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Brief report

Reproduction and metabolism at −10°C of bacteria isolated from Siberian permafrost

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Summary

We report the isolation and properties of several species of bacteria from Siberian permafrost. Half of the isolates were spore-forming bacteria unable to grow or metabolize at subzero temperatures. Other Gram-positive isolates metabolized, but never exhibited any growth at −10°C. One Gram-negative isolate metabolized and grew at −10°C, with a measured doubling time of 39 days. Metabolic studies of several isolates suggested that as temperature decreased below +4°C, the partitioning of energy changes with much more energy being used for cell maintenance as the temperature decreases. In addition, cells grown at −10°C exhibited major morphological changes at the ultrastructural level.

Introduction

Permafrost is a low temperature (−1 to −12°C) ecosystem that, at depth, has remained frozen for 10^4–10^6 years. Despite subzero temperatures, 2–7% of the water within permafrost persists as briny liquid films and lenses (cryopegs) that form as a result of salting out of the soil as the *in situ* temperature drops and remains at about −10°C (Andersen, 1967; Brown, 1969; Gilichinsky *et al*., 1993; Ershov, 1998). The presence of liquid water and the thermostability of permafrost make it the ideal environment for studying low-temperature growth mechanisms of microorganisms.

Psychrotolerant microorganisms, including spore-forming and non-spore-forming bacteria, are easily isolated from ancient (10^3–10^6 years) permafrost samples (Gilichinsky and Wagener, 1995). Spore-forming bacteria are expected in this soil-like environment given their resilience under conditions of extreme temperatures, salinity, water activity, nutrient levels and UV radiation (Nicholson *et al*., 2000). Although it is not unusual to isolate non-spore-forming organisms from permafrost (Shi *et al*., 1997), their survival mechanisms remain unknown because growth and metabolism have not been examined in detail at subzero temperatures. To date, the lowest temperatures reported for bacterial reproduction are −5 to −8°C as determined by colony formation from permafrost and frozen food (Gilichinsky *et al*., 1993; 1995; Geiges, 1996). Whereas, *in situ* microbial activity has been reported at temperatures from −10 to −17°C in Siberian permafrost, South Pole snow, lichens and cryptoendoliths (Friedmann *et al*., 1993; Schroeter *et al*., 1994; Finegold, 1996; Kappen *et al*., 1996; Lang, 1996; Carpenter *et al*., 2000; Rivkina *et al*., 2000; Russell, 2000).

In an effort to understand better the cellular processes at subzero temperatures, we have recovered both spore-forming and non-spore-forming bacteria as viable cells from highly saline cryopeg samples that were buried within permafrost for *c.* 40 000 years (Shi *et al*., 1997; Brinton *et al*., 2002). We determined the phylogenetic identity of isolates and assessed their tolerance to low temperatures and to high salt concentrations. We measured their activity at −10°C by monitoring metabolism via resazurin reduction and growth via optical density and cell counts. The relationship between energy used per cell division (as estimated by growth and resazurin reduction rates) and temperature was examined. In addition, temperature correlated changes in cellular structure were visualized via transmission electron microscopy of cells grown at 22°C and −10°C.

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Results and discussion

Isolation and identification of bacteria from cryopeg samples

Cryopeg samples were obtained from the Kolyma Lowland region of Siberia, Russia, a tundra zone with a harsh climate and continuous permafrost that has remained frozen for at least 40 000–50 000 years (Shi *et al*., 1997). Samples were recovered from depths of 11–24 m below the surface. The ^{14}C age of samples was estimated at 43 000 ± 1000 years based on the age of adjacent permafrost cores (Brinton *et al*., 2002). *In situ* temperatures remain constant at about −12°C and cryopeg salinity was 12–14%. Forty-six eubacterial isolates were obtained, were screened by RFLP analysis of 16S rRNA genes, and represented 17 distinct patterns (Table 1). The most common isolates (5, 2 and 3a) were cultured under all initial isolation conditions. More than half (53%) of the patterns were represented by only one isolate.

Sequence analysis showed that the 17 patterns represented the following nine genera: *Psychrobacter, Arthrobacter, Frigoribacterium, Paenibacillus, Bacillus, Subtercola, Microbacterium, Rhodococcus* and *Erwinia* (Table 1). Most isolates were related to common soil organisms; whereas eight were related to known psychrotolerant strains of bacteria often found in polar regions. For example, species of *Psychrobacter, Frigoribacterium,* and *Paenibacillus* have been isolated from the North Sea and Antarctica (Bowman *et al*., 1997a, b; Brambilla *et al*., 2001; Christner *et al*., 2001). Isolate 11 was also related (95% identity) to bacteria isolated from Artic and Antarctic seawater (Mergaert *et al*., 2001). In general, the isolates could be organized into two broad groups: spore-forming and non-spore-forming bacteria.

Spore-forming bacteria were easily isolated and presumably survived the >40 000 years permafrost burial as spores. Non-spore-forming bacteria were also easily recovered, although their survival is more problematic given the long-term, low-temperature conditions. The most abundant isolate was a non-spore-forming Gram-negative bacterium (isolate 5), suggesting that this isolate survived very well, and perhaps even grew.

Activity of isolates at low temperatures and at high salt concentrations

To determine the tolerance of isolates to *in situ* conditions, we examined the activities and growth of actively growing vegetative cells over a range of temperatures and salinities. Isolates were found to be psychrotolerant, but not

Table 1. Summary of isolate characterization.

Isolate	Number of isolates	16S rDNA affiliation (closest relative)	Identity (%)	Optimum growth temp[a] (°C)	Salt tolerance[b] (% NaCl)	Specific Growth rate[c] at −10°C (day^{-1})	Resazurin reduction rate[c] at −10°C (fmol $cell^{-1}day^{-1}$)
Non-spore-forming bacteria							0.58 ± 0.18[d]
5	10	*Psychrobacter* sp. St1	98	24.3	**0,3**,10	0.016	6.01
2	8	*Arthrobacter citreus*	98	[22]	**0**.3*		0.18
2b	1	*Arthrobacter citreus*	99	22.0	0,3	0	0.66
3b	1	*Arthrobacter citreus*	99	[22]	0,3,10		0.64
3a	5	*Frigoribacterium aff. faeni* A-1/C-an/E	97	28.3	**0**,3,10	0.0034	0.72
4a	1	*Subtercola* sp. P229/10	97	[22]	0,**3**,10	0	0.67
8	1	*Microbacterium* sp. VA8728	95	29.0	0,**3**	0	0.61
11	1	*Rhodococcus* sp. strain: SGB1168-118	96	25.5	**0**,3*	0.0027	0.70
12	1	*Erwinia persicinus*	96	[22]	0,3*		0.47
Spore-forming bacteria							0.12 ± 0.18
1a	3	*Paenibacillus* sp. DSM 1352	98	[22]	0		0
1b	1	*Paenibacillus chondroitinus*	96	27.1	0		0
6	2	*Paenibacillus chondroitinus*	96	[22]	0		0.08
9b	3	*Bacillus psychrosaccharolyticus*	98	[32]	0*		0
4c	1	*Bacillus psychrosaccharolyticus*	99	[22]	0,**3***	No growth in liquid media	
7	3	*Bacillus psychrophilus*	99	25.7	**0**,3		0.46
4b	3	*Bacillus psychrodurans*	98	24.4	**0**,3		0.16
9	1	*Bacillus psychrodurans*	99	[22]	0,**3**	No growth in liquid media	

a. Data in brackets indicate the maximum temperature at which growth was observed when the optimum was not determined.
b. Preferred salt concentration is indicated by bold type; whereas starred (*) entries indicate that tolerance to 10% NaCl was not tested.
c. Rates were determined in R2A broth plus 3% NaCl (for isolates intolerant to 3% NaCl, rates were determined in R2A).
d. Data for isolate 5 was not included in the calculation of this average.

psychrophilic. Optimum growth temperatures ranged from 24°C to 33°C (Table 1). All isolates could grow at 10.5°C and most could grow at 4°C. Several isolates were halotolerant (5, 3a, 3b, 4a), but none were halophilic. On average, non-spore-forming isolates were more tolerant to low temperatures and salt than the spore-forming isolates. Tolerance to conditions that reflect major environmental constraints suggests that these isolates may be survivors only and may not have had sufficient time, nutrients, and/or activity levels to evolve additional adaptations to their environment.

To determine if activity levels were limiting organisms at low temperatures, we examined the metabolic activity of isolates via the reduction of resazurin (Venkitanarayanan *et al.*, 1997). The kinetics of resazurin reduction for four different isolates (2b, 3a, 5 and 8) at four different temperatures (22, 10.5, 4, −10°C) were determined. As expected, resazurin reduction was fastest at 22°C (16–29 times faster than at −10°C) and slowest at −10°C; whereas autoclaved controls showed no change in absorbance (data not shown). In addition, reduction rate varied by isolate and salt concentration. The rate of resazurin reduction correlated with growth rate in the presence of 3% NaCl with correlation coefficients of 0.99, 0.99, 0.99 and 0.95 for isolates 2b, 3a, 5 and 8 respectively.

Subsequently, resazurin reduction was used to assess the activity of all isolates at −10°C (Table 1). Spore-forming isolates showed little or no resazurin reduction although all were capable of resazurin reduction at higher temperatures (data not shown). In contrast, all of the non-spore-forming isolates exhibited resazurin reduction at −10°C, with an average rate of 0.6 fmol cell^{-1}day^{-1}. Isolate 5 reduced resazurin (6.0 fmol cell^{-1}day^{-1}) 10 times faster than any other isolate.

At the low-temperature limit for life, metabolic and growth rates are low and individual metabolic processes are usually measured as proxies for cell division (Karl and Bossard, 1985a,b; 1986; Carpenter *et al.*, 2000; Rivkina *et al.*, 2000). Unfortunately, the validity of using individual metabolic processes (i.e. DNA or protein synthesis) in the absence of other criteria to estimate growth rate at low temperatures has not been demonstrated. At subzero temperatures, significant amounts of energy may be required to simply maintain cell viability and prohibit cell reproduction (Stouthamer, 1977; Friedmann *et al.*, 1993). Without direct evidence for cell division, rates of metabolic processes can indicate the presence of viable cells, but not necessarily cell reproduction.

Growth of isolates at −10°C

Because low temperature cell maintenance energy requirements are not well understood; careful interpretation must be made of metabolic activity measurements at low temperatures. Hence, the growth of several isolates at −10°C was monitored over time via culture turbidity (Fig. 1A). One isolate, *Psychrobacter* sp. 5 had a detectable growth rate of 0.016 day^{-1}. Resazurin reduction rates measured on day 0 (Table 1) and day 75 (0.24, 2.1 and 0.27 fmol cell^{-1}day^{-1} for 2b, 5 and 8 respectively) were not appreciably different, indicating that cells were still metabolically active. Growth of isolate 5 at −10°C was confirmed by monitoring plate counts as well as optical density over time (Fig. 1B). The ability of isolates 2b, 3b, 4a, 8 and 12 to reduce resazurin but not reproduce at their low temperature limits indicated that survival is possible

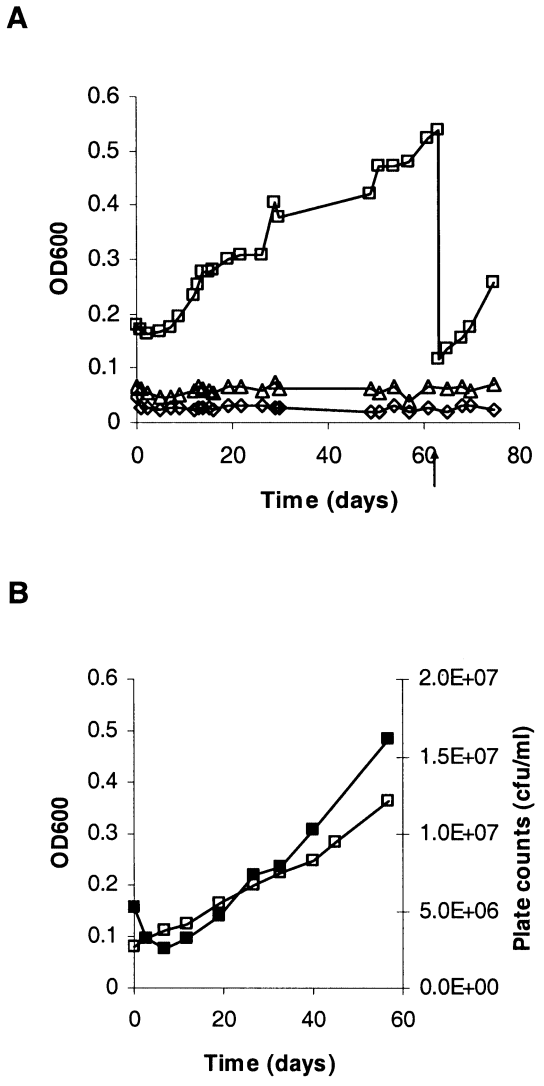

Fig. 1. Growth of selected isolates at −10°C.
A. Plot of optical density at 600 nm versus time for isolates 2b (◇), 5 (□), and 8 (△) in R2A plus 3% NaCl. On day 63 (arrow), 1 ml of the isolate 5 culture was diluted into 4 ml of fresh media.
B. Plot of optical density at 600 nm (□) and plate counts (■) versus time for isolate 5 in R2A plus 10% NaCl.

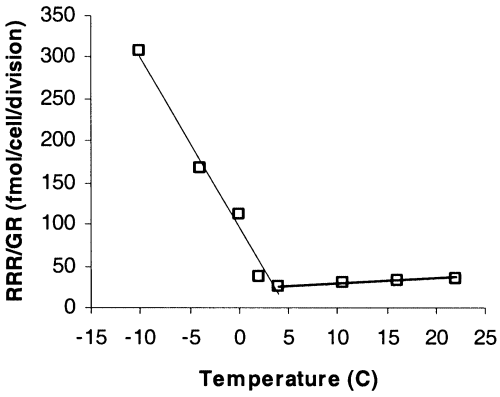

Fig. 2. Plot of temperature versus the ratio of resazurin reduction rate (RRR) to growth rate (GR) for isolate 5 grown in R2A plus 3% NaCl. Lines of best fit are shown for temperatures −10 to 4°C and 4 to 22°C.

without reproductive growth. Altogether, these data emphasis the fact that at subzero temperatures the estimation of growth rates from metabolic rates is not straightforward.

Further characterization of isolate 5

Because of the high metabolic and growth rates at −10°C, isolate 5 was further characterized. DNA:DNA hybridization confirmed that isolate 5 was a new species of *Psychrobacter* (degree of binding equalled 33% and 37% with *Psychrobacter glacincola* ATCC700754 and *Psychrobacter immobilis* ATCC43116, respectively). We therefore name isolate 5: *Psychrobacter cryopegella*. The ratio of resazurin reduction rate (respiration or energy use) to growth rate (cell yield) provided an estimate of the amount of energy required per cell division at a given temperature. When this ratio was plotted versus temperature (Fig. 2), a sharp break in the energy use curve occurred at 4°C. These data indicated that at 4°C cells grow most efficiently, above 4°C the energy required per cell division is relatively constant, and below 4°C the energy required per cell division increases dramatically as temperature decreases. The same trend was apparent for isolates 2b, 3a, and 11 (data not shown). These data indicated that below 4°C cells funnel energy primarily into repair and maintenance rather than division and biosynthesis.

Transmission electron microscopy images of isolate 5 revealed differences in cell structure that correlated with growth temperature (Fig. 3). Cells grown at 22°C appeared normal; whereas cells grown at −10°C were smaller and exhibited centrally located DNA, different cell wall structure, more outer membrane vesicles and many intracellular membrane inclusions. Centrally located DNA and intracellular membrane inclusions could be associated with cell division and may be more abundant at −10°C because slow growth rates may cause cellular processes to be more discrete. Despite these structural differences, cells appeared healthy and were still actively dividing as evidenced by the plethora of cells caught mid-division.

Conclusions

In the quest for defining the growth limits of life on Earth, microbiologists are constantly in search of extreme environments where bacteria not only survive, but also successfully reproduce. Although we have not demonstrated *in situ* activity of these organisms, the significance of isolating bacteria with detectable levels of activity and growth at −10°C after surviving for 43 000 years at −10°C can not be overlooked. Potentially, bacteria remain viable for tens of thousands of years by actively reproducing or by maintaining activity levels sufficient only to repair cellular damage, such as amino acid racemization or DNA damage from background radiation. Energy for cell maintenance or reproduction may come from nutrients that leach into the water from the surrounding frozen sediments. Evidence for *in situ* microbial activity in adjacent permafrost core samples has recently been reported

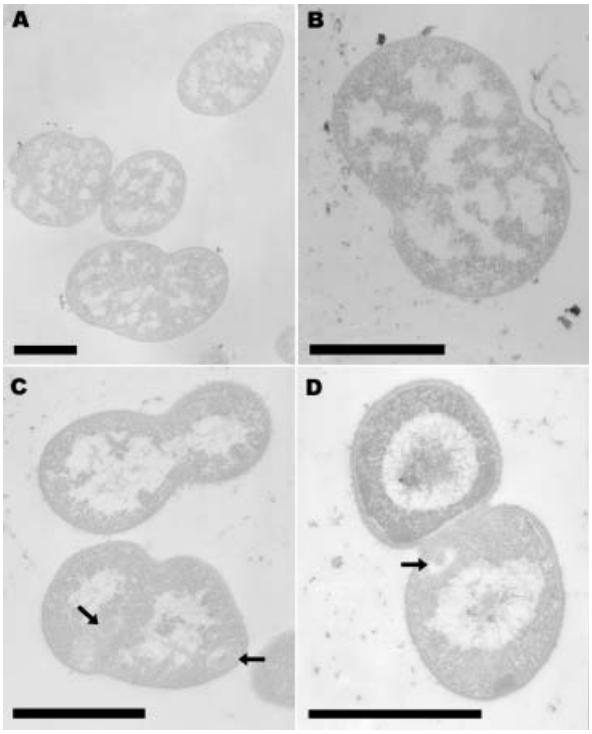

Fig. 3. Transmission electron microscope images of isolate 5.
A and B. Grown at 22°C.
C and D. Grown at −10°C.
Scale bar is 1 μm in all panels. Arrows point to intracellular membrane inclusions.

(Brinton *et al*., 2002). Understanding the low temperature adaptations and growth mechanisms of microorganisms has implications for general survival strategies of microorganisms, geochemical cycling in low temperature ecosystems, and the physical limitations of terrestrial life.

Experimental procedures

Sample collection

Samples were obtained from the Kolyma Lowland (70°N, 160°E). To avoid contamination, cores were obtained by drilling using the column rotation method without washing or drilling fluid (Shi *et al*., 1997). When the borehole reached a cryopeg, water was collected with a sterile bottle placed inside a core device. Samples contained some debris from the overlying permafrost layer. Samples were stored for 1.5 years at −10°C in a Model 2005 low-temperature incubator (VWR Scientific Products).

Isolation of bacteria

Samples were spread directly on plates of R2A (±3% NaCl) or LB and incubated at 4, 10.5 or 22°C. R2A media consisted of 0.5 g l^{-1} yeast extract, proteose peptone no. 3, casamino acids, dextrose and soluble starch; 0.3 g l^{-1} sodium pyruvate and dipotassium phosphate; and 0.05 g l^{-1} $MgSO_4$. The LB media consisted of 10 g l^{-1} pancreatic digest of casein, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl. Plates included 15 g l^{-1} agar. Based on colony morphology, 46 different colonies were picked for further analysis.

16S rDNA amplification, digest and sequencing

16S rDNA was PCR amplified from cells using universal bacterial primers 11f (5′-GTTTGATCMTGGCTCAG-3′) and 1512r (5′-ACGGYTACCTTGTTACGACTT-3′) and standard procedures. Amplification products were digested and analysed as in (Bakermans and Madsen, 2002). One isolate was chosen to represent each pattern for further analysis. Amplification products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for sequence analysis by MWG Biotech Inc (High Point, NC). Identity with closest relatives was determined by BLAST (Altschul *et al*., 1997). DNA:DNA reassociation was determined by standard techniques (De Ley *et al*., 1970).

Growth rate

Cultures were grown overnight at room temperature in R2A broth with or without 3% NaCl, diluted 1:10 into fresh media in sterile test tubes, and incubated at various temperatures (22, 10.5, 4 or −10°C) with shaking at 150 r.p.m. Absorbance at 600 nm was monitored over time. Growth rates at exponential phase were calculated by standard methods. Optimal growth temperature was determined by incubation in R2A in a temperature gradient block. The halotolerance of isolates was analysed by incubation in R2A broth with 0, 3 or 10% NaCl at 10.5 or 22°C with shaking at 150 r.p.m.

Resazurin reduction

In this assay, resazurin (blue) is reduced to resorufin (pink). Cultures were grown overnight and diluted into fresh media as above. After equilibration to the desired incubation temperature (22, 10.5, 4 or −10°C), resazurin was added (final concentration 0.001%) and incubated without shaking. Absorbance at 600 nm was monitored over time. Controls included samples killed by heat sterilization and reagents only.

Transmission electron microscopy

Cultures were grown in R2A + 3% NaCl at 22 or −10°C for 1 or 66 days respectively. Cultures were collected by centrifugation, washed, and resuspended in fresh media plus 3% glutaraldehyde for 1 h. Cells were collected by centrifugation and resuspended in fresh media plus 3% glutaraldehyde and 1.5% agarose, and stored at 4°C for 4–5 days. After primary fixation, agarose pellets were rinsed with fresh media and post-fixed for 4 h at room temperature in 1% osmium tetroxide. Unreacted osmium tetroxide was removed by several rinses in distillate water. Samples were dehydrated in a graded series of ethanol (30, 50, 70, 90, 100%) with 2% uranyl acetate added to the 70% ethanol step. Progressive infiltration of the samples with LR-White resin was done for 72 h. Final polymerization was performed in gelatine capsules at 65°C for 24 h. A Sorval Porter-Blum MT2-B ultramicrotome was then used to cut sections of *c*. 75 nm. The ultrathin sections were stained with uranyl acetate for 25 min followed by lead citrate for 30 min and viewed on a Jeol 100 CX II at 80keV.

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Note added in proof

Interpretation of compact DNA structures visible in TEM images of isolate 5 grown at −10°C was modified following the 10 January 2003 publication of ‘Ringlike structure of the *Deinococcus radiodurans* genome: a key to radioresistance?’ by S. Levin-Zaidman, J. Englander, E. Shimoni, A. K. Sharma, K. W. Minton, and A. Minsky in *Science* **299:** 254–256. The compact DNA structures may represent stationary phase dormant cells; alternatively, these structures may form in response to stresses inherent to cell reproduction at low temperatures.

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