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ORIGINAL PAPER

Metabolic activity of Siberian permafrost isolates, *Psychrobacter arcticus* and *Exiguobacterium sibiricum*, at low water activities

Monica A. Ponder · Michael F. Thomashow · James M. Tiedje

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Abstract The Siberian permafrost is an extreme, yet stable environment due to its continuously frozen state. Microbes maintain membrane potential and respiratory activity at average temperatures of −10 to −12°C that concentrate solutes to an $a_w = 0.90$ (5 osm), The isolation of viable *Psychrobacter arcticus* sp. 273-4 and *Exiguobacterium sibiricum* sp. 255-15 from ancient permafrost suggests that these bacteria have maintained some level of metabolic activity for thousands of years. Permafrost water activity was simulated using ½ TSB + 2.79 m NaCl (5 osm) at and cells were held at 22 and 4°C. Many cells reduced cyanotetrazolium chloride (CTC) indicating functioning electron transport systems. Increased membrane permeability was not responsible for this lack of electron transport, as more cells were determined to be intact by LIVE/DEAD staining than were reducing CTC. Low rates of aerobic respiration were determined by the slope of the reduced resazurin line for *P. arcticus*, and *E. sibiricum*. Tritiated leucine was incorporated into new proteins at rates indicating basal level metabolism. The continued membrane potential, electron transport and aerobic respiration, coupled with incorporation of radio-labeled leucine into cell material when incubated in high osmolarity media, show that some of the population is metabolically active under simulated in situ conditions.

Keywords *Psychrobacter* · Siberian permafrost · *Exiguobacterium* · Salt tolerance · Low temperature · Low water activity

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Introduction

The majority of the Earth's surface is cold, with approximately 70% covered by oceans with an average temperature of 4°C and over 20% of land surfaces subjected to consistently cold conditions (Pewe 1995). Permafrost, consisting of soils, sediments and rock, are exposed to temperatures of 0°C or below for a period of at least 2 years and longer depending on the region. Depending on the geologic stratum sampled, permafrost microbes from the Kolyma region of northeast Siberia have been subjected continuously to frozen conditions (−10 to −12°C) for 20,000 to 3 million years (Vishnivetskaya et al. 2000).

The majority of microorganisms isolated from buried permafrost are bacteria, although fungi, algae and Archaea have also been isolated (Gilichinsky et al. 1995). In contrast to many ocean isolates, most permafrost isolates are not

psychrophilic, but psychro-tolerant as they can grow at low, even subzero temperatures and also above 20°C (Gilichinsky et al. 2007). Bacteria are more numerous in permafrost than in sea ice, with 10^8 colony forming units per gram of soil isolated from permafrost soils up to 3 million years old (Vishnivetskaya et al. 2000). Studies of permafrost microbes have generally focused on strains that were isolated and grown at room temperature in nutrient-rich media. We previously isolated 238 bacterial strains from different strata of 20–30,000 year old Kolyma region permafrost using several isolation strategies, including low nutrient media and cryoprotectants with care to prevent exposure to temperatures above 4°C (Vishnivetskaya et al. 2000). Previous characterization has indicated that these isolates are psychroactive because they grow at −2.5°C, and are predicted to grow at temperatures as low as −12°C, the ambient temperature of the Kolyma region of permafrost (Ponder et al. 2005).

Bacteria are theorized to survive in the permafrost either by a very slow metabolic rate, or by existing in a state of anabiosis. Both theories have supporting and contradictory evidence, and presumably both are true for different microorganisms. The presence of such large numbers of viable microorganisms suggests DNA and membrane repair mechanisms must exist to protect the cells from damage by free radicals produced during long term exposure to γ- radiation emitted from the surrounding rock (Friedmann 1994).

Several recent studies indicate that, in addition to repair and basal metabolism, microbial growth occurs at permafrost temperatures. Colony formation has been shown to occur in Siberian permafrost isolates incubated at −8 to −10°C over an 18-month period (Gilichinsky 1993). In vitro doubling rates for a mixed Siberian permafrost community of 1 day at 5°C, 20 days at −10°C, and 160 days at −20°C were measured by membrane lipid incorporation (Rivkina et al. 2000). The Siberian permafrost isolate *Psychrobacter cryohalolentis* (formerly *cryopegella*) has a growth rate, determined by plate count method, of 0.16 day^{-1} at −10°C (Bakermans et al. 2003). *P. cryohalolentis* is closely related to the *Psychrobacter arcticus* sp. 273-4 the strain used in our study, (Bakermans et al. 2006), suggesting that the latter may be active within the permafrost.

Low temperature is not the only limiting stress within the Siberian permafrost. Ice formation increases solute concentration by decreasing the amount of free water available. This produces an environment with low water activity, similar to desiccated and salt-stressed environments. The low water activity of 0.90 encountered in permafrost corresponds to an increase in solute concentration to 2.79 m NaCl (5 osm) extrapolated by Rand et al (2004). Low water activity requires an increased amount of turgor pressure to maintain cellular respiration and thus provide energy necessary for cellular processes.

We investigated the ability of two phylogenetically unrelated Siberian permafrost-isolates *Psychrobacter arcticus* sp. 273-4 and *Exiguobacterium sibiricum* sp. 255-15 to maintain metabolic activity in high osmotica media by evaluating membrane permeability and electron transport shuttling. These are necessary to maintain essential membrane potential. Metabolic activity measurements were compared to the mesophilic, low salt tolerating *E. coli* and the obligate halophile *Dehalobacterium* DSM 7990. The permafrost cells' aerobic respiration and incorporation of radio-labeled leucine suggest that they produce enough energy to maintain basal metabolism, repairing damage, though perhaps not enough to divide.

Materials and methods

Isolation and phylogenetic characterizations

Psychrobacter arcticus sp. 273-4 and *Exiguobacterium sibiricum* sp. 255-15 were isolated from Kolyma-Indigirka lowland permafrost samples 20,000–30,000 YBP and 2–3 million YBP, respectively (Vishnivetskaya et al. 2000). These two isolates, 255-15 and 273-4 were chosen for further studies based on ability to grow at −2.5°C, growth in increased salinities (to 1 M NaCl), ease of culturability at 4°C, and age of permafrost strata (Ponder et al. 2005). *Psychrobacter arcticus* sp. 273-4, a Gram negative γ-Proteobacterium, has been described as the type strain and deposited into the DSMZ collection as isolate DSM 17307 (Bakermans et al. 2006). Strain 255-15 is a Gram positive member of the *Firmicutes* phylum, Order *Bacillales* and has been described as the type strain of *Exiguobacterium sibiricum* and deposited as DSM 17290 in DSMZ (Rodrigues et al. 2006).

Culture conditions

Psychrobacter arcticus sp. 273-4 and *E. sibiricum* sp. 255-15 were acclimated to low water activity and low temperature through four serial cultures in ½ TSB + 0.91 m NaCl (1.61 osm) at 4 or 22°C. The permafrost isolates were grown shaking at 250 rpm to an absorbance (600 nm) of 0.3, cells were pelleted and re-suspended in the same volume of ½ TSB + 2.79 m NaCl or ½ TSB + 3.86 m sucrose. These concentrations correspond to internal osmotic pressures of 5 osm, as extrapolated from Rand et al (2004) *E. coli* B was grown in 1/10 TSB to an $OD_{600} = 0.3$ and centrifuged to collect cells which were then re-suspended in the same volume of ½ TSB + 2.79 m NaCl. *Dehalobacterium* DSM 7990 was grown to an $OD_{600} = 0.3$ in *Marinococcus albus* medium (DSMZ

medium 434) and then transferred to an equal volume of ½ TSB + 2.79 m NaCl.

Electron shuttling activity by CTC

Acclimated bacteria (10^8 CFU ml^{-1}) were used to inoculate ½ TSB, ½ TSB + 2.79 m NaCl and ½ TSB + 3.86 m sucrose and were incubated in the dark at 22°C for 10 days or 4°C for 14 days to be certain that cells acclimated to the new conditions and no residual activity remained. Cyanotetrazolium chloride (CTC) (Polysciences, Warrington, PA) was added at inoculation to achieve a total concentration of 5 mM. To determine whether CTC reduction was an artifact of residual electron transport activity occurring immediately after osmotic shock, two parallel batches of cells were inoculated for 4 and 24 h prior to addition of CTC. Cells were fixed with formalin (37%) overnight at the end of the incubation, concentrated onto black MilliPore GTBP filters, counterstained with DAPI (Sigma-Aldrich, St. Louis, MO), and visualized with a Leitz fluorescence microscope. Three slides for each of three biological replicates per sample were counted for the number of red (active) and blue (inactive) cells (minimum of 100 cells per slide) in ten fields per slide. The percent active cells were then calculated and a student's *t* test was used to determine statistical significance (Statview version 5.0, SAS institute).

Membrane permeability by LIVE/DEAD staining

Membrane permeability was assessed using the LIVE/DEAD BacLight Bacterial viability kit (Molecular Probes, Eugene, OR). Samples were incubated at the original growth temperature (4 or 22°C). Ten milliliters of cells were assayed, as described by the manufacturer, immediately before transfer, and every 16 and 48 h after transfer to 5 osm medium at 22 and 4°C, respectively. Three biological replicates per treatment were used and three slides were viewed for each replicate. The numbers of red (DEAD) and green (LIVE) cells (minimum of 200 cells per slide) for ten fields per slide were counted using a Leitz fluorescence microscope.

Aerobic respiration by resazurin reduction

Cells were grown overnight and diluted 1:100 into fresh media (½ TSB + 0.91 m NaCl, ½ TSB + 2.79 m NaCl) and then resazurin (Kodak, NY) was added (final concentration 0.001%). Resazurin (purple) reduction was measured by recording the colorimetric shift at OD600 to its product, resorulin (pink) (Bakermans et al. 2003). Measurements were made every 20 min at 22°C and every 4 h at 4°C. Controls consisted of samples killed by autoclaving and reagents only.

Tritiated leucine incorporation assay

The *P. arcticus*, *P. cryohalolentis* and *E. sibiricum* were grown to an $OD_{600} = 0.3$ in ½ TSB + 0.91 m NaCl and transferred to the same volume of ½ TSB + 2.79 m NaCl. Ten microCuries of ^{3}H- leucine (40–60 mCi, Sigma-Aldrich) was added to each sample, and incubated at 4 or 22°C. Radiolabel incorporation was measured every 6 h at 22°C for the first 72 h, then every 24 h for 7 days, while the 4°C cultures were sampled daily for 30 days. Samples were pelleted by centrifugation (incorporated) and the supernatant (unincorporated) filtered onto nitrocellulose filters (Millipore, Billerica, MA). Two extractions with ice-cold 5% trichloroacetic acid were performed on both the pellet and filter separately. The filter and pellet were then washed separately with ice-cold 80% ethanol. The filters were dissolved in an alkaline solution (0.05 N NaOH, 25 mM EDTA, 0.1% SDS) for 60 min at 90°C (Buesing and Gessner 2003). After cooling and centrifugation at 14,000×*g* for 10 min, 100 µl samples of supernatant were aliquoted to 6 ml of Safety Solve scintillation fluid (Research Products International Corp, Mount Prospect, IL). Radioactivity of the filtrate and dissolved filters (100 µl) was measured with a Hewlett-Packard liquid scintillation counter for 10 min. Protein concentrations were determined for each sampling time point using the BioRad Quick protein determination assay kit (BioRad, Hercules, CA).

Transmission electron microscopy

Negative staining, without any other pretreatment, was used to determine the intact morphology of cell surface structures. A drop of cell suspension (10 ml of culture concentrated to 1 ml) was deposited onto a formvar and carbon coated grids for 30 s. After that, the excess sample was removed with a filter paper. Samples were then stained with a drop of 1% phosphotungstic acid (pH 7.0) for 15–3 s. The excess stain was wicked away with filter paper.

For ultra-structural studies, cells were suspended and fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C. After fixation, cells were pelleted and re-suspended in 2% agarose. Agarose blocks containing the cells were fixed 2 h at room temperature. The plugs were then washed three times for 15 min in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and washed an additional three times for 15 min in 0.1 M cacodylate buffer. The plugs were then dehydrated for 10 min in series of acetone (30, 50, 70, 95, 100, 100, 100%). Infiltration was then performed using 3:1

acetone: Poly/Bed 812, 1:1 acetone: Poly/Bed 812 and 1:3 acetone: Poly/Bed 812 followed by infiltration overnight in 100% Poly/Bed 812. Samples were embedded in silicone molds and polymerized at 60°C for 24 h. Blocks were sectioned with a Power Tome XL ultramicrotome (RMC, US); 70 nm sections were stained with 2% uranyl acetate and lead citrate (Reynolds formulation) and then observed using a JOEL 100 CX transmission electron microscope (Japan) at an accelerating voltage of 100 kV. Images were taken digitally with a Mega View II system. All sample preparation and TEM was performed by the Center for Advanced Microscopy at Michigan State University.

Results

Culture in 1/2 TSB + 2.79 m NaCl

An increase in turbidity was not detected in ½ TSB + 2.79 m NaCl for *E. coli*, *P. arcticus* or *E. sibiricum* during a 3-month incubation period at 22 or 4°C (results not shown).

Electron transport

Electron shuttling activity, indicated by the red fluorescence of CTC, was detected in 5 osm media (2.79 m NaCl or 3.86 m sucrose) for *P. arcticus* and *E. sibiricum*. A substantially higher percentage of active cells were detected in the permafrost isolates when compared to *E. coli* under all conditions (Table 1). In the absence of salt stress, the percentage of active cells in both permafrost isolates did not differ between 22 and 4°C, while *E. coli* was significantly inhibited by the lower temperature ($P < 0.05$). Incubation in 5 osm medium resulted in a significant reduction of active cells in all strains, with *P. arcticus* and *E. coli* exhibiting a difference with both temperature and increased osmoticum. The largest decrease in percent of active cells in high osmoticum occurred for *P. arcticus* at 4°C (50% loss), and *E. sibiricum* at 22°C (34% loss). The use of both sucrose and salt as osmolytes gave comparable decreases in activity ($P < 0.05$) for all organisms, suggesting that the decreased metabolic activity was not due to ion toxicity as the salt was not more severe (Table 1).

Membrane permeability

Overall, permafrost isolates show greater membrane longevity in high osmotica compared to *E. coli*, but more membrane permeability than the obligate halophile, *Dehalobacterium.* The majority of the permafrost isolates and the obligate halophile membranes, *Dehalobacterium*, were intact when incubated in the 5 osm media for 6 days at 22 or 4°C (Fig. 1). Incubation at lower temperatures resulted in a statistically significant decrease in live cells in 5 osm media for both *P. arcticus* and *E. coli* ($P < 0.004$); however, the incubation at 4°C increased survival for *E. sibiricum* cells after 6 days ($P < 0.009$) in 5 osm media

Table 1 Percent of the metabolically active cells measured by CTC reduction vs. total cells DAPI stained for two permafrost isolates and *E. coli* at different temperatures and osmotica after 10 days at 22°C and 14 days at 4°C

Medium	Temperature (°C)	Organism	Percent of cells that reduced CTC
½ TSB	4	*E. coli*	5.9
		E. sibiricum	64.2[b]
		P. arcticus	77.6[b]
	22	*E. coli*	28[a]
		E. sibiricum	62.3[b]
		P. arcticus	87.0[b]
½ TSB + 2.79 m NaCl (5 osm)	4	*E. coli*	1.9[a]
		E. sibiricum	35.1[b]
		P. arcticus	27.2[b]
	22	*E. coli*	1.8[a]
		E. sibiricum	28.2[b]
		P. arcticus	57.0[b]
½ TSB + 3.86 m sucrose (5 osm)	4	*E. coli*	1.6
		E. sibiricum	47.9[b]
		P. arcticus	11.8[b]
	22	*E. coli*	2.6[a]
		E. sibiricum	21.5[b]
		P. arcticus	45.6[b]

The median difference between percent active cells based on temperature was significant ($P < 0.05$) for *E. coli* in ½ TSB media and for *P. arcticus* at 5 osm.

[a] Median difference in percent active cells between ½ and 5 osm media, also different from other organisms: $P < 0.05$

[b] Median difference in percent active cells between ½ TSB and 5 osm media, no difference between organisms: $P < 0.05$

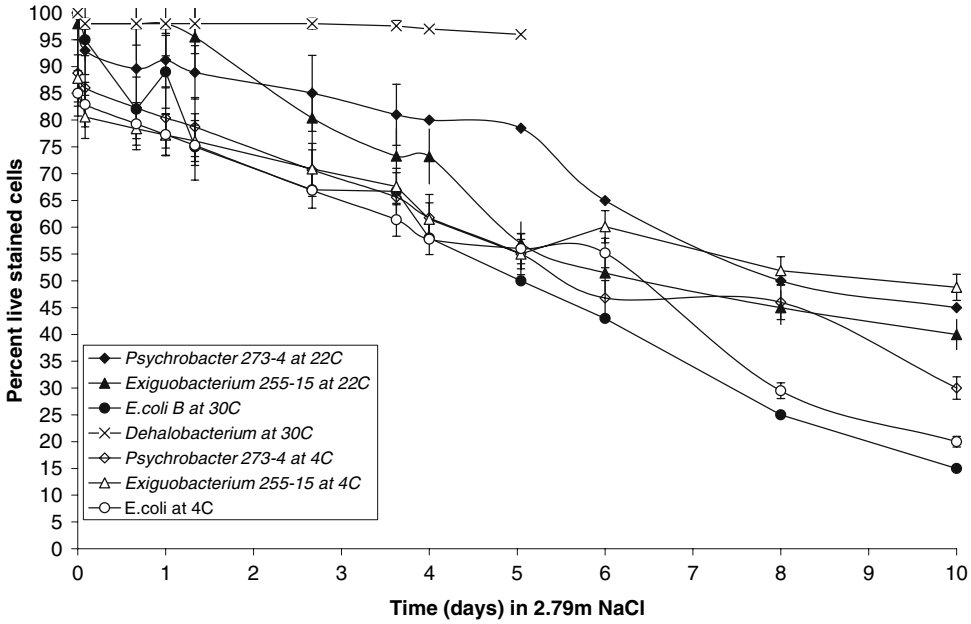

Fig. 1 The average percent of cells with intact membranes after 10 days incubation in ½ TSB + 2.79 m NaCl at different temperatures

(when compared to 22°C). There was no significant difference ($P = 0.89$) in the number of intact membranes between the halotolerant permafrost isolates and *E. coli* until day 7 when significantly more *E. coli* membranes lost integrity. Permafrost cell membranes were maintained longer within 5 osm media compared to *E. coli*, with 20–35% more permafrost cells intact at day 10. All the cells of the obligate halophile, *Dehalobacterium* DSM 7990, retained intact membranes until day 7, when death and lysis occurred due to nutrient exhaustion. The slight differences in membrane permeability with differing temperatures follow the same trend as the electron transport results. The 4°C incubation showed increased metabolic activity for *E. sibiricum* sp. 255-15 and slightly decreased activity for *P. arcticus*. However, more cell membranes remained intact than reduced CTC indicating the lack of electron transport in non-CTC stained cells was not due to loss of membrane integrity.

Aerobic respiration

The aerobic respiration rates, as measured by resazurin reduction in 5 osm medium were higher for *P. arcticus* than *E. sibiricum* (Figs. 2a, b). Lower rates of respiration, determined by the slope of the reduced resazurin line, resulted after incubation at 4°C compared to 22°C for both *P. arcticus* and *E. sibiricum* in 5 osm medium. At 4°C, 5 osm respiration rates reduced twofold in *E. sibiricum*, while *P. arcticus* respiration rates were comparable to *Exiguobacterium* rates at 22°C (Figs. 2a, b). Autoclaved controls showed no change in absorbance (data not shown). In ½ TSB, without added salt, respiration and growth rates significantly correlated ($r^2 = 0.97$ and 0.98 for *E. sibiricum* and *P. arcticus*, respectively) (data not shown).

Incorporation of tritiated leucine into protein

Incorporation of tritiated leucine as measured by disintegrations per minute (DPM) increased in all the organisms tested at both 22 and 4°C in ½ TSB + 2.79 m NaCl (Figs. 3a, b). There was no significant increase in protein number at 22°C for *P. cryohalolentis* or *E. coli* after 2 h in high osmotica media, suggesting these may be shock proteins that either retained stability or were necessary to re-synthesize continually. However, protein number increased with time at 4°C for *P. arcticus*, *P. cryohalolentis* and *E. sibiricum. P. arcticus* showed larger net dpm incorporation (6,388 dpm) at 22°C after 7 days, which based on extrapolation, would require 198 days at 4°C. Incorporation for *P. cryohalolentis*, *E. sibiricum* and *E. coli* were not significantly different from each other at 22°C. The approximate numbers of proteins synthesized were calculated based on a specific activity of 40–60 mCi and the average protein size of 36 kDa for *E. coli*. After 7 days at 22°C in 5 osm media ~300, 52, 40 and 30 protein molecules were synthesized for *P. arcticus*, *P. cryohalolentis, E. coli* and *E. sibiricum*, respectively. Protein molecules synthesized at 4°C after 30 days were ~275, 320 and 200 for *P. arcticus, P. cryohalolentis* and *E. sibiricum* (Fig. 3b). Incorporated ^{3}H-leucine values were significantly different with temperature, with 4°C improving incorporation for only *P. cryohalolentis* and *E. sibiricum* based on net dpm. The maximal incorporation of leucine for *Dehalobacterium* was larger compared to the permafrost isolates (data not shown).

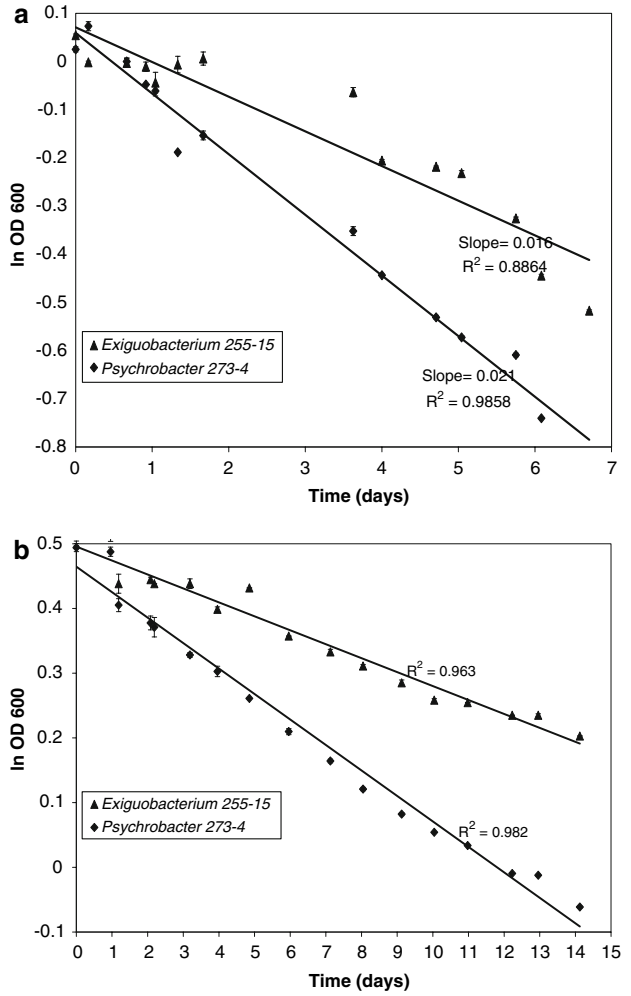

Fig. 2 **a** Resazurin reduction at 22°C by *E. sibiricum* sp. 255-15 and *P. arcticus* sp. 273-4 in ½ TSB + 2.79 m NaCl. The slope of the reduced resazurin line is 0.021, corresponding to a mean reduction in 48 h for *P. arcticus* sp. 273-4. The rate of reduction for *E. sibiricum* sp. 255-15 is 0.016, corresponding to a mean rate of 63 h. Error bars are smaller than symbol except where shown; **b** resazurin reduction measured by absorbance decrease at 4°C by *E. sibiricum* sp. 255-15 and *P. arcticus* sp. 273-4 in ½ TSB + 2.79 m NaCl. Error bars are smaller than symbol except where shown

Transmission electron microscopy

The TEM images of cells stained with phosphotungstic acid revealed the presence of capsules surrounding *P. arcticus* cells grown in the presence of high salt at both 22 and 4°C (Fig. 4a, b). *P. arcticus* grown at 22°C in ½ TSB showed no capsule (Fig. 4c), while cells grown at 4°C in ½ TSB showed a polysaccharide coating that allowed cells to adhere to each other but no capsule layer (Fig. 4d). Cross sections of cells grown at 4°C in the presence and absence of high salt reveal that the cells have dispersed cytoplasm (Fig. 4e, f). Some cells grown in ½ TSB + 2.79 m NaCl showed shrunken cytoplasm and all showed thickened cell membranes.

Discussion

Siberian permafrost isolates, *Psychrobacter arcticus* sp. 273-4 and *Exiguobacterium sibiricum* sp. 255-15 maintain membrane potential and electron transport, respire, and synthesize new proteins when incubated in high osmolarity media. These results provide evidence that a portion of the cell population is metabolically active under simulated in situ conditions, despite the lack of cell growth. The culture of microorganisms from ancient Siberian permafrost supports the hypothesis that some low level of metabolic activity must have continued during burial in this frozen, low water activity environment. On-going repair processes have been recently demonstrated in 35,000-year-old Siberian permafrost by amino acid racemization studies. The estimated ratio of D-aspartic acid (resulting from dead or inactive cells) to L-aspartic acid was smaller than predicted by mathematical models, allowing the authors to conclude that some metabolic activity was occurring within the permafrost environment to convert the D form to the L form (Brinton et al. 2002). Furthermore, membranes of native Siberian permafrost communities are continually synthesized at permafrost temperatures (5 to −20°C). There is a direct correlation between the decreasing incorporation of ^{14}C-acetate into lipids and the thickness of the unfrozen water layer surrounding the loamy soil (Rivkina et al. 2000). The presence of unfrozen water would allow for mass transfer of nutrients and elimination of waste products, until the diffusion gradients slow overall movement (Ostroumov and Siegert 1996)

Despite recent reports of growth of *Psychrobacter cryohalolentis* (Bakermans et al. 2003) at permafrost temperatures (−10°C), repeated attempts to grow the closely related *P. arcticus*, *P. cryohalolentis* and phylogenetically distant *E. sibiricum* at the corresponding water activity in NaCl medium were unsuccessful. These permafrost isolates were capable of growth on solid or liquid media containing up to 1.0 m NaCl at 4°C (Ponder et al. 2005) but no growth was seen during a 3-month incubation period at 22 or 4°C in medium with 2.79 m NaCl. The high NaCl concentration may be toxic for growth, despite no major differences between electron transport activity with sucrose as an alternative osmolyte. More electron transport activity at 2.79 m NaCl was indicated by the red fluorescence of CTC than those recently reported for particle-attached cells isolated from sea ice, which are surrounded by only 10% brine (equivalent to 1.7 m NaCl) (Junge et al. 2004), suggesting permafrost microbes may be better adapted to maintain electron transport at low water activities.

To maintain electron transport and cellular viability, bacteria must maintain turgor pressure. Gram positive bacteria, such as *E. sibiricum* sp. 255-15, have thicker cell walls,

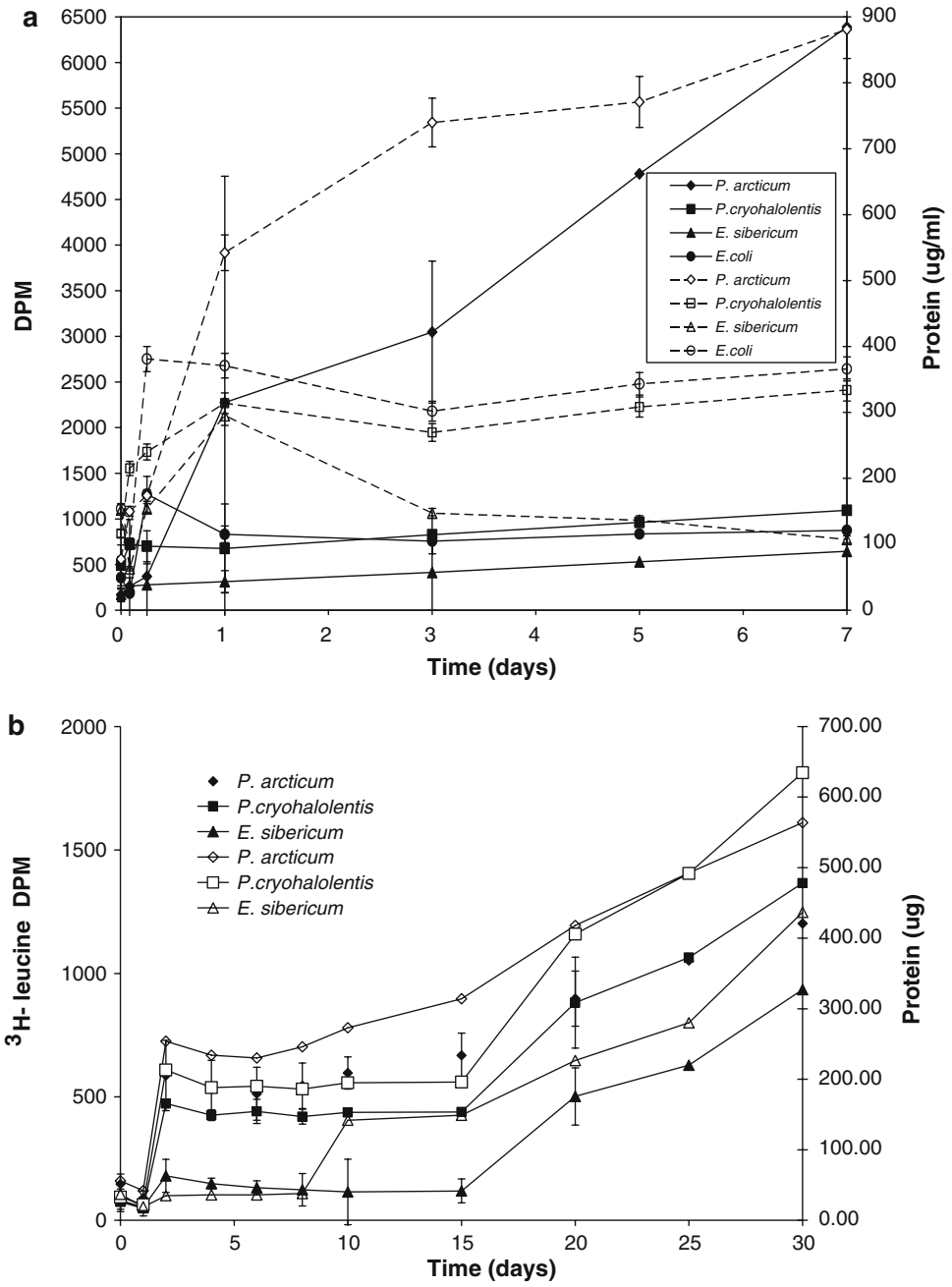

Fig. 3 **a** Incorporation of ^{3}H- leucine into TCA precipitable fraction of cells grown at 22°C for permafrost isolates and 37°C for *E. coli* in ½ TSB + 2.79 m NaCl. The amount of protein (*dashed lines*) extracted is shown in the secondary axis with *open symbols*. Triplicate time-points for each time. Y-error bars denote standard deviation from mean; **b** incorporation of ^{3}H- leucine into the TCA precipitable fraction of cells over 30 days in ½ TSB + 2.79 m NaCl at 4°C in permafrost isolated *P. arcticus* and *E. sibiricum* . The secondary axis (*open symbols*) indicate µg protein from which the ^{3}H was extracted. Triplicate time-points for each time. Y-error bars denote standard deviation from mean

allowing them to withstand greater turgor pressures than Gram negative cells, in which volume changes provide turgor pressure control. Despite the difference in cell wall and membrane composition between the Gram positive *E. sibiricum* cells and its Gram negative counterpart, *P. arcticus*, there were no significant differences in membrane permeability in 5 osm media. The dead cells were likely plasmolysed. Loss of membrane integrity was not the exclusive cause of the decreased electron transport in permafrost cells in 5 osm media, as more cells remained intact than reduced CTC in the case of the permafrost isolates and *E. coli*. Incubation in 5 osm media may decrease the turgor potential maintained in the permafrost isolates to the point that cellular division is inhibited but proton motive force continues. Maintenance of internal turgor pressure by extrusion of Na^{+} via H^{+}/Na^{+} antiporters occurs in several bacteria and Archaea (Albers et al. 2001) and would be vital in the permafrost environment. Recent genome sequencing of *P. arcticus* and *E. sibiricum* sp. 255-15 by the Joint Genome Institute reveals the presence of these H^{+}/Na^{+} antiporters, perhaps allowing the maintenance of membrane potential detected in LIVE/DEAD stained cells.

Transmission electron microscopy of *P. arcticus* confirmed the majority of cells appeared to be intact after

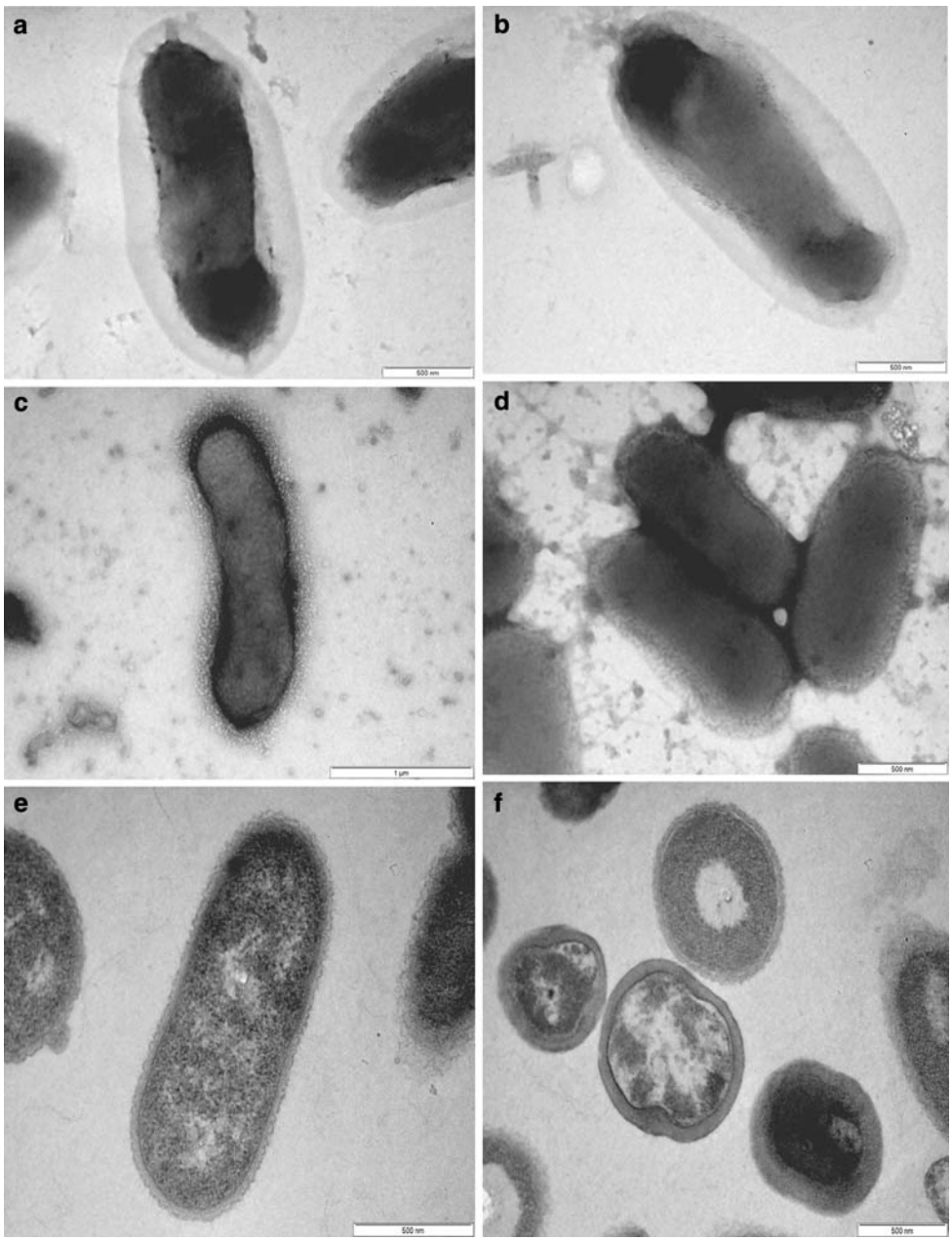

Fig. 4 Negative stained transmission electronmicrographs of *P. arcticus* sp. 273-4. **a** Capsule surrounding cells grown at 22°C in ½ TSB + 2.79 m NaCl; **b** capsule surrounding cells grown at 4°C in ½ TSB + 2.79 m NaCl; **c** no capsule surrounds cells grown at 22°C in ½ TSB; **d** no capsule surrounds cells grown at 4°C in ½ TSB, however a polysaccharide coating is visible that allows cells to adhere to each other; **e** cross section of cell grown at 4°C in ½ TSB, **f** cross sections of cells grown at 4°C in 2.79 m NaCl reveal some cells with compacted cytoplasm, with all cells showing an increased membrane thickness

5 days. Furthermore, a capsule layer was visible around all cells in 5 osm medium, while no cells showed capsules when incubated in ½ TSB at 4 or 22°C (Fig. 4a, b). The presence of capsular layers surrounding non-spore-forming bacteria occurs frequently in permafrost soils, and are believed to aid in formation of cyst-like forms (Soina et al. 2004). TEM images of *P. arcticus* do not show the altered cytoplasm and compact nucleoids typical of resting cells. Pleomorphy and encapsulation of cells in extracellular polymers is common and has been recently been reported for unknown cells isolated from sabkhas incubated at low water activities (Krumbein et al. 2004). Extracellular polysaccharides serve to bind water, allowing access to nutrients for continuing exchange of metabolic end products. For this reason, microbes isolated from hypersaline environments are commonly found in biofilms (Litchfield 1998). The presence of capsules surrounding *P. arcticus* 273-4 may be an adaptation to the decreased water activity of the permafrost and may allow for the adherence to soil particles and, therefore, the access to unfrozen water layers for metabolic activity.

Respiration within soils decreases with water activity, the resulting energy must maintain a favorable osmotic balance within the cells and the remaining energy is available for growth and division. In vitro nucleic acid synthesis has been reported at low temperatures, with water

activity equivalent to the levels tested within this study, for both mixed microbial communities and pure culture. Low rates of nucleic acid synthesis indicated by biological incorporation of ^{3}H leucine (protein) and methyl ^{3}H thymidine (nucleic acid) have been detected in arctic snow at temperatures between −12 and −17°C (Carpenter et al. 2000) and in pure cultures inoculated into sea ice incubated at −15°C (Christner 2002). Furthermore, radioisotopic studies of Lake Vostok (Antarctica) accretion ice indicate the presence of metabolic activity at −20°C, with only a small portion resulting from macromolecular synthesis; the remaining activity was used for cellular repair (Karl et al. 1999). In this study, the continuing incorporation of radiolabeled leucine into new proteins indicates that a low rate of metabolic activity occurs in 5 osm medium. The low number of proteins synthesized by the *Psychrobacter* isolates in the 5 osm media with time correspond with those protein numbers determined for an Antarctic sea ice isolated *Psychrobacter* incubated at −15°C, which would have an even lower associated water activity than this experiment (Christner 2002). The energy necessary for this continual protein production likely stems from aerobic respiration, which is reduced in 5 osm media and would result in less energy available for growth and division. The increase in protein numbers at 4°C with time may not indicate actual cell growth, as previous studies have shown that *P. arcticus* increases in cell biovolume with increased salinity (Ponder 2005). The finding of the low numbers of proteins synthesized in 5 osm media suggest that the microbes maintain only enough basal metabolism to allow repairs to membranes and DNA molecules and do not actively divide in this oligotrophic, freezing environment as described by Price and Sowers (2004). This continued metabolic activity under low water activity stress may be a survival mechanism for these ancient permafrost isolates.

Measuring activity at low water activity, equivalent to −10°C, supports the concept that the liquid inclusions present in permafrost (Gilichinsky et al. 2003) may provide an adequate habitat for active microbial populations on Earth and possibly elsewhere. The Siberian permafrost bacteria have been within low temperature soil with a high concentration of NaCl salts for thousands of years (Vishnivetskaya et al. 2000). Despite the constant low water activity, obligate halophiles were not exclusively selected, supporting continued metabolic activity in a resting state. Investigation of mechanisms of microbial persistence within permanently cold environments is of great importance to the fields of astrobiology and polar research. The remarkable ability of phylogentically diverse microbial populations to survive in a continuously frozen matrix of permafrost for millions of years makes the permafrost community unique. Our results raise questions on the survival time of a cell in continually frozen conditions; the level of metabolic activity necessary to retain viability; and the possibility of life existing on other cryogenic astral bodies.

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