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Low-temperature recovery strategies for the isolation of bacteria from ancient permafrost sediments

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Abstract Permafrost represents a unique ecosystem that has allowed the prolonged survival of certain bacterial lineages at subzero temperatures. To better understand the permafrost microbial community, it is important to identify isolation protocols that optimize the recovery of genetically diverse bacterial lineages. We have investigated the impact of different low-temperature isolation protocols on recovery of aerobic bacteria from northeast Siberian permafrost of variable geologic origin and frozen for 5000 to 3 million years. Low-nutrient media enhanced the quantitative recovery of bacteria, whereas the isolation of diverse morphotypes was maximized on rich media. Cold enrichments done directly in natural, undisturbed permafrost led not only to recovery of increased numbers of bacteria but also to isolation of genotypes not recovered by means of liquid low-temperature enrichments. On the other hand, direct plating and growth at 4°C also led to recovery of diverse genotypes, some of which were not recovered following enrichment. Strains recovered from different permafrost samples were predominantly oligotrophic and non-spore-forming but were otherwise variable from each other in terms of a number of bacteriological characteristics. Our data suggest that a combination of isolation protocols from different permafrost samples should be used to establish a culture-based survey of the different bacterial lineages in permafrost.

Key words Arctic · Permafrost · Subsurface · Microorganisms · Psychrotolerant organism · Low temperature · Subzero

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Introduction

The permafrost microbial community has been described as "a community of survivors" (Friedmann 1994). Viable bacterial populations in permafrost sediments can be viewed as the result of a continuous process of selection that keeps eliminating those unable to withstand prolonged exposure to subzero temperatures (Gilichinsky et al. 1993). At temperatures that remain at −10°C or lower for periods as long as 2–3 million years, microbial growth rates would be expected to be extremely low. As such, permafrost may represent an example of an "absolute extreme environment" (Friedmann 1994) where survival of certain life forms continues and the stringency of the environmental conditions is such that the continuous evolution of successful adaptations is hindered.

During the past 15 years, Russian and American scientists have pursued the characterization of microbial communities from permafrost cores in the tundra zone of the Kolyma lowland (North-Eastern Siberia) (Zvyagintsev et al. 1985, 1990; Gilichinsky et al. 1988, 1992, 1993; Gilichinsky and Wagener 1995). The temperature in these sediments is −10° to −12°C. These studies have suggested that bacterial recovery by cultural methods decreases with increasing permafrost age, and that aerobic bacteria were mostly non-spore-formers, nonhalophilic, and psychrotolerant. True psychrophiles were rarely isolated. Cultural and physiological studies with microorganisms from these sediments suggest that the ratio of readily culturable (hypometabolic) bacteria to those that may represent viable but nonculturable forms (deep resting cells) may be determined by the extent and duration of subzero

exposure (for review, see Vorobyova et al. 1997). As with other natural environments, a large portion of the permafrost microbial community remains uncultured. Furthermore, some cells may have died when exposed to the stresses of thawing and oxygen exposure when samples were melted in the laboratory for microbial study. The physiological and phylogenetic diversity of this community has only recently begun to be addressed (Shi et al. 1997; Rivkina et al. 1998; Tiedje et al. 1998). Isolation and characterization of additional bacteria from permafrost is important to identify physiological themes that may be relevant to the survival properties of the permafrost community. These organisms must not only survive in a frozen world but also withstand cell aging and starvation.

Previous culture-based methods employed relatively high temperatures of incubation (~22°C) and rich media. It is evident from studies of bacteria in the deep subsurface, however, that the nutrient content of the media may strongly influence both the yield and the diversity of the organisms that can be cultured (Palumbo et al. 1996). In addition, the prolonged exposure of the bacteria in permafrost to subzero temperature and the expected very low growth rate suggests that sudden exposure to rich media and relatively high temperatures might represent a stress that can inhibit recovery of a fraction of the community. In this work, we have evaluated the impact of different low-temperature recovery methods, including media of different composition and low-temperature enrichments directly in the permafrost sediment, on bacterial recovery.

Materials and methods

Sampling sites and core recovery procedures

The Kolyma lowland permafrost samples used in this study are listed in Table 1. Nine samples were collected in August 1997 from the late Pleistocene stratum (20000–30000 YBP) from different depths in one borehole (I/97) at site B. Other samples were collected at sites A, C, D, and E (Fig. 1) in the summers between 1991 and 1997. The samples were obtained by slow rotary drilling to avoid melting of the sediment ice. Contamination with organisms nonindigenous to the frozen core sample can be excluded on the basis of previous method validation (Zvyagintsev et al. 1985; Gilichinsky et al. 1988; Khlebnikova et al. 1990). Briefly, the extracted cores always remained frozen, surface material of the core was trimmed away with a sterile knife, and core sections (5–7 cm in diameter; Fig. 2) were placed in presterilized aluminum tins, sealed, and kept frozen during storage and transport. In the laboratory, the frozen core sample was fractured in a hood with a sterile knife and only internal fragments were taken by sterile forceps for analysis (Rivkina et al. 1998).

The core sections were stored in Pushchino (Russia) at −18°C until October 1997 when they were transported to East Lansing (MI, USA) and stored at −10°C, a temperature nearer the in situ conditions. The samples collected in 1997 had only a brief exposure (1 month) to the slightly

Table 1. Sites and cores

Site	Location	Sample (box no.)	Well no. (collection date)	Depth (m)	Age (years)	Genesis and lithology
A	Arctic Coast, East Siberian Sea, Point Chukochil	2671	4/91 (1991)	17.4	Middle Pleistocene (100000)	Marine (littoral) sands
		1359	5/91 (1991)	1.2	Holocene (7000–10000)	Lake–swamp, loam
B	Mouth of Malaya Kon'kovaya River, Right bank	392	1/97 (1997)	0.15	Modern tundra soil	
		348		3.0	Late Pleistocene (20000–30000)	Alluvium sandy loam
		130		4.5		
		109		6.5		
		293		8.0		
		262		12.0		
		273		12.5		
		215		13.0		
		235		13.5		
C	Middle part of Bol'shaya Chukochya river, right bank	190	5/94 (1994)	5.5	Middle Pleistocene (200000–600000)	Lake–alluvium, loam and sandy loam
		255	2/94 (1994)	43.6	Late Pliocene–early Pleistocene (2–3 million)	
		3361	1/91 (1991)	35.8		
D	Arctic Coast, East Siberian Sea, mouth of Kuropatochya River	309	3/96 (1996)	8.0	Late Pleistocene (20000–30000)	
E	Khalerchinskaya Tundra	0198	17/91 (1991)	7.5		Channel-fill sands

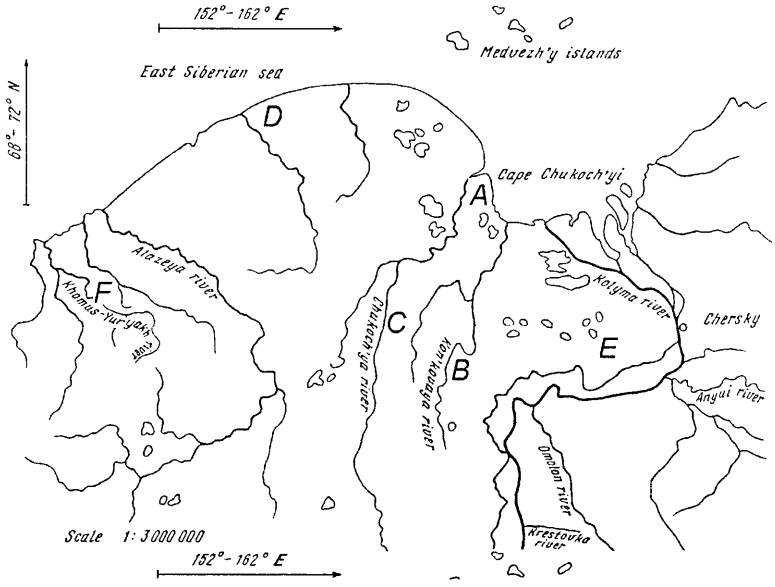

Fig. 1. Kolyma lowland study sites are indicated by *letter* (*A–F*)

Fig. 2. Sandy core with detritus (*dark layers*). The measured core temperature after extraction was −7°C

colder laboratory storage temperature (−18°C) before initiating these experiments.

Culture media and enrichment procedures

Tryptic soy broth (TSB, Difco) was used at full, 1/2, and 1/10 strength as indicated, and solidified with 1.5% agar (TSA). Because liquid water films in frozen soil are of high osmolarity, NaCl (2% and 10%) and sucrose (0.1 M and 0.5 M) were used in media as osmoprotectants in combination with varying strength TSB, as indicated. Selected samples were also plated on R2A agar (Difco).

Three strategies were used to initially cultivate the permafrost microorganisms: (i) direct plating of diluted cell suspensions on agar media; (ii) enrichment in liquid media before plating; and (iii) enrichment in thawed but otherwise undisturbed sediments (natural permafrost sediment [NPS] enrichment) held at 4°C for 2, 6, and 12 weeks before plating. The latter was pursued to give the organisms a chance to grow without the surrounding ice but otherwise in their native environment.

For direct plating, material (1 g) from the center of the core was added to sterile 15-mm polypropylene tubes (Falcon) and mixed with 10 ml sterile 1/10 strength TSB. The suspension was shaken for 2–3 min, and serial tenfold dilutions were plated (50 µl) in duplicate on the different media. The plates were incubated aerobically at 4°C for 2 weeks, and colony-forming units (cfu) were calculated as averages of duplicate plates. All plates used for viable counts were pooled for diversity determinations. Plates were re-examined weekly for 8 weeks for the appearance of new colonies.

For broth enrichments, 50 µl of permafrost suspension (prepared as described above) was added to 2.5 ml of the different media in sterile polypropylene tubes and incubated at 4°C. After 8 weeks, the enrichments were diluted and aliquots plated on the same TSB-osmoprotectant combination agar that was used in the enrichment. cfu were determined as before.

For NPS enrichment, the permafrost sediment (1 g) was added to sterile polypropylene tubes and incubated at 4°C without further disturbance for 2, 6, and 12 weeks. At each time point, the samples were plated on the indicated media and incubated at 4°C. cfu were determined as described.

Bacterial characterization and preservation

Altogether, 238 individual strains were purified and characterized. Duplicate 1/2 TSB agar plates were analyzed by determining the number of each colony type present using colony morphology and the color and cell morphology, motility, and Gram reaction of representative colonies of each type (Balkwill et al. 1989). The isolates were purified by subculturing on 1/2 TSA, and purity was confirmed by visual examination of the plates and by phase-contrast microscopy. Gram stain status was determined by lysis with 0.2 N KOH (Manafi and Kneifel 1990). To minimize genetic changes that might occur during repeated transfer of the bacterial isolates on laboratory media, we preserved all isolates by transfer to TSB containing 15% sterile glycerol and freezing (−70°C) immediately after purification.

The proportion of oligotrophic cells was estimated following the concept outlined by Hattori (1980), who described oligotrophs as strains that were isolated on diluted media and which could not grow on full-strength media. In this case we estimated the percent oligotrophs as the difference between cfus on diluted (1/10 TSA) versus full-strength TSA.

Diversity and equitability indices were calculated by using a modified version of Simpson's index (Haldeman and Amy 1993), and the colony type and number and cell information were as described earlier from duplicate 1/2 TSA plates.

Rep-PCR genomic fingerprinting

The REP primers, cycling programs, and reaction mixture composition were as previously described, using a small portion of a single colony directly in the PCR reaction (Rademaker et al. 1998). Eight microliters of the reaction mixture was loaded onto 18-cm-long 1.5% agarose gels and run at 4°C in 0.5× Tris-acetate-EDTA buffer for 7 h at 4 V/cm. A 1-kb ladder (GIBCO-BRL) was included as a size reference. Gel images were digitized with a Video Graphic camera and stored as TIFF files. The band positions were normalized with the above-mentioned molecular size markers, and analyzed with GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium) using the pairwise Pearson's product-moment correlation coefficient (*r*-value). Cluster analysis of similarity matrices was performed by the pair group method using arithmetic averages (UPGMA) (Rademaker et al. 1998). A similarity value of 90% or less was used to define different genotypes; genotype diversity is reported as number of different genotypes per total isolates examined.

Direct cell counts

Cell counts were determined by counting cells in melted permafrost after staining with 5-(4,6-dichlorotriazin-2-yl)-aminofluorescein (DTAF) following the protocol of Bloem et al. (1995). Microscopy was done with a Leitz Ortholux fluorescence microscope using the 63× objective and a filter with a bandpass of 450–490 nm. The images were collected using a 10-s exposure on a digital camera and analyzed by IPLab Spectrum computer software for Macintosh (Scanalytics, Billerica, Mass., USA).

Results

The permafrost samples were derived from sediments that differed in lithology, physicochemical composition, and age since deposition (Tables 1, 2). DTAF staining showed numerous, intact, brightly staining cells in all samples, and DTAF-based direct cell counts did not vary significantly among samples (2.1×10^7–1.2×10^8 cfu/g) (Table 3).

Direct plating at 4°C on different media

All permafrost samples, except two (samples 0198 and 2671) yielded bacterial colonies following direct plating and incubation for 2–8 weeks at 4°C. The two oldest samples in the collection, 3361 and 255 (0.6–1.8 and 2–3 million years, respectively), yielded no colonies on TSA and only a small number on 1/10 TSA (Table 3). It was also apparent that samples collected in the period 1991–1994 yielded noticeably fewer colonies than samples of equivalent geologic age collected in 1997. In fact, the relatively young sample 0198 (25 000–40 000 years), collected in 1991, failed to yield any colonies on plating (Table 3). The 1991–1994 samples were all stored at −18°C; perhaps this colder temperature affected recovery.

Overall, the plate count of bacteria from permafrost samples was higher on diluted (1/10) TSA than on full-strength media, in contrast to modern tundra soil, which yielded similar numbers of bacteria on full-strength or 1/10

Table 2. Physicochemical characteristics of permafrost samples

Sample	Ice content (%)	Organic C (%)	pH	Aqueous extract (mg Eq/100 g)							Solid residual (%)	Particle size (%)	
				HCO_3^-	Cl^-	SO_4^{2-}	Ca^{2+}	Mg^{2+}	K^+	Na^+		>0.01 mm	<0.01 mm
2671	27		7.4	0.52	9.38	0.75	0.8	0.12	0.3	9.38	0.58	72.3	27.7
1359	47	3.2	7.2	0.2	0.6	0.08	0.2	0.15	0.05	0.41	0.06	73.3	26.7
392	24	4.1	5.6	0.1	0.2	0.02	0.08	0.08	0.02	0.23	0.03	70.4	29.6
348	28												
130	40												
109	47												
293	31	0.6–1.0	6.8–7.1	0.2–0.9	0.4–1.3	0.4–1.3	0.2–0.8	0.3–0.8	0.02–0.05	0.7–1.3	0.08–0.15	85.9–71.7	14.1–28.3
262	26												
273	22												
215	24												
235	27												
190	17	0.9	7.3	0.5	0.4	0.05	0.28	0.44	0.06	0.4	0.03	ND	ND
255	21	1.1	7.3	0.7	0.52	0.18	0.37	0.25	0.06	0.66	0.05	ND	ND
3361	33	3.2	7.0	0.4	0.15	0.34	0.3	0.28	0.1	0.17	0.08	64.6	36.4
309	ND	1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0198	22	1.2	7.8	0.25	0.2	0.15	0.15	0.22	0.02	0.26	0.05	92.4	7.6

Table 3. Direct and viable counts of bacteria from permafrost sediment samples collected at the Kolyma lowland

Sample no.	Direct count (log)	Viable count (log CFU/g of sediment) at 4°C on	
		TSA	1/10 TSA
392[a]	8.49	5.66	5.51
1359	7.89	2.78	3.54
309	7.93	3.30	3.90
348	8.26	4.52	5.34
130	8.08	4.88	5.04
109	8.41	5.79	6.52
293	8.45	5.83	6.36
262	8.66	5.82	6.43
273	8.38	4.49	5.98
215	8.10	4.38	5.26
235	8.51	4.57	5.23
0198	7.69	None	None
190	7.91	2.30	None
2671	7.87	None	None
3361	7.36	None	2.60
255	7.32	None	2.30

TSA, tryptic soy broth
[a] modern tundra soil

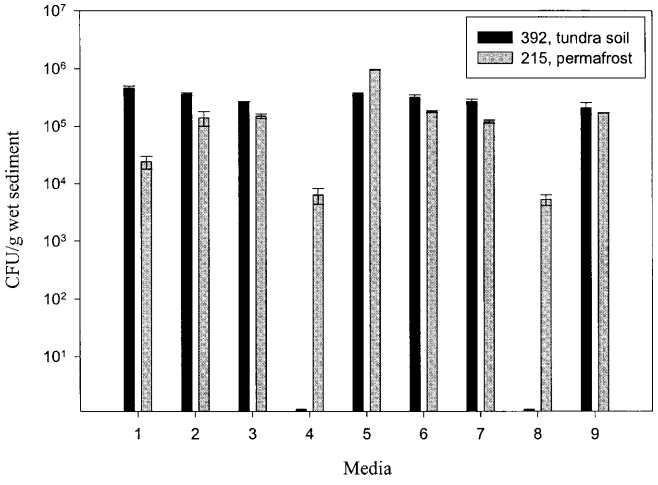

Fig. 3. Effect of media on recovery of bacteria from permafrost versus surface tundra soil. Data are mean and range of two replicate plate counts on the following media: *1*, TSA (tryptic soy agar); *2*, 1/2 TSA; *3*, 1/2 TSA + 2% NaCl; *4*, 1/2 TSA + 10% NaCl; *5*, 1/2 TSA + 0.1 M sucrose; *6*, 1/10 TSA; *7*, 1/10 TSA + 2% NaCl; *8*, 1/10 TSA + 10% NaCl; *9*, 1/10 TSA + 0.1 M sucrose

TSA (Table 3). Although yield from permafrost was higher on diluted media, colony diversity was greater on less-diluted media. For example, the maximum number of distinct colony types isolated from sample 215 was obtained on full-strength TSA, 1/2 TSA, and 1/2 TSA with 0.1 M sucrose (average of 7 and range 6–9, as compared to average of 3 and range 2–5 on the 1/10 strength media). Medium with 1/2 TSA was routinely used in later studies because it gave higher counts than TSA yet revealed more colony diversity than 1/10 TSA.

The effect of nutrient richness plus osmoprotectants is illustrated for one permafrost sample, no. 215, in comparison with that for modern tundra soil, no. 392. These two samples had similar total direct counts (~2×10^8 cells/g). With the exception of media containing 10% NaCl, medium type affected recovery of CFUs to a greater extent for permafrost samples than for modern tundra soil (Fig. 3). Sample 215 gave noticeably variable CFUs, with the highest counts on 1/2 TSA + 0.1 M sucrose, while the counts for the surface soil were about the same on all media. An interesting exception was observed with the two media containing 10% NaCl, on which only the permafrost sample yielded significant numbers of colonies (Fig. 3). In general, however, the inclusion of 2% NaCl or 0.1 M sucrose did not have marked influence on yield in any samples.

Although certain strains (e.g., gram-negative yellow or dark-yellow rods) were isolated on all media, others (e.g., gram-positive orange micrococci) were isolated only on full-strength TSA and 1/2 TSA or on 1/10 TSA containing either 2% or 10% NaCl. White colonies of gram-negative, variable-length rod-shaped bacteria were the other cell type isolated on the 10% NaCl media. Both cell types from the 10% NaCl media could grow in the absence of salt and thus appear strongly halotolerant, although not halophilic.

Bacteriological heterogeneity of communities from different permafrost samples

Two common themes were identified from the bacteriological analysis of the permafrost-derived communities: The majority (70% or more of the isolated microorganisms) appeared to be oligotrophic, and spore-forming bacteria were only rarely encountered. Table 4 summarizes the characteristics of the communities isolated from eight different permafrost samples derived from different depths of a single borehole. All samples had approximately the same direct cell counts, 1.2×10^8–4.6×10^8 cell/g, similar to that of modern tundra soil (see Table 3). The fraction of the total bacterial population that could be recovered on 1/2 TSA varied, ranging from 0.01% in the deepest sample (sample 235; 13.5 m) to 1.5% midway in the column (sample 109; 6.5 m) (Table 4). Of the eight samples of this borehole, three (293, 262, 273) yielded approximately equal numbers of gram-positive and gram-negative bacteria, whereas either gram-positive or gram-negative bacteria predominated in the others. More than 50% of isolates in four samples (130, 293, 262, 273) produced pigments, mostly yellow or orange. Microscopic examination revealed that most organisms were rod shaped and, as mentioned previously, endospore-forming bacteria were rare, isolated in low frequency only from one sample (sample 348).

Natural permafrost sediment (NPS) enrichment at 4°C

We incubated permafrost samples at 4°C without any additional media to minimize sample disturbance and the shock from the nutritional shift to complex media. As one indication of whether changes in cfu were caused by bacterial growth in situ or by increased culturability of the resident bacteria, total bacterial counts were monitored during the

Table 4. Characteristics of culturable bacteria that grew at 4°C on 1/2 TSB agar in 4 weeks from different layers in the 20000- to 30000-year stratum

Sample no.	Percent of the total						Bacterial diversity		
	Viable bacteria	Oligotrophic bacteria	Gram negative	Rod shaped	Motile	Pigmented	Distinct colony types	Simpson's index	Equitability
348	0.10	85	2	99	99	2	6	1.2	0.2
130	0.26	76	11	34	12	89	4	2.3	0.57
109	1.51	82	32	40	1	17	6	2.7	0.45
293	0.27	71	54	90	90	98	4	2.5	0.63
262	0.41	76	46	46	46	54	3	2.2	0.73
273	0.25	97	47	40	40	66	7	1.4	0.2
215	0.08	87	99	99	1	12	7	2.1	0.3
235	0.01	78	99	99	1	2	5	1.1	0.21

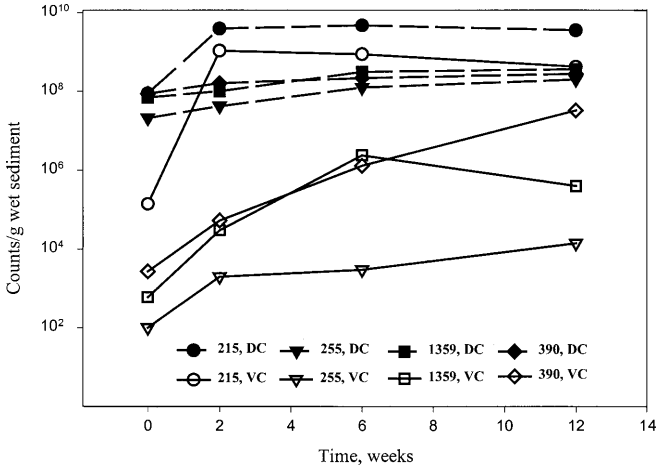

Fig. 4. CFU (colony-forming units) (*open symbols*) and direct counts (*solid symbols*) of bacteria during NPS enrichment at 4°C. CFUs are means of two replicate plate counts, and direct counts are means of counts from ten microscope fields for each of two replicate soil smears

enrichment period using DTAF staining (Fig. 4). In the case of some samples (e.g., 390 and 1359), the increase in plate counts was not accompanied by significant increases in total bacterial counts, suggesting that the fraction of culturable bacteria had increased during the NPS enrichment. In the case of sample 215, a sharp increase in plate counts within the first 2 weeks of NPS enrichment was paralleled by increased total bacterial counts, suggesting that in this case the changes in cfu were caused by growth of the bacteria in situ. In the case of the ancient sample 255 (2–3 million years old), which yielded few cfu before enrichment, the rate of increase during the first several weeks was slow but cfu continued to increase throughout the entire NPS enrichment period. In most samples, cfu increased during NPS enrichment only for the first 2–6 weeks. NPS enrichment did not significantly increase recovery of bacteria from those samples that yielded few or no colonies before the enrichment (e.g., samples 0198, 2671, 3361; see Table 3). Samples 2671 and 3361 failed to yield any colonies during the entire period of NPS enrichment, and sample 0198 yielded limited bacterial growth only after 6 weeks of enrichment.

The colony diversity of the NPS isolates at week 2 was 6 ± 3 and the genotype diversity 0.88, which is similar to the diversity values obtained by direct enrichment, 7 ± 3 and 0.74, respectively.

Liquid enrichment at 4°C

Liquid enrichment resulted in a pronounced increase in cfu in most samples, except for those with low original plate counts (e.g., sample 255). The inclusion of osmoprotectants in the liquid enrichments affected bacterial recovery in an osmoprotectant- and sample-specific manner. For example, liquid enrichment cultures of sample 190 with 1/2 TSB, 1/10 TSB, 1/2 TSB + 0.1 M sucrose, and 1/2 TSB + 0.5 M sucrose yielded substantial counts (10^7–10^8 cfu/ml), whereas few or no cfu were obtained in enrichments using other media. In contrast, liquid enrichments of sample 1359 only yielded appreciable plate counts on 1/2 TSB, 1/10 TSB, and when 2% NaCl was added to 1/2 TSB or 1/10 TSB (plate counts in these enrichment cultures were 1.2×10^8, 2×10^4, 1.2×10^8, and 3.1×10^8 cfu/ml, respectively), whereas diluted TSB supplemented with sucrose did not consistently result in a plate count increase in this sample (data not shown). The inclusion of 10% NaCl in diluted TSB enrichment cultures was accompanied by low (e.g., sample 215) or no plate counts in other samples, or by the appearance of mostly fungal colonies (e.g., sample 309).

Liquid enrichments yielded more cfu than NPS enrichments, as expected, but resulted in the predominance of only a few morphotypes (average, 2, and range 1–4). Liquid enrichments of sample 215 (using 1/2 TSB and 1/10 TSB) were dominated by light-yellow, gram-positive coccoidal rods (*Arthrobacter*-like). Only two to four colony types were typically observed in liquid enrichments that contained osmoprotectants.

Isolation protocol affects recovery of certain genotypes from permafrost

Sample 215 was used as a model to determine whether different genotypes were isolated by the three different

isolation strategies. In this case, multiple colonies resembling those isolated by direct plating were picked following NPS enrichment at 2, 6, and 12 weeks (30, 8, and 8 strains, respectively) and following liquid enrichment at 8 weeks (26 strains). Figure 5 shows the REP-PCR patterns of 42 gram-positive strains isolated from sample 215. It is apparent that most strains grouped into several distinct genotypic clusters. Several strains isolated from different media (e.g., with or without NaCl or sucrose) had identical REP-PCR patterns and likely represented the same microorganism (e.g., 215-96 and 215-100, isolated on 1/2 TSA and on 1/10 TSA + 2% NaCl, respectively), suggesting that the presence of NaCl or sucrose was not essential for the recovery of such strains.

An important finding was that certain clusters (e.g., the clusters 215-96/215-100/215-99, and 215-112/215-101/215-116, respectively) consisted of strains isolated only following NSP enrichment and not following liquid enrichment or direct plating at time zero. Similarly, some genotypic clusters (e.g., 215-79/215-87, 215-80/215-86, and 215-81/215-75, respectively) were isolated following liquid enrichment but not following NSP enrichment or by direct plating before enrichment. Thus, NSP enrichment and liquid enrichment may favor the isolation of nonoverlapping genotypes. On the other hand, certain genotypic clusters (e.g., 215–11/215-3/215-68 and 215-25/215-14/215-38) were isolated only by direct plating at zero time and not following NSP or liquid enrichment. Interestingly, the strains in these last two clusters were gram-positive orange cocci, which, on the basis of 16*S* rDNA sequencing of representative strains, appeared to be in the genus *Planococcus*, suggesting the existence of at least two distinct *Planococcus* lineages in this permafrost sample (represented by strains 215-68 and 215-14 in Fig. 5).

Although direct plating isolation appeared to be required for the recovery of these *Planococcus* lineages, other genotypes (e.g., the cluster 215-91/215-119) could be recovered both by direct plating and following NSP enrichment (Fig. 5). Genomic fingerprinting of 13 gram-negative strains from sample 215 produced similar findings (data not shown). In summary, our findings suggest that NSP and liquid enrichment can lead to isolation of different genotypes, whereas other lineages are best recovered by direct plating in the absence of either enrichment protocol.

Discussion

The intrinsically interesting features of permafrost microorganisms suggest the need for continued development of improved protocols for their recovery from ancient frozen sediments. Multiple and diverse adaptive strategies may have evolved in different lineages of permafrost bacteria. A comprehensive library of strains would facilitate the identification of such strategies for prolonged survival at subzero temperatures.

Low-temperature cultivation on diluted media appeared to be quite effective for recovery of bacteria from several permafrost samples, yielding, in some cases (e.g., sample 215), approximately the same numbers of cfu as modern tundra soil (~10^5 cfu/g; see Table 3). Permafrost bacteria isolated in rich media were less numerous than those in diluted media but had greater morphological diversity. Interestingly, similar results were obtained during isolation of bacteria from the deep subsurface (Palumbo et al. 1996). Although our data indicated that the inclusion of osmoprotectants in the culture media did not consistently enhance recovery of bacteria from permafrost, inclusion of osmoprotectants during the thawing stage of the samples may prove to enhance survival and recovery and will be investigated in future studies. Our results suggested that medium composition affected recovery of aerobic psychrotolerant microorganisms from the permafrost to an extent greater than that observed with modern tundra soil (see Fig. 3). Interestingly, permafrost samples (but not modern tundra soil) yielded bacterial colonies on media with high salt content, suggesting the existence of strongly halotolerant microbes in the permafrost. It remains to be determined whether the salt tolerance of these strains may also be associated with cold and freeze tolerance.

In previous isolations of aerobic bacteria from permafrost at 20°C, a significant fraction were found to be spore-formers (Shi et al. 1997). In contrast, spore-formers were only rarely obtained from our isolations, which were all done at 4°C, perhaps reflecting an intriguing paucity of psychrotolerant and psychrophilic spore-forming bacteria in the permafrost. NPS enrichments at 4°C, without any further manipulation of the permafrost sample, resulted in enhanced recovery without obvious compromises in diversity. In contrast, liquid enrichment resulted in predominance of a limited number of types (often an apparent monoculture). These findings may reflect the fact that, during enrichment in the soil matrix, diverse microbial communities can develop independently in separate soil microenvironments, which is not the case in liquid media.

As an isolation protocol, NPS enrichment had two additional important consequences concerning (i) the yield of bacteria recovered from these enrichments and (ii) the genotypes that were isolated. Bacterial recovery from several samples was markedly increased, suggesting that this method may be useful as a general isolation protocol for microorganisms from permafrost. Microscopic monitoring of DTAF-stained cells during the enrichment period suggested that, in some samples at least, the increased cfu were caused by changes in the relative frequency of viable but not culturable forms in the resident bacterial population. In other samples, DTAF-staining bacteria appeared to multiply rapidly in the sample during the first 2 weeks of the cold enrichment period, suggesting that most cells in these samples are alive although not isolatable. Gaining further information about this dominant, viable population would be particularly interesting.

Genomic fingerprinting of a number of strains by *rep*-PCR suggested that NPS enrichment, liquid enrichment, and direct isolation resulted in the isolation of non-overlapping lineages. The *Planococcus* isolates were not recovered following NPS or liquid enrichment but were repeatedly isolated following direct plating of the sediments. *rep*-PCR

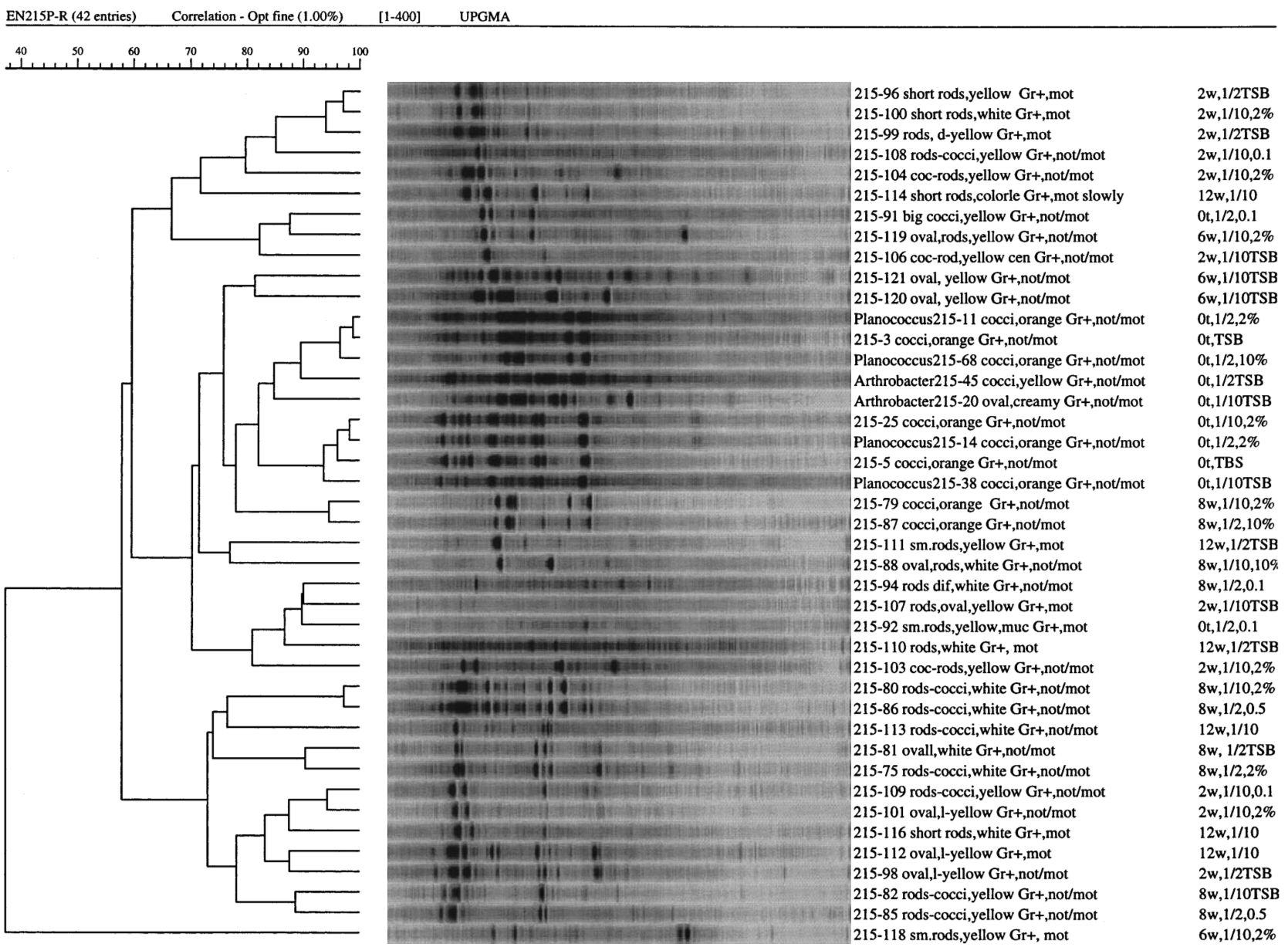

Fig. 5. *rep*-PCR patterns of the gram-positive isolates from sample 215. Strain number and characteristics are shown to the *right* of the gel image. Genus names, where shown, were determined by partial 16*S* rDNA sequence analysis (unpublished data). The *right-hand column* indicates the medium and isolation strategy used to obtain the isolates: *0*t, direct isolation; 2, 6, 12 weeks (*w*), NPS (natural permafrost sediment); 8 weeks, liquid enrichment

patterns can change as the result of genomic rearrangements that may take place during resuscitation and culture of the microorganisms. However, the observed differences between genotypes were greater than can be easily accounted for by such putative rearrangements. Our findings, therefore, suggest that NPS enrichments not only can enhance quantitative recovery of bacteria from permafrost but can also facilitate the isolation of genetically distinct lineages, several of which apparently could not be obtained by the other isolation protocols which we used here. However, to maximize the retrieval of genetically diverse lineages, NPS enrichment should be supplemented by direct plating protocols and alternative enrichment procedures for aerobic bacteria. Furthermore, the psychrotolerant permafrost community may also include anaerobes and Archaea, which have recently been detected in Siberian permafrost (Rivkina et al. 1998; Tiedje et al. 1998). Future studies are needed to more fully characterize the composition and adaptive physiology of the prokaryotic community in permafrost.

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