ELSEVIER

Biophysical Chemistry 89 (2001) 13–24

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Re-evaluation of osmotic effects as a general adaptative strategy for bacteria in sub-freezing conditions

Carol A. Mindock[a], Mayya A. Petrova[c], Rawle I. Hollingsworth[a,b,*]

[a] *Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319, USA*
[b] *Department of Chemistry, Michigan State University, East Lansing, MI 48824-1319, USA*
[c] *MSU / NSF Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1319, USA*

Received 8 June 2000; received in revised form 19 September 2000; accepted 25 September 2000

Abstract

We studied the molecular mechanisms of adaptation of a Siberian psychrophilic bacterial strain. Upon adaptation to 4°C from 24°C, the major changes observed were in the membrane and cell surface chemistry. There was no evidence for the formation of so-called 'compatible metabolites' that are thought to be responsible for the survival at very low to sub-freezing temperatures. The membrane fatty acids were shorter with an increased amount of unsaturation in the 4°C cells compared to the 24°C cells. The absence of a significant amount of phosphorylation in the membrane lipids at 4°C compared to the levels at 24°C was another significant difference. At 4°C, the cell size was reduced in volume by a factor of ~ 14 compared to its size at 24°C. The polar polysaccharide capsular layer was also significantly reduced. There were no significant changes in the protein profiles indicating that antifreeze proteins were not being produced. The results obtained here are consistent with observations and established theories and principles on and about the behavior of water in confined spaces. These indicate that ordering effects, surface charge and polarity are the key determinants of the freezing point and the type of ice structure that will be formed when water is confined to an area of the size of a bacterial cell.

Keywords: Ordered water; Low temperature adaptation; Psychrophile; Membrane; Osmolyte

* Corresponding author. Tel.: +1-517-353-0613; fax: +1-517-353-9334.
E-mail address: holling7@pilot.msu.edu (R.I. Hollingsworth).

0301-4622/01/$ - see front matter
PII: S0301-4622(00)00214-3

1. Introduction

The processes bacteria use to allow them to survive and function at close to freezing temperatures and below is a mystery that has fueled much interest and spawned several theories. It is generally believed that there are two major physical or physico-chemical threats that the bacterium needs to respond to in these temperatures regimes. The first is ice formation within the cell which might lead to cell lysis because of the volume increase on expansion of water as ice is formed. The second is the increased salinity outside the cell as ice formation leads to the separating out of pure water (as ice) and a corresponding increase in salt concentration leading to an osmotic gradient across the cell membrane. It is thought that a general mechanism to counteract both of these phenomena would be an increase of solute concentration inside of the bacterial cell. The generally accepted adaptative response is an increase in the intracellular amounts of certain metabolites, especially glycine betaine, glycerol, mannitol, and sorbitol both as cryo-protectants and as osmolytes [1–3].

The underlying assumption in the popular theory on how bacteria adapt to sub-freezing conditions is that water in the confined spaces of a cell has the same physico-chemical properties of bulk water. Contrary to this notion, it is a well established fact that water in confined spaces (such as in a bacterial cell) has very different properties to those of bulk water. At the heart of this are the ordering effects some surfaces have on adsorbed water. When in close proximity to an ordered surface, water can become highly ordered and, as a result, have a lower freezing point, altered ionic solubilities, increased viscosity, and reduced dielectric constants compared to bulk water [4–10]. This ordering effect can extend as far as 1 μm from the surface [5,7]. The typical bacterial cell is only a few microns in its smallest dimension and its interior is, therefore, a highly confined space. Because of the profound effects of surfaces on the physical properties of water and on the phase transition between liquid water and ice, the discussion of low temperature adaptation and freeze resistance of bacteria is one of the properties of confined water and not of the bulk properties of water.

The freezing point of water is a function of the structure of the ice that will be formed. When bulk water freezes it typically forms a crystalline ice having a hexagonal packing arrangement (ice I_h). This is the most stable form of ice. The situation with ice formation on surfaces is different. When ice films are deposited at temperatures below 120°K on ultra-clean platinum surfaces, this non-charged ordered surface causes a net alignment of the water molecules resulting in polarization of the ice. This form of ferroelectric ice is called ice XI, and unlike the ice formed in bulk water is amorphous rather than crystalline in nature [11–15]. Several groups have shown that the ability to form ice on a surface, as well as the structure of ice formed, is dependent upon the characteristics of that surface. The ice crystal structures formed in various porous sol-gel silicas and ordered aluminosilicates was shown to be highly influenced by the pore size, pore shape, filling factor, and thermal history. Also, the neutron diffraction patterns often indicated the presence of a hybrid having both cubic (a metastable form of ice) and hexagonal ice characteristics [16].

Self-assembled amphiphilic systems such as long-chain alcohol's are good model systems for studying the effects of biomembranes on ice-formation. The ability of self-assembled amphiphilic alcohols to cause ice nucleation was found to be dependent on the aliphatic chain length. It was found that the longer chain alcohols form more crystalline monolayers and caused ice nucleation at higher temperatures than the shorter chain alcohols. Short chain alcohols have a higher degree of tilt away from the surface plane and are much more fluid, which decreases the temperature at which they cause ice nucleation. The freezing point of water in contact with functionalized hydrocarbon layers with 14–16 carbons in the chain was found to be −14°C [17]. Mixtures of chain lengths have even lower freezing point depression effects.

As discussed above, the ordering of water on a surface, especially an ordered, non-polar hydrocarbon, can dramatically reduce the tendency of water to freeze thus, affording a high level of

cryoprotection. Quite apart from depressing the freezing point of water, a highly ordered water surface has reduced solvent properties for salts and metabolites. This effectively forces solutes away from the surface and (in the case of a cell) towards the center causing an artificially high solute concentration there. There are, therefore, two water phases within the cell. The water layer near the surface is highly ordered and therefore, has a lower freezing point. The water in the middle has a very high solute concentration and also has a lower freezing point due to colligative effects. The various alterations in bacterial cell surface chemistry, especially at the level of the membrane lipids can thus play key roles in survival of cells at sub-freezing temperatures. The foregoing discussion also indicates that the production of osmolytes might not necessarily be an appropriate response.

In this report, we study the molecular mechanisms of low temperature adaptation in a psychrophilic bacterial strain. The organism was isolated from a permafrost sediment core of age 60–100 thousand years from the Siberian tundra zone on Kolyma-Indigirka Lowland (152–162°E and 68–72°N). Soil temperatures in this region are typically below −10°C. We conclude that during low temperature adaptation, changes in membrane chemistry and no increase in osmolyte concentrations are important for adaptation.

2. Materials and methods

2.1. Isolation and culture

The core sample was obtained by rotary drilling without any solution or chemical reagents, and the extracted core temperature was no higher than −7°C. Using the previously described methods of sampling, storage, transportation, and contamination control [18], it was shown that the micro flora in the samples were native and not the result of contamination. The organism studied (designated 45-3) was isolated from a sample at a core depth of 4.0 m. The bacterium was a Gram positive, existed as rods and V-forms in young cultures and coccus type in old cultures. Based on its morphological properties and 16S rRNA sequence it was classified as an *Arthrobacter* sp. (Jim Tiedje, Center for Microbial Ecology, Michigan State University, Michigan; personal communication). For the adaptation studies, the strain was cultured in trypticase soy broth, shaking at moderate speeds at 4 and 24°C, but was metabolically active at temperatures well below 4°C.

2.2. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR-500 spectrometer (500 MHz). Chemical shifts for samples in d_4-methanol are quoted relative to the proton resonances at 4.78 and 3.30 ppm, and for samples in D_2O relative to the proton resonance at 4.65 ppm. For the double quantum filtered-correlated spectroscopy (DQF-COSY) experiments, a total of 256 data sets with 24 transients at 2048 points each were acquired. The total correlated spectroscopy (TOCSY) experiments were also performed by using a total of 256 data sets with 24 transients at 2048 data points each and with a mixing time of 90 ms.

2.3. Hydrophobicity analysis

Cells from 4°C and 24°C cultures were diluted to the same optical density. Glass coverslips coated with chlorotrimethylsilane were dipped into the cultures for several minutes and then rinsed thoroughly in a stream of fresh media. The coverslips were placed on slides and the cells were then stained with several drops of 0.1% acridine orange for 5 min. The coverslips were again washed thoroughly using fresh media. The cells were viewed using rhodamine iso-thiocyanate (RITC) optics with excitation and primary barrier filters at 529 and 550 nm, respectively.

2.4. Total lipid extraction

Cells from the 4 and 24°C cultures were stirred with 60 ml water, 130 ml chloroform, and 15 ml methanol, at 30°C for 12 h. The layers were separated by centrifugation. The aqueous layers

were extracted once more with 30 ml chloroform for 15 min, and the organic layers were then combined. The aqueous layers were again centrifuged and the clear solutions were kept. The organic layers were analyzed by NMR spectroscopy (using d_4-methanol as the solvent) and by thin layer chromatography (TLC) using 10:4:2:2:1 chloroform/acetone/methanol/acetic acid/water. TLC analyses were performed on silica gel plates. The spots were visualized by spraying either with orcinol or 10% phosphomolybic acid in ethanol and heating at 120°C to visualize the organic components. Dissolution of the total lipids in d_4-methanol resulted in a methanol insoluble oily residue. This residue was dissolved in d-chloroform and analyzed by NMR spectroscopy and electrospray mass spectrometry.

2.5. Polysaccharide isolation

The aqueous layers form the total lipid extracts were concentrated to 4 ml and 12 ml of ethanol was added. The polysaccharide which precipitated was recovered with a glass rod and dissolved in 10 ml of water, and 10 mg $MgCl_2$, and 10 units each of RNase A and DNase were added. The solutions were kept at room temperature for 2 h. They were then dialyzed for 4–5 h, changing the water twice during that time, lyophilized and the resulting solids were weighed. These polymers were analyzed by NMR spectroscopy (using D_2O as the solvent) and by gas chromatography-mass spectrometry (GC-MS) after converting them to alditol acetate derivatives [19]. The remaining aqueous alcoholic solution was centrifuged to remove any precipitated solids. The supernatants which contained free amino acids, carbohydrates, and the like were removed and analyzed by NMR spectroscopy (using D_2O as the solvent) and a sample subjected to amino acid analysis on a Waters Millipore system which included a WISP 710B autosampler, a Waters 510 pump, and a Waters 440 absorbance detector.

2.6. Fatty acid methyl ester analysis

Total cells from cultures grown at 4 and 24°C were methanolysed in 2% HCl in methanol at 75°C for 30 h. The suspensions were blown to dryness, and 1 ml H_2O and 2 ml hexane were added. After vigorous shaking, the mixture was centrifuged and the hexane layer was removed and concentrated to dryness. The resulting fatty acid methyl esters were analyzed by gas chromatography (GC) and GC-MS using a 30-m DB1 column. The temperature program was a ramp from 160°C to 320°C at 3°/min. The final temperature of 320° was held for 20 min.

2.7. Lipid headgroup analysis

Approximately 5 mg of total lipids (isolated as described above) from cells cultured at 4 and 24°C were dried, and then 0.5 ml of 2 M trifluoroacetic acid was added. The lipids were hydrolyzed at 120°C for 1.5 h. The lipid headgroups were obtained by adding 0.5 ml of H_2O and extracting twice with two volumes of $CHCl_3$. The chloroform layers were removed and combined. The aqueous layers, containing the lipid headgroups, were dried and analyzed by NMR spectroscopy (using D_2O as the solvent).

2.8. Protein analysis by SDS-PAGE

Total cell lysates were prepared from 4 and 24°C cultures by adding two volumes of 10 mM Tris–HCl, pH 8.0 and 3 mg/ml lysozyme and incubating for 1 h on ice. One volume of 2X sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer solution [0.125 M Tris–HCl (pH 6.8), 0.04% sodium dodecylsulphate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.0025% bromophenol blue] was then added. The samples were mixed well, boiled for 5 min, and centrifuged briefly to remove any precipitate. The lysates from 4 and 24°C cultures were separated using Laemmli's discontinuous buffer system [20] on 12% polyacrylamide slab gels containing 0.32% bisacrylamide, 0.375 M Tris–HCl (pH 8.8), and 0.1% SDS. The stacking gel contained 4% polyacrylamide, 0.1% bisacrylamide, 0.125 M Tris–HCl (pH 6.8), and 0.1% SDS. Electrophoresis was carried out at 80 V for approximately 3 h. Gels were stained in a solution of 0.1% Coomassie

blue in 40% methanol/10% acetic acid, and destained in a solution of 40% methanol/10% acetic acid.

2.9. Transmission electron microscopy

Arctic strain 45-3 cells from 4 and 24°C cultures were negatively stained with 1.5% potassium phosphotungstate and viewed by transmission electron microscopy.

3. Results and discussion

The major changes observed in the permafrost bacterium 45-3 on adaptation to low temperatures were alterations in its size and cell surface structures. Transmission electron microscopy revealed that there was a dramatic difference in cell sizes for bacteria grown at the two temperatures. Those grown at the higher temperature had twice the length and more than twice the diameter of the cells grown at the lower temperature. Since the bacteria were rods at both temperatures, this represented a difference in volume of a factor of ~ 14. Electron microscopy also indicated that the 4°C cells had only a very small capsular polysaccharide layer and therefore, a very non-polar membrane surface since these took up the ionic stain only with difficulty. The hydrophobicity assay confirmed this. Cells from the 4°C culture (Fig. 1a) attached to the silanized coverslips with a coverage per unit area of 18 times those from the 24°C culture (Fig. 1b). The mass of the capsular polysaccharide recovered from the cells cultured at 4°C was less by a factor of 4.8 compared to that recovered from the 24°C cells. This reduction in capsule size at the lower temperature is very beneficial from another perspective. At low temperatures, the presence of a thick capsular polysaccharide coating would impede water flux through the cell and prevent nutrients from entering or metabolic waste from leaving due to decreased motional dynamics and slower diffusivity of molecules. In addition to this, Vogler [21,22] has pointed out that water on hydrophobic surfaces is less dense with a more open hydrogen-bonded network than water in contact with hydrophilic surfaces and is less likely to freeze.

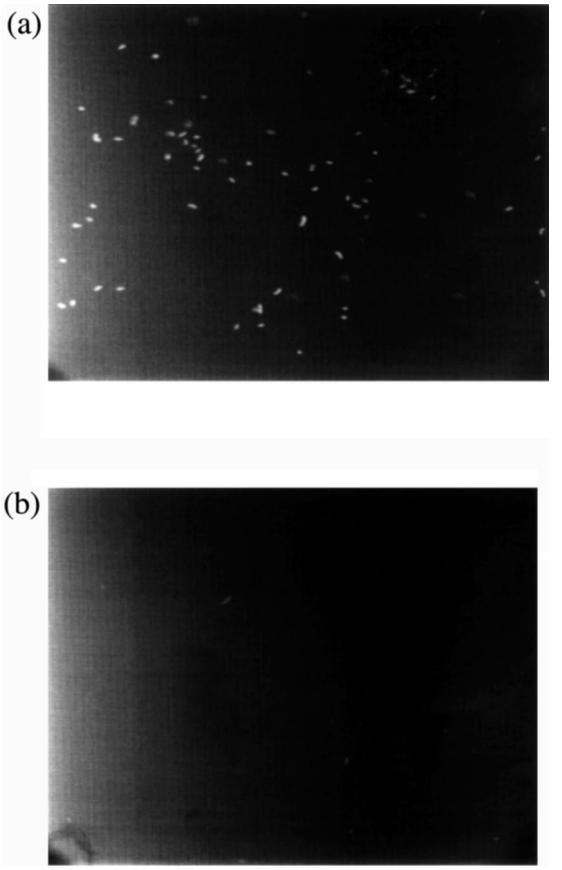

Fig. 1. Fluorescent micrographs using RITC optics (at the same magnification) of Arctic strain 45-3 stained with 0.1% acridine orange attached to silanized coverslips. (a) Cells cultured at 4°C. (b) Cells cultured at 24°C.

Significant alterations also occurred in the membrane lipid structures. Thin layer chromatography of the total lipids from the 4 and 24°C cells showed three major components which, based on standards, were identified as triacylglycerol, diacylglycerol and free fatty acids. The identies were later confirmed by NMR spectroscopy. The levels of triacylglycerol were much higher in the cells grown at 4°C and appeared as a methanol insoluble oil that was soluble in chloroform or hexane. In the case of the 4°C cells, triacylglycerols accounted for more than half the total lipids. Dia-

cylglycerol was the next predominant component, while only traces of phosphoglycerol were present. Other minor components were observed in the lipid preparations from the 24°C cells but the quantities were too small to allow characterization. Triacylglycerol probably serves as an energy source and not as a membrane component. Its presence, though, precluded a meaningful estimate of the total amount of membrane lipids obtained from the 4°C cells. Triacylglycerol is only sparingly soluble in methanol, however, and this was used as a way of separating it from the total lipids for NMR analyses. Its identity was confirmed by electrospray mass spectrometry. The mass spectrum showed a peak at m/z 605 due to the protonated form of a triacylglycerol species containing two C12 and one C10 fatty acid with a total of 3 double bonds between them. Signals for other homologous species separated by 14 or 28 mass units i.e. 1 or 2 methylene groups (e.g. m/z 591 and 619) were also present. An independent NMR spectroscopy analysis was also performed. The recovery of lipids (excluding triacylglycerol) from the 4°C cells was $\sim$6 mg per gram (wet weight) and approximately half this amount for the 24°C cells.

Proton NMR and two-dimensional DQF-COSY and TOCSY experiments (not shown) of the total lipids from the 4 and 24°C cells were performed. The two dimensional experiments indicated that the signals between 5.30 and 5.43 ppm were due to vinyl protons because of correlations with those at $\sim$2.05 ppm. These were much more abundant in the lipids from the 4°C culture than from the 24°C culture. Connectivities between signals assignable to diacylglycerol and triacylglycerol were also evident. In the spectrum of the 24°C lipids, extra signals due to the glycerol group of phosphatidylglycerol at 3.71 and 4.12 ppm were also observed.

The ^{1}H NMR spectra of the isolated lipid headgroups of the 4°C cells (after precipitating triacylglycerol with methanol) showed definitively that the only lipid headgroup was glycerol (Fig. 2a). The two doublet of doublets at 3.47 and 3.57 ppm were assigned to the C1 and C3 protons of glycerol. The multiplet at 3.7 ppm was assigned to the proton on carbon 2 of glycerol. The lipid headgroup components of the 24°C cells were glycerol and phosphoglycerol (Fig. 2b). The chemical shifts of the glycerol signals were the same as mentioned above. Two doublet of doublets at 3.65 ppm and 3.76 ppm were assigned to the C1 and C3 protons of phosphoglycerol. The multiplet at 3.84 ppm was assigned to the proton on carbon 2 of phosphoglycerol. From this data the proportion of phosphoglycerol was determined to be 40% by integration of the peaks. This method is much more reliable than charring the TLC plates and integrating densitometer traces. Gas chromatography and gas chromatography-mass spectrometry of the fatty acid methyl esters of the membrane lipids from the 4 and 24°C cells indicated the major lipid in both cell types was an *ante-iso* C_{15} lipid. The 4°C cells, however, contained a large amount of a $C_{15:1}$ lipid, and in general had shorter chain lengths and a greater degree of unsaturated and branched chains (Table 1). The cells cultured at 24°C contained a much smaller amount of the $C_{15:1}$ lipid, while the relative proportion of *iso* C_{16}, *ante-iso* C_{16} and C_{17} increased over the 4°C cells.

These results show that the synthesis of charged lipid components was almost totally suppressed during low temperature adaptation in this bacterium. Only diacylglycerols and triacylglycerols (which were likely contaminants of the membrane preparation) were found in the membrane of the 4°C cells, but the 24°C cells contained both phosphatidylglycerol and diacylglycerol. Smaller amounts of triacylglycerol were also found. The degree of unsaturation was higher and the average length shorter in the fatty acid chains of 4°C cells compared to 24°C as was expected if the average motional dynamics (fluidity) of the membrane lipids was to be conserved. Because no charged lipids are present in the membranes of the 4°C cells, there is no need for counter ions to be localized there to stabilize the lipid headgroups. The incorporation of shorter acyl chains and the decrease in lipid headgroup stabilization maintains a more fluid membrane such that the membrane surface is less likely to act as a template for ice formation whilst the uniformity of the membrane surface could serve to maintain a highly ordered layer of water which, as discussed

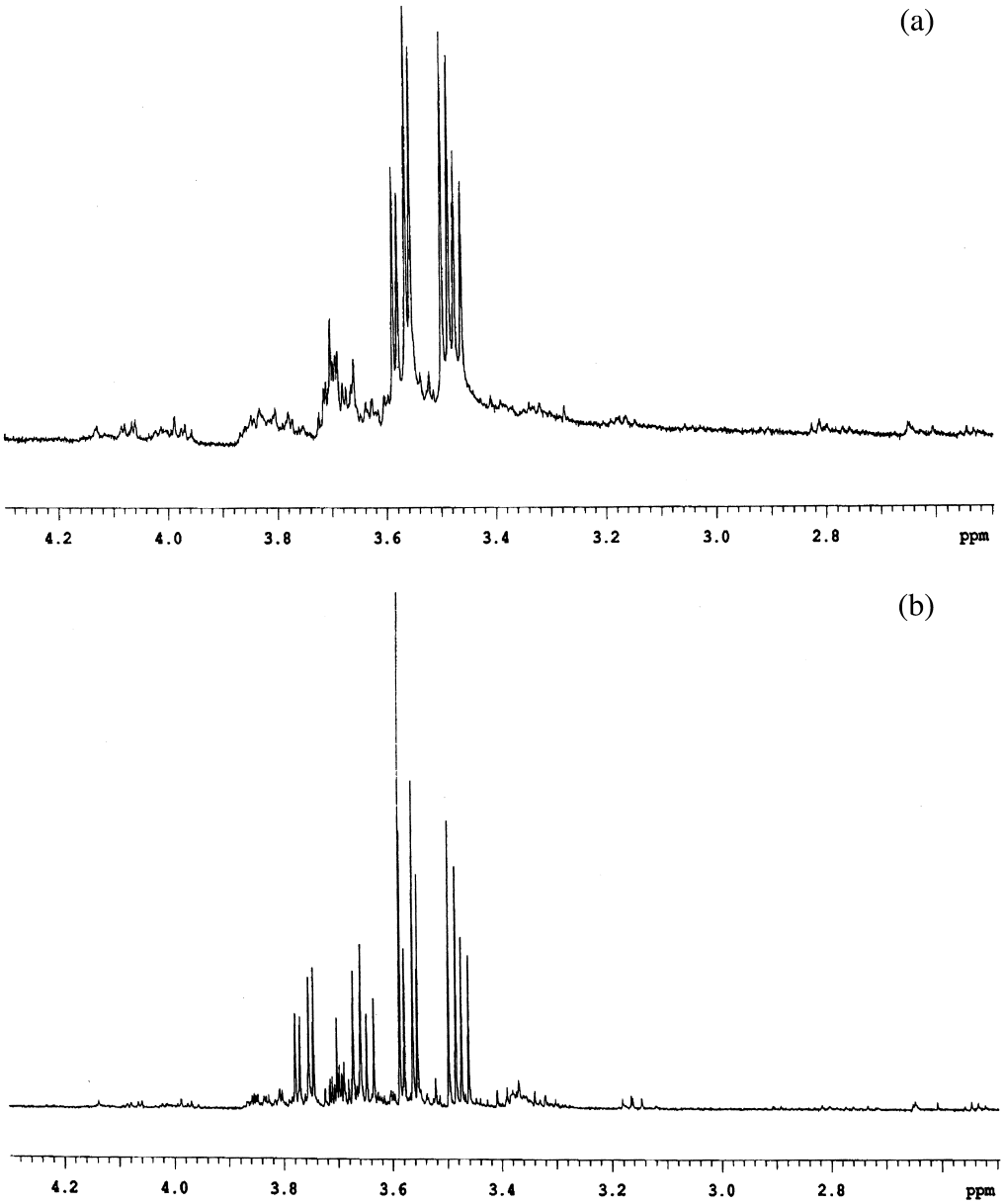

Fig. 2. ^{1}H NMR spectra of the lipid headgroups from the bacteria grown at (a) 4°C and (b) 24°C. The only lipid headgroup in the 4°C cells was glycerol. The two doublet of doublets at 3.47 and 3.57 ppm were assigned to the C1 and C3 protons of glycerol. The multiplet at 3.7 ppm was assigned to the proton on carbon 2 of glycerol. The lipid headgroups of the 24°C cells were glycerol and phosphoglycerol. The chemical shifts of glycerol were the same as mentioned above. The two doublet of doublets at 3.65 and 3.76 ppm were assigned to the C1 and C3 protons of phosphoglycerol. The multiplet at 3.84 ppm was assigned to the proton on carbon 2 of phosphoglycerol.

Table 1
Percent of fatty acid methyl ester derivatives in the total methanolysate of the cells grown at 4 and 24°C in order of elution.[a]

Structure	% in 4°C cells	% in 24°C Cells
$C_{14:1}$	1.75	0.00
iso-C_{14}	3.55	1.89
n-C_{14}	2.38	1.78
$C_{15:1}$	17.82	0.87
anteiso-C_{15}	58.95	67.04
iso-C_{16}	7.90	10.39
n-C_{16}	4.70	8.72
anteiso-C_{17}	2.96	9.31

[a] Trace amounts ($< 0.1\%$) of other fatty acids were seen by GC-MS, namely *anteiso*-C_{13} in the 4°C culture and *n*-C_{15} and *n*-C_{18} in both cultures.

earlier, would be more resistant to freezing as the temperatures decreases.

To determine whether other proposed mechanisms of low temperature adaptation (such as the production of osmolytes and/or anti-freeze proteins) are utilized by this bacterium, analysis of the cytoplasmic components as well as the total protein profiles were carried out for cells cultured at 4 and 24°C. Proton NMR spectroscopy was used to provide a comprehensive determination of the relative amounts of the various metabolic intermediates elaborated by strain 45-3 at 4°C and 24°C. Analyses were carried out on the supernatant of the ethanol precipitation of the aqueous layer which would contain free amino acids, sugars, sugar alcohols, and nucleotides. Proton NMR spectra of these fractions from the 4 (Fig. 3a) and 24°C cells (Fig. 3b) indicated that the major components were amino acids. Two-dimensional DQF-COSY (Fig. 4) and TOCSY (data not shown) experiments showed that these were glutamate, valine, glycine, methionine, threonine, and a compound tentatively identified as 3-aminobutyric acid. These results were in agreement with amino acid analyses. From the NMR spectra, the relative proportions of the amino acids in each culture did not change significantly between cells cultured at the two temperatures. Signals for common osmolytes such as free sugars and sugar alcohols such as glycerol, sorbitol and mannitol, which have proton shifts in the 3.0–4.5 ppm region were noticeably absent or at most present in only trace amounts. Signals for the methyl group of glycine betaine (~ 3.2 ppm), another common osmolyte was also absent.

Total lysates from cells cultured at 4 and 24°C were run on 12% SDS-PAGE gels (Fig. 5). There were no significant changes in the protein profiles, either in new proteins produced or suppressed, from the two cell cultures. Also there were no significant changes in the amounts of the various proteins produced at both temperatures, as was confirmed by running subsequent gels of total cellular proteins from other cultures grown at these two temperatures.

These results show no evidence for the presence of such common compatible solutes as glycine betaine, glycerol, glucose, sorbitol, and mannitol in the 4°C cells. This was definitively determined by amino acid analysis and by NMR spectroscopy. Signals for these metabolites were completely absent from the NMR spectra of the 4°C cells. Therefore, in this bacterium, the induction of catalytic systems (enzymes) involved in the synthesis of metabolites is not triggered by low temperature when within a few degrees of freezing. This was also clear from SDS-PAGE where there were no new protein bands, and no increase in the relative amounts of bands found in 24°C cultures was observed for the 4°C cells. The lack of a dramatic increase in new protein synthesis on going from 24 to 4°C indicates that anti-freeze proteins, which would need to be produced in large quantities, cannot be involved as well.

We have shown here that colligative (bulk) effects are not necessarily important for adaptation to low temperatures that can result in freezing. Surface ordering effects and the exclusion of solutes in aqueous solutions from ordered surfaces are, however, extremely important and powerful effects. These are well recognized phenomena that are utilized in desalination systems. In a membrane-based desalination system, water is passed through membranes and only pure water flows through because the water close to the pores in the membrane is ordered and excludes solutes [4]. Organization of water at the membrane surface in a manner that still allows flow

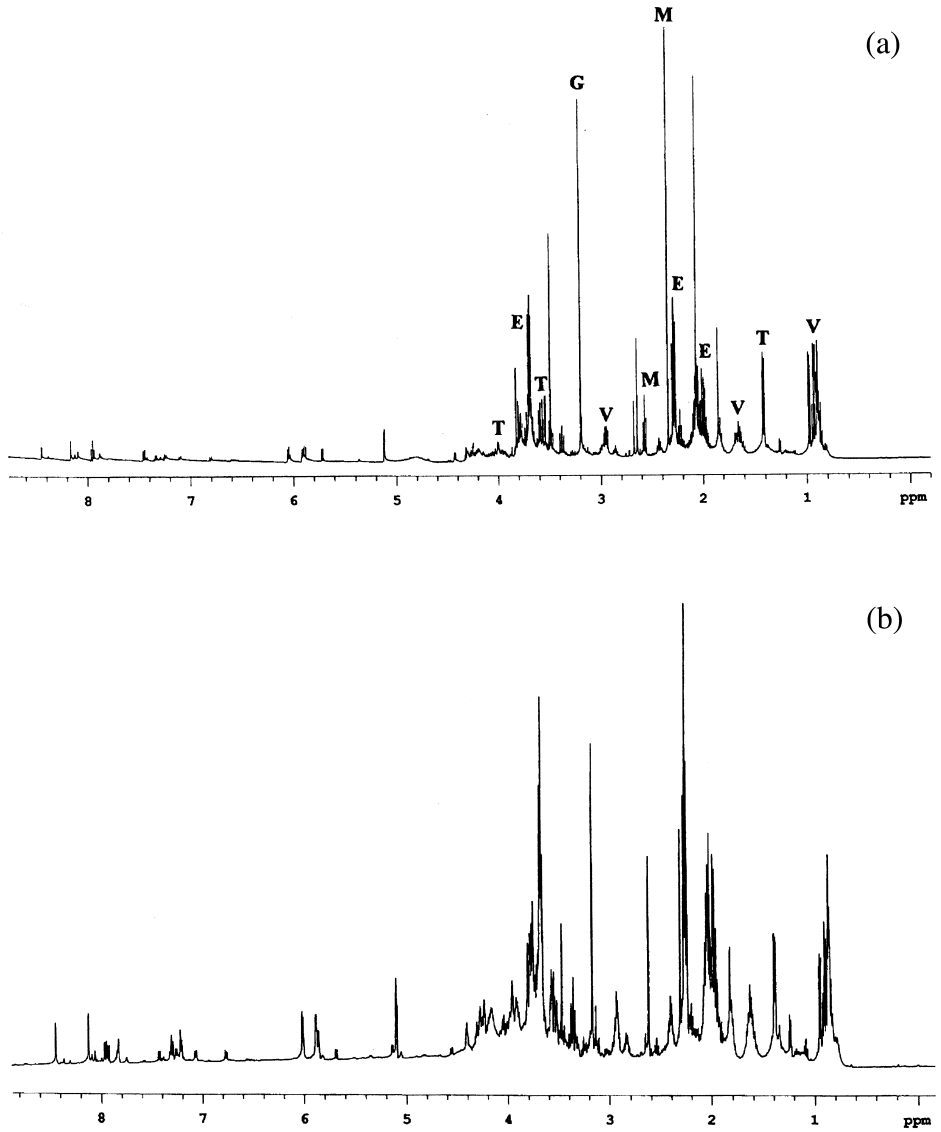

Fig. 3. ^{1}H NMR spectra of the cytoplasmic metabolites. (a) Cells cultured at 4°C. (b) Cells cultured at 24°C. The peaks are labeled with the one letter amino acid codes. The major components were glutamate (E), valine (V), glycine (G), methionine (M), threonine (T), and a compound tentatively identified as 3-aminobutyric acid (also see Fig. 4). Note that there are no significant differences in the relative proportions of amino acid components.

(as in desalination membranes) and reduces the freezing point is an excellent adaptative strategy. One way of accomplishing this would be for the cell to increase the relative amount of (membrane) bound water by reducing the cell size to increase confinement, and changing the membrane surface chemistry so that the proportion of ions and polar molecules on its surface is reduced. The water thus, arranged will not freeze and the small amount of un-ordered water in the

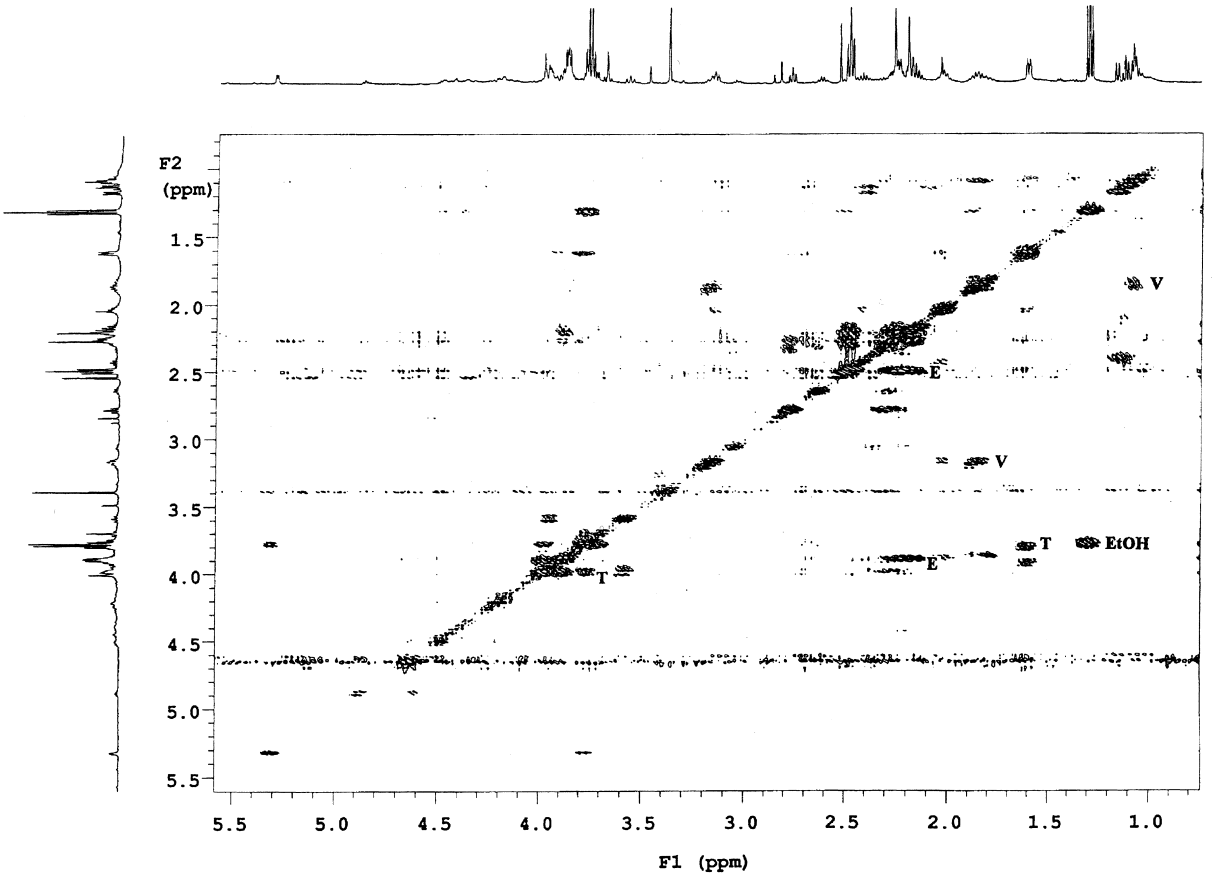

Fig. 4. Two-dimensional DQF-COSY spectrum of the cytoplasmic metabolites from cells cultured at 4°C. The cross peaks are labeled with one letter amino acid codes.

center of the cell (if any) will contain all of the electrolytes and will not freeze because of colligative effects.

This Siberian permafrost strain 45-3 was chosen for these adaptation studies because of its broad growth range of 0–30°C. It is adapted to an environment with a consistently lower temperature than the more modern day bacteria that now live on the permafrost surface. This strain has a 96% 16S rRNA sequence identity with *Arthrobacter pascens*. It also has similar rod/coccus growth cycles, cell size, and fatty acid profiles to this and other modern day *Arthrobacter* strains from various habitats including farm and deep subsurface terrestrial environments and human clinical materials [23–26]. This indicates that the adaptation mechanism described here is not a peculiarity of ancient bacterial strains but should also be found in current *Arthrobacter* species.

Acknowledgements

We thank Dr Vera Soina for isolating the Siberian permafrost strain 45-3, and Dr Yin Tang (from our laboratory) for doing the transmission electron microscopy. This work was supported in part by the Center for Microbial Ecology, a National Science Foundation Science and Technology Center (NSF grant BIR 912-0006) and by the National Science Foundation (Grant IBN 9507189 to RIH).

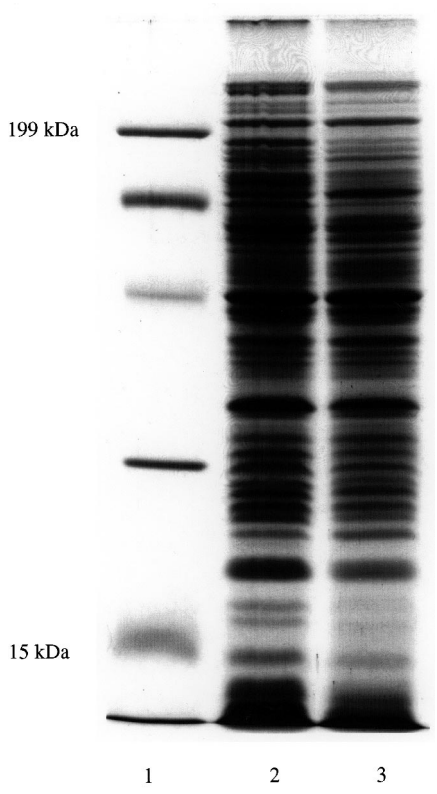

Fig. 5. SDS-PAGE gel of total proteins of lysates from cells cultured at 4 and 24°C. Lane (1) Low moleucular weight standards, lane (2) 4°C lysate, and lane (3) 24°C lysate.

References

[1] F. Franks, S.F. Mathias, R.H.M. Hatley, Water, temperature and life, Phil. Trans. R. Soc. Lond. B 326 (1990) 517–533.
[2] G.W. Gould, J.C. Measures, Water relations in single cells, Phil. Trans. R. Soc. Lond. B 278 (1977) 151–164.
[3] R. Ko, L.T. Smith, G.M. Smith, Glycine beatine confers enhanced osmotolerance and crytolerance on *Listeria monocytogenes*, J. Bacteriol. 176 (1994) 426–431.
[4] R.D. Schultz, S.K. Asunmaa, in: J.F. Danielli, A.C. Riddiford, M.D. Rosenberg (Eds.), Recent Progress in Surface Science, vol. 3, Academic Press, New York and London, 1970, p. 291.
[5] G. Peschel, P. Belouschek, in: W. Drost-Hansen, J. Clegg (Eds.), Cell-Associated Water, Academic Press, New York, 1979, p. 3.
[6] R.M. Hurtado, W. Drost-Hansen, in: W. Drost-Hansen, J. Clegg (Eds.), Cell-Associated Water, Academic Press, New York, 1979, p. 115.
[7] F.M. Etzler, A statistical thermodynamic model for water near solid interfaces, J. Colloid Interface Sci. 92 (1983) 43–56.
[8] W. Drost-Hansen, J.L. Singleton, Liquid asset: How the exotic properties of cell water enhance life, Sci. 29 (1989) 38–42.
[9] P.M. Wiggins, Role of water in some biological processes, Microbiol. Rev. 54 (1990) 432–449.
[10] F.M. Etzler, R.F. Ross, R.A. Halcomb, The structure and properties of vicinal water: lessons from statistical geometry, Phys. A 172 (1991) 161–173.
[11] S.T. Bramwell, Ferroelectric ice, Nat. 397 (1999) 212–213.
[12] M.J. Iedema, M.J. Dresser, D.L. Doering, J.B. Rowland, W.P. Hess, A.A. Tsekouras, J.P. Cowin, Ferroelectricity in water ice, J. Phys. Chem. B 102 (1998) 9203–9214.
[13] X.C. Su, L. Lianos, Y.R. Shen, G.A. Somorjai, Surface-induced ferroelectric ice on Pt(111), Phys. Rev. Lett. 80 (1998) 1533–1536.
[14] S.M. Jackson, V.M. Nield, R.W. Whitworth, M. Oguro, C.C. Wilson, Single-crystal neutron diffraction studies of the structure of ice XI, J. Phys. Chem. B 101 (1997) 6142–6145.
[15] S.M. Jackson, R.W. Whitworth, Evidence for ferroelectric ordering of ice Ih, J. Chem. Phys. 103 (1995) 7647–7648.
[16] J.M. Baker, J.C. Dore, P. Behrens, Nucleation of ice in confined geometry, J. Phys. Chem. B 101 (1997) 6226–6229.
[17] R. Popovitz-Biro, J.L. Wang, J. Majewski, E. Shavit, L. Leiserowitz, M. Lahav, Induced freezing of supercooled water into ice by self-assembled crystalline monolayers of amphiphilic alcohols at the air-water interface, J. Am. Chem. Soc. 116 (1994) 1179–1191.
[18] G.M. Khlebnikova, D.A. Gilichinsky, D.G. Fedorov-Davidov, E.A. Vorobyeva, Quantitative evaluation of microorganisms in permafrost deposits and buried soils, Microbiol. 59 (1990) 148–155 (in Russian).
[19] R.I. Hollingsworth, M. Abe, J.E. Sherwood, F.B. Dazzo, Bacteriophage-induced acidic heteropolysaccharide lyases that convert the acidic heteropolysaccharides of Rhizobium trifolii into oligosaccharide units, J. Bacteriol. 160 (1984) 510–516.
[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nat. 227 (1970) 680–685.
[21] E.A. Vogler, Water and the acute biological response to surfaces, J. Biomater. Sci.-Polym. Ed. 10 (1999) 1015–1045.
[22] E.A. Vogler, Structure and reactivity of water at biomaterial surfaces, Advan. Colloid Interface Sci. 74 (1998) 69–117.
[23] J. Loveland-Curtze, P.P. Sheridan, K.R. Gutshall, J.E. Brenchley, Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the Arthrobacter subgroup and description of Arthrobacter psychrolactophilus, sp. nov, Arch. Microbiol. 171 (1999) 355–363.

[24] T.L. Kieft, D.B. Ringelberg, D.C. White, Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium, Appl. Environ. Microbiol. 60 (1994) 3292–3299.

[25] G. Funke, M. Pagano-Niederer, B. Sjödén, E. Falsen, Characteristics of Arthrobacter cumminsii, the most frequently encountered Arthrobacter species in human clinical specimens, J. Clin. Microbiol. 36 (1998) 1539–1543.

[26] P. Kämpfer, R.M. Kroppenstedt, Numerical analysis of fatty acid patterns of coryneform bacteria and relaxed taxa, Can. J. Microbiol. 42 (1996) 989–1005.