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Physiological characteristics of bacteria isolated from water brines within permafrost

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Abstract: In the Arctic there are lenses of overcooled water brines (cryopegs) sandwiched within permafrost marine sediments 100–120 thousand years old. We have investigated the physiological properties of the pure cultures of anaerobic *Clostridium* sp. strain 14D1 and two strains of aerobic bacteria *Psychrobacter* sp. isolated from these cryopegs. The structural and physiological characteristics of new bacteria from water brines have shown their ability to survive and develop under harsh conditions, such as subzero temperatures and high salinity.

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Introduction

Perspectives for discovering life on extraterrestrial bodies force many researchers to study terrestrial objects with subzero temperature. The presence of free water is an absolute requirement for the existence of life both on and beyond the Earth. Under conditions of subzero temperature, water may only be present in the form of highly mineralized brines.

All known salt ecosystems on the Earth are open water reservoirs characterized by above zero temperature, with the only exception being the Antarctic lake Don Juan Pont, which has permanent subzero temperature. With a high concentration of salts (45% $CaCl_2$), the lake becomes frozen only at a temperature of -48 °C (Meyer *et al*. 1962).

In the Arctic there are lenses of sodium-chloride water brines (cryopegs) with constant temperatures of (-9)–(-11) °C and mineralization of 170–300 g l^{-1} sandwiched within permafrost marine sediments 100–120 thousand years old (Gilichinsky *et al*. 2003). Studies of this unique ecological niche have found that water brine lenses in permafrost form a habitat for psychrophilic and psychrotrophic halotolerant microorganisms (Gilichinsky *et al*. 2003). A pure culture of *Clostridium* sp. strain 14D1 and two strains of psychrotrophic aerobic bacteria *Psychrobacter* sp. were isolated. The objective of the present work is to study the structural and physiological characteristics of new bacteria from water brines in arctic permafrost, which give them their ability to survive and develop under harsh conditions such as subzero temperatures and high salinity.

Materials and methods

Bacterial strains

Clostridium sp. strain 14D1 and *Psychrobacter* sp. strains 1pS and 2pS were isolated from water brines in permafrost (Gilichinsky *et al*. 2003). The new isolates were deposited in the Russian Collection of Microorganisms (VKM B-2271, VKM B-2269 and VKM B-2270, respectively).

Culture media

Anaerobic heterotrophic bacterium *Clostridium* sp. strain 14D1 was cultivated following the Hungate anaerobic technique (Hungate 1969) on a liquid nutrient medium of the following content (g l^{-1}): KH_2PO_4, 0.7; K_2HPO_4, 0.7; NH_4Cl, 0.5; $MgSO_4 \times 7H_2O$, 0.1; NaCl, 1.0; ascorbate Na, 1.0. Glucose was generally used as a source of carbon and energy, at 2.0 g l^{-1}. *Psychrobacter* sp. strains 1pS and 2pS were cultivated on the medium containing (g l^{-1}): Na_2HPO_4, 11.2; KH_2PO_4, 4.0; NH_4Cl, 2.0; NaCl, 4.0; $MgCl_2 \times 6H_2O$, 0.1; $CaCl_2$, 0.01; $FeSO_4 \times 7H_2O$, 0.005; solution of microelements trace elements solution (Balch 1979), 10 ml. Acetate in the concentration of 10 g l^{-1} was used as a substrate for growth. To assess the utilization of various carbon sources, substrates were introduced in the quantity of 0.1%.

Bacterial growth was found by measuring the optical density at 600 nm using a 'Specol-221' (Germany) spectrophotometer. The doubling time (t_d) was calculated to be the time necessary for the optical density to double (Pirt 1975). The growth rate was calculated to be $\mu = \ln 2/t_d$.

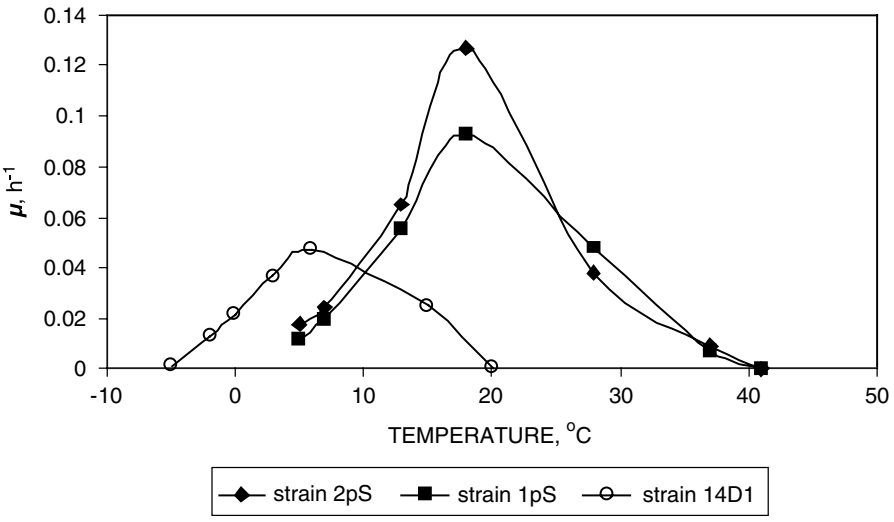

Fig. 1. Temperature effect on the isolates growth.

Resistance to heat and freezing/thawing, spore formation

Actively growing liquid cultures were tested for their resistance to heating by incubation at 20, 30 and 37 °C (24 h) and at 50, 60, 70 and 80 °C (20 min). Following the treatments, growth was monitored during incubation under standard conditions. The effects of freezing/thawing were tested by placement of actively growing cultures in a freezer at −40 °C for 1 month, followed by incubation under standard conditions.

For detection of spores a low nutrient medium was used based on DSMZ medium 63 (Anonymous 2001), which was modified by adding yeast extract and trypticase peptone, each in the concentration of 2 g l^{-1}.

Quantitative analysis of substrates and products in culture media

Alcohols were analyzed using a Pye-Unicam 304 gas chromatograph equipped with a (1 m × 2 mm ID) glass column packed with Porapak QS, 80–100 mesh (Fluka, Germany). The temperatures of the column, injector and flame-ionization detector were 90, 150 and 180 °C, respectively. The carrier gas was nitrogen at a flow rate of 20 ml min^{-1}. Fatty acids were analyzed using the same chromatograph equipped with a (2 m × 2 mm ID) glass column packed with Chromosorb W/AW-DMCS + 5% neopenthylglycolsuccinate, 100–200 mesh (Fluka). The pH of samples was adjusted to 4.0 with orthophosphoric acid. The temperature of the column was raised from 80 to 175 °C at a rate of 6 °C min^{-1}. The injector and detector were kept at 150 and 180 °C, respectively. The carrier gas was CO_2. Lactate was determined with lactate dehydrogenase by a colorimetric method (Hohorst 1970).

Lipid analysis of bacterial fatty acids

Lipids were extracted from the cell biomass that was dried in a stream of helium at 80 °C and then placed under vacuum. To 30 mg of dry biomass, 0.4 ml of 1 N solution of hydrogen chloride in methanol was added, and the mixture was heated at 80 °C for 3 hours. The methyl esters of fatty acids and other lipid components were extracted twice with hexane. The extract was dried, silylated in 20 μl of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide for 15 min at 80 °C and diluted with hexane to 100 μl. A 1 μl portion of the reaction mixture was analyzed with a model HP-5973 GC-MS system (Hewlett-Packard). Separation was carried out on a fused quartz capillary column (25 mm × 0.25 mm) with the immobile phase HP − 5 ms Hewlett-Packard (with a layer thickness of 0.2 μm). Chromatography was conducted in temperature programming mode from 120 to 280 °C with a rate of 5 degrees per minute. The temperature of the injector and interface was 280 °C. Data processing was carried out using standard programs of the GC-MS system.

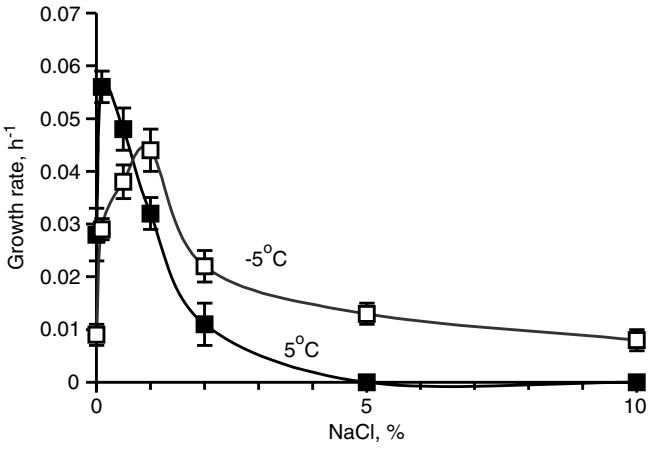

Fig. 2. Shift of the 14D1 strain optimum salinity for growth at +5 and −5 °C.

Results

As a result of our microbiological studies on cryopegs (Gilichinsky *et al.* 2003) we have isolated a pure culture of

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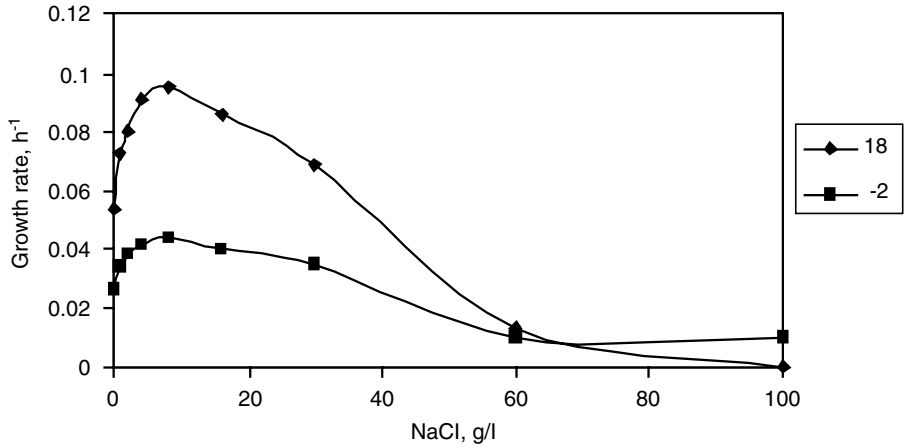

Fig. 3. Effect of NaCl concentration on the *Psychrobacter* sp. 2pS growth.

anaerobic bacterium strain 14D1 and two strains of aerobic bacteria. Cells of strain 14D1 were gram-positive motile bacilli with central endosporum. The bacterium fermented sugars, di- and polysaccharides, under strongly anaerobic conditions. Based on genetic tests, the bacterium was classified as belonging to genus *Clostridium* (Gilichinsky *et al.* 2003). We have also found that two other strains, 1pS and 2pS, gram-negative nonmotile cocci and coccobacilli, represent the genus of *Psychrobacter*. They were growing on mono- and dicarboxylic acids under aerobic and microaerophilic conditions.

Considering the characteristics of the habitat, we were specifically interested in looking at bacterial characteristics such as growth temperature, tolerance to salinity in the culture medium and correlation between substrate choice and temperature of cultivation.

Growth temperature

Figure 1 presents the results of temperature impact on the growth rate of strain 14D1 and strains 1pS and 2pS of *Clostridium* sp. 14D1 was growing in the temperature range −5 to 20 °C with an optimal growth temperature of 5 °C. The 1pS and 2pS strains of *Psychrobacter* sp. were growing in the temperature range 0 to 37 °C with an optimal growth temperature of 18–20 °C. 14D1 and 2pS strains were able to grow at sub-zero temperatures with very low growth rate.

NaCl effect on isolates growth

We have tested the effect of NaCl on the growth of the 14D1 strain at the optimal growth temperature (5 °C) and at a temperature close to the natural temperature of the habitat (−5 °C). At 5 °C the optimal concentration of NaCl was 0.5% and at a concentration of 5.0% the isolate growth ceased (Fig. 2); at −5 °C the optimum concentration of NaCl was 1.0% and growth was observed in the range 0 to 10.0%.

The effect of NaCl concentration on *Psychrobacter* sp. growth was studied for the 2pS strain. The strain was tolerant to a concentration of NaCl of 0–3.0% in the cultivation medium (Fig. 3). At the optimal growth temperature, introduction of 1.0% of NaCl to the medium inhibited 2pS strain growth, while at a temperature of −2 °C there was no inhibition observed.

Table 1. *Utilization of some organic compounds as sole carbon and energy sources by novel isolates at 18, 5 and −2 °C. '−' represents no growth after 42 days of incubation*

Substrates	Temperature, (°C)	*Clostridium* sp. 14D1	*Psychrobacter* sp. 1pS	*Psychrobacter* sp. 2pS
Xylan	18	−	−	−
	5	+	−	−
	−2	+	−	−
Cellobiose	18	−	−	−
	5	+	−	−
	−2	+	−	−
D-Glucose	18	+	−	−
	5	+	+	+
	−2	+	n.d.	+
Sucrose	18	+	−	−
	5	+	−	+
	−2	+	n.d.	+
Trehalose	18	+	−	−
	5	+	+	+
	−2	+	n.d.	+
L-Glutamate	18	−	−	−
	5	−	+	+
	−2	+	n.d.	+
L-alanine	18	−	−	−
	5	−	+	+
	−2	−	n.d.	+

Use of carbon and energy sources

We have tested the ability of the new isolates to utilize organic compounds at three temperatures, including temperatures different from the optimal temperature for each species. As may be seen from Table 1, the 14D1 strain did not use xylan and cellobiose at $t = 18$ °C, but it did grow on these substrates at 5 and −2 °C. The microorganism used L-glutamate only at −2 °C. *Psychrobacter* did not use D-glucose, sucrose, trehalose, L-glutamate or L-alanine substrates at the optimal

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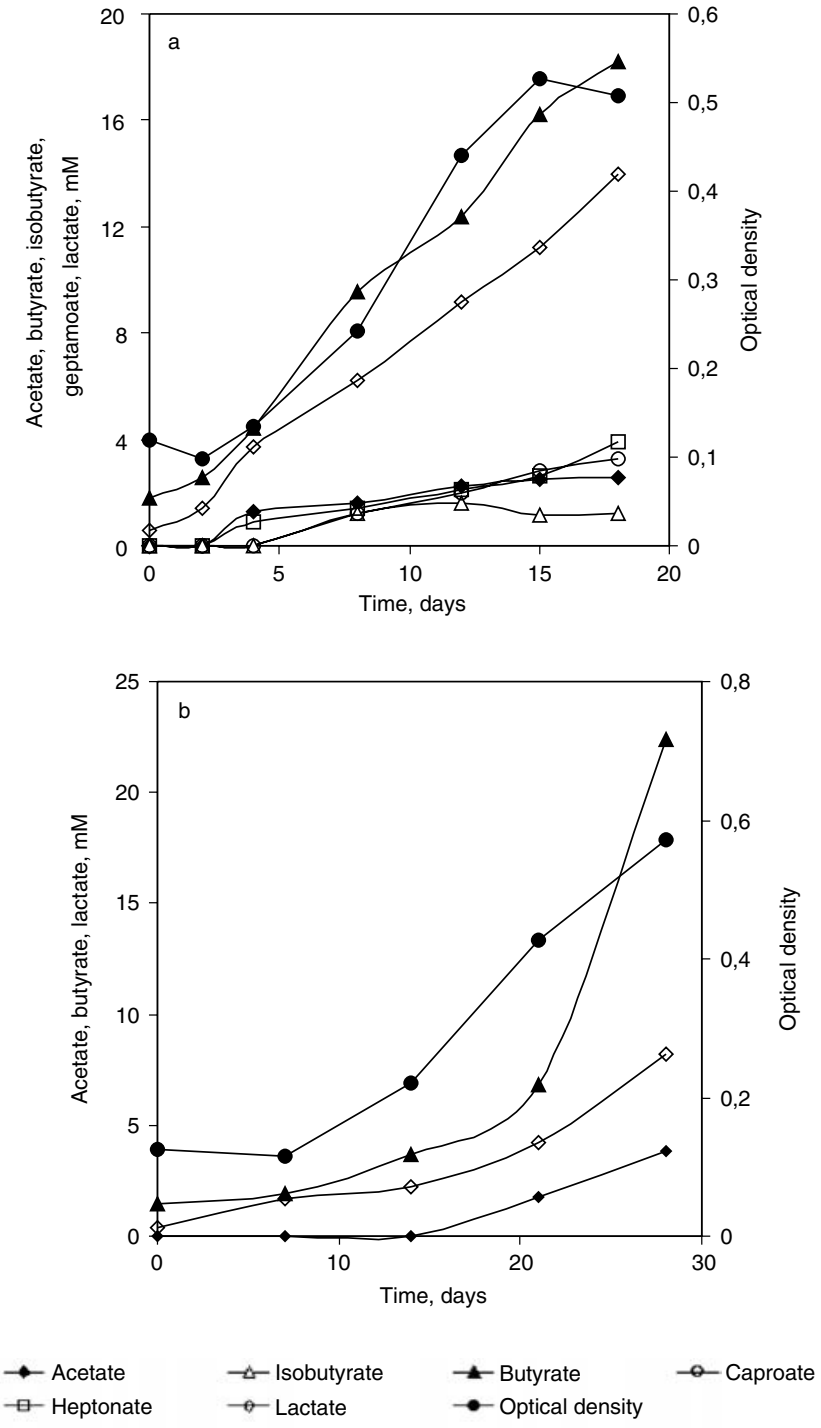

Fig. 4. Clostridium (strain 14D1) growth at different temperatures: (a) +5 °C, (b) −2 °C.

growth temperature (18 °C), but grew on these compounds at minimal growth temperatures.

As stated above, the 14D1 strain represents saccharolytic clostridia and ferments a wide spectrum of sugars producing fatty acids, lactate and small quantities of ethanol. We studied the dynamics of formation of non-gaseous products from glucose by the 14D1 strain at two temperatures.

In the cultivation of the 14D1 strain at the optimal growth temperature (5 °C), amongst the non-gaseous metabolic products butyrate and lactate prevailed (Fig. 4(a)), approximately in the ratio 1:1. In addition, small quantities of acetate, isobutyrate, caproate and heptanoate were formed. Formation of butyrate and lactate in the ratio 3:1 (Fig. 4(b)) as well as small quantities of ethanol (data not shown) was typical for the growth of this strain at subzero temperature (−2 °C).

Cryopegs are characterized by a low content (0.046%) of organic carbon (Gilichinsky *et al.* 2003). We studied the

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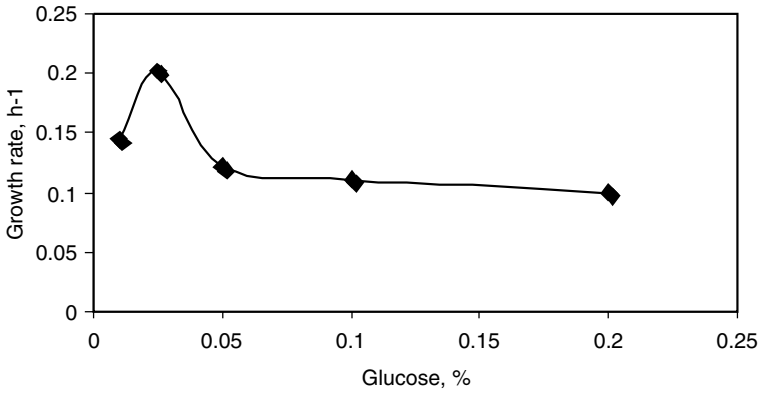

Fig. 5. Strain 14D1 growth at various glucose concentrations.

dependence of the 14D1 strain growth rate on the concentration of carbon source taking glucose as an example. The change in the concentration of glucose from 0.01 to 0.025% at the optimal growth temperature of 5 °C led to an increase in the growth rate of the studied strain from 0.14 to 0.20 h^{-1} (Fig. 5). Furthermore, an increase in glucose concentration led to a decrease in growth rate.

Both the psychrotrophic 1pS and 2pS strains and the psychrophylic 14D1 strain after freezing at −40 °C for 1 month had been growing under the optimal conditions without a lag-period.

Lipid content of cell wall

Analysis of the cell wall lipids of the 1pS and 2pS strains of *Psychrobacter* sp. and the 14D1 strain of *Clostridium* sp. detected the presence of fatty acids, aldehydes and hydroxy-acids with an even number of atoms from C_{14} to C_{18} (Table 2). The lipid complex of the 14D1 strain is characterized by the prevalence of mono-unsaturated hexadecane acid $C_{16:1}$, with double bond positioned by the 9th carbon atom, and myristame acid $C_{14:0}$. In the cell wall of both strains of *Psychrobacter* we found significant quantities of hydroxy-acids with various lengths of carbon chain. The content of hydroxy-acids makes up 34.0% for the 1pS strain and 30.8% for the 2pS strain.

Spore-formation of the 14D1 strain

While there was no spore formation observed during exponential growth, old cultures did contain single spores. Acidation of the medium up to pH 3.5–4.0, freezing/thawing or warming up to 50, 60, 70 and 80 °C did not induce spore formation. There was partial spore formation on poor medium. Introduction of vegetative isolate cells into cryopeg water without adding nutrients stimulated spore formation by the 14D1 strain. After this procedure, a spore was formed virtually in every cell.

Discussion

There are two known classes of microorganisms capable of growing at temperatures below 5 °C: the first class, adapted to steady, cold conditions, is primarily represented by bacteria isolated from sea and marine sediments; the second class, microorganisms inhabiting unsteady, cold conditions, is characterized by a wider range of growth temperatures, which often exceed the maximum temperature of the environment by 20–30 °C (Barros & Morita 1978). The cryopegs that we studied are an econiche with constant sub-zero temperature over the geological time scale. However, we isolated both psychrophilic and psychrotrophic bacteria. Isolated *Clostridium* sp. 14D1 is an obligate psychrophil according to the Morita classification (Morita 1975). By its phenotypical properties it is close to psychrophilic clostridium *C. lacusfryxellense*, isolated from the Antarctic microbial mat (Spring *et al.* 2003). It has the lowest known minimal growth temperature (−43 °C) predicted theoretically by the Raktovsky model (Ratkovsky *et al.* 1983). Almost all known representatives of the genus *Psychrobacter* are marine inhabitants (Juri 1991; Maruyama *et al.* 2000). Isolated psychrobacters by their chemotaxonomic and genetic characteristics are most close to *Psychrobacter glacincola*, which are halotolerant psychrophilic bacteria found in the marine ice of Antarctica (Bowman *et al.* 1997). *Psychrobacter* sp. 1pS and 2pS are psychrotrophic bacteria. We have experimentally shown that these bacteria are able to slowly develop at −2 and −5 °C (Table 3).

Changes in the ratios of the metabolic products (Fig. 4) during 14D1 strain cultivation at different temperatures show changes in the metabolism with a decrease of temperature. Presumably, most of the energy in this case is spent on maintenance of the population growth. Additional studies are required for a final conclusion. The lipid content of cell walls is an important index of cell adaptation (Inniss & Ingraham 1978), both to growth at sub-zero temperatures (Vreeland 1987) and to high salinity of the medium (Vreeland 1987). There is direct evidence that membranes with a higher content of unsaturated fatty acids function better under conditions of low temperatures (Wilson *et al.* 1970). Another method of cell adaptation to low-temperature growth is a higher content of fatty acids with a shorter chain length (Russell & Hamamoto 1998). The content of unsaturated compounds in the lipid complex of the cell walls of the 14D1 strain is much higher (58.07%) than in the cell walls of the 1pS (37.95%) and 2pS (38.4%) strains. Possibly, the high content of myristame acid ($C_{14:0}$) and unsaturated compounds

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Table 2. *Composition of lipids fatty acids and aldehydes of microorganisms from water brines*

Compound*		*Psychrobacter* sp. 1pS	*Psychrobacter* sp. 2pS	*Clostridium* sp. 14D1
Acid	Aldehyde	Contents (mol. %)		
$C_{12:0}$				0.53
$C_{12:0}$ 3 OH		3.0	2.4	
$C_{14:0}$		0.4	0.3	32.6
$C_{14:1\Delta9}$				1.4
$C_{14:1\Delta\ 11}$				0.7
$C_{14:0}$ OH		0.9	0.6	
	$C_{14:1\Delta9}$			0.36
	$C_{14:1\Delta\ 11}$			0.15
$C_{16:0}$		8.1	6.4	6.6
$C_{16:1}$		3.95		
	$C_{16:1}$			12.51
$C_{16:1\ \Delta9}$			1.3	37.0
$C_{16:1\ \Delta\ 11}$			0.8	5.1
C_{17cyc}				0.6
$C_{18:0}$		18.2	19.6	1.51
$C_{18:1\ \Delta9}$		30.2	18.1	0.57
$C_{18:1\Delta11}$		3.8	18.2	0.28
$C_{16:0}$ 10 OH		1.3	1.6	
$C_{18:0}$ 10 OH		28.8	26.2	
$\sum$ unsaturated compounds:		37.95	38.4	58.07

* First number – length of carbon bond, second – number of binary bonds, third – location of binary bond on carbon chain.
Δ- Symbol of binary bond.

Table 3. *Doubling times of bacterial generation at different temperatures*

	Doubling time (days)	
Temperature (°C)	*Clostridium* sp. 14D1	*Psychrobacter* sp. 2pS
−2	2.2	3.6
−5	18.05	n.d.

(Table 2) increases the fluidity of the membranes of the new bacterium *Clostridium* sp. which explains the lower optimum temperature and lower growth temperature in the 14D1 strain.

The high content of hydroxylated fatty acids in the lipids of the 1pS and 2pS strains indicates their adaptation to the higher salinity of the medium (Vreeland 1987).

Traditionally, the ability of bacteria to utilize certain substrates as carbon and energy sources are estimated under optimal growth conditions. When the cultivation temperature approached that of the habitat, the spectrum of utilized substrates expanded, enhancing the chances of the bacteria to survive in the econiche. These data correspond to the results obtained earlier for deep-sea bacteria of genii *Alteromonas*, *Bacillus* and *Vibrio* (Ruger 1988). To simulate the conditions of the natural habitat (Weibe *et al.* 1992) in our experiments, we have cultivated the 14D1 strain in media with various glucose contents. A decrease in glucose in the cultivation medium resulted in an increase in growth rate. This indicates that the new isolate is adapted to a low content of nutrients in the natural econiche.

Conclusion

The long-time survival of bacterial cells is connected to the successful occurrence of a secondary metabolism, including the formation of resting forms (Smith *et al.* 1974), that has its own optimum temperature different from the optimum growth temperature. To date, we can not yet estimate the optimum temperature of spore formation in the new bacterium *Clostridium* sp. 14D1. Nevertheless, the experiments we have carried out have shown that spore formation is a response of an organism to the absence of nutrients in the medium.

The bacteria studied not only adapted themselves to subzero temperatures, that is they survived freezing, grew at subzero temperatures and expanded the spectrum of consumed substrates with a temperature decrease (Table 1), but they were also tolerant to high salt content. As shown by experiments, temperature decrease was accompanied by an increase of salt concentration (Figs 2 and 3), which did not inhibit microbial growth. The other interesting fact was a shift of optimum salinity in the 14D1 strain with a decrease of cultivation temperature. The complex influence of low temperature and salinity on the metabolism of cryopeg inhabitants has yet to be studied.

The study of the physiological characteristics of microorganisms under conditions of sub-zero temperatures is complicated due to the low rate of metabolic processes. Nevertheless, active adaptation of the bacteria already studied to such conditions gives one the hope to explore the full vital activity at subzero temperatures in saline habitats. Generally, the study of the metabolism of living beings at temperatures below zero has just been launched. One may state that, even though the cryolithosphere value in the solar system is huge, we do not possess knowledge of the regularity of microbial functioning under these conditions. Bacteria isolated from water brines in permafrost may be a useful model for studies of the adaptation strategies of biological objects to subzero temperatures and consequently for solving the problem of exobiology.

Acknowledgments

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