E., Gevertz, D., and Voordouw, G. 1997. Effect of nitrate injection on the microbial community in an oil field as monitored by reverse sample genome probing. Appl. Environ. Microbiol. **63**(5): 1785–1793. PMID:16535595.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**(22): 4673–4680. doi:10.1093/nar/22.22.4673. PMID:7984417.

Tomczak, M.M., Marshall, C.B., Gilbert, J.A., and Davies, P.L. 2003. A facile method for determining ice recrystallization inhibition by antifreeze proteins. Biochem. Biophys. Res. Commun. **311**(4): 1041–1046. doi:10.1016/j.bbrc.2003.10.106. PMID:14623287.

Vali, G. 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. J. Atmos. Sci. **28**(3): 402–409. doi:10.1175/1520-0469(1971)028<0402:QEOERA>2.0.CO;2.

Walker, V.K., Palmer, G.R., and Voordouw, G. 2006. Freeze–thaw tolerance and clues to the winter survival of a soil community. Appl. Environ. Microbiol. **72**(3): 1784–1792. doi:10.1128/AEM.72.3.1784-1792.2006. PMID:16517623.

Wilson, S.L., and Walker, V.K. 2010. Selection of low-temperature resistance in bacteria and potential applications. Environ. Technol. **31**(8–9): 943–956. doi:10.1080/09593331003782417. PMID:20662383.

Wilson, S.L., Kelley, D.L., and Walker, V.K. 2006. Ice-active characteristics of soil bacteria selected by ice-affinity. Environ. Microbiol. **8**(10): 1816–1824. doi:10.1111/j.1462-2920.2006.01066.x. PMID:16958762.

Xiao, N., Suzuki, K., Nishimiya, Y., Kondo, H., Miura, A., Tsuda, S., and Hoshino, T. 2010. Comparison of functional properties of two fungal antifreeze proteins from *Antarctomyces psychrotrophicus* and *Typhula ishikariensis.* FEBS J. **277**(2): 394–403. doi:10.1111/j.1742-4658.2009.07490.x. PMID:20030710.

Xu, H., Griffith, M., Patten, C.L., and Glick, B.R. 1998. Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Can. J. Microbiol. **44**: 64–73. doi:10.1139/w97-126.

Yankofsky, S.A., Levin, Z., Bertold, T., and Sandlerman, N. 1981. Some basic characteristics of bacterial freezing nuclei. J. Appl. Meteorol. **20**(9): 1013–1019. doi:10.1175/1520-0450(1981)020<1013:SBCOBF>2.0.CO;2.

Yergeau, E., and Kowalchuk, G.A. 2008. Responses of Antarctic soil microbial communities and associated functions to temperature and freeze–thaw cycle frequency. Environ. Microbiol. **10**(9): 2223–2235. doi:10.1111/j.1462-2920.2008.01644.x. PMID:18479442.

Zachariassen, K.E., and Hammel, H.T. 1976. Nucleating agents in the haemolymph of insects tolerant to freezing. Nature, **262**(5566): 285–287. doi:10.1038/262285a0. PMID:958371.