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Biodiversity of cryopegs in permafrost

David Gilichinsky [a,*], Elizaveta Rivkina [a], Corien Bakermans [b], Viktoria Shcherbakova [c], Lada Petrovskaya [d], Svetlana Ozerskaya [c], Natalia Ivanushkina [c], Galina Kochkina [c], Kyastus Laurinavichuis [c], Svetlana Pecheritsina [c], Rushania Fattakhova [a], James M. Tiedje [b]

[a] *Institute of Physicochemical and Biological Problems in Soil Science, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russian Federation*

[b] *Center for Microbial Ecology, Michigan State University, USA*

[c] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Russian Federation*

[d] *Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Russian Federation*

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Abstract

This study describes the biodiversity of the indigenous microbial community in the sodium-chloride water brines (cryopegs) derived from ancient marine sediments and sandwiched within permafrost 100–120,000 years ago after the Arctic Ocean regression. Cryopegs remain liquid at the in situ temperature of −9 to −11 °C and make up the only habitat on the Earth that is characterized by permanently subzero temperatures, high salinity, and the absence of external influence during geological time. From these cryopegs, anaerobic and aerobic, spore-less and spore-forming, halotolerant and halophilic, psychrophilic and psychrotrophic bacteria, mycelial fungi and yeast were isolated and their activity was detected below 0 °C.

Keywords: Permafrost; Water brines; Viable microorganisms; Ancient ecosystem; Biodiversity; Metabolic activity

1. Introduction

Most of the known and studied saline aquatic ecosystems on the Earth are open water reservoirs characterized by temperatures above zero (Dead Sea, Mono Lake, Great Salt Lake) with the only exception being the Antarctic lake Don Juan Pond, which has temperatures permanently below zero. Because of high concentration of salts (45% $CaCl_2$), Don Juan Pond freezes only at −48 °C [1]. The largest Antarctic subglacial lake, Lake Vostok, at a depth of 4 km and an age of about 400,000 years [2], also has temperatures above 0 °C (based on the near 0 °C temperature of the bottom of the Ice Sheet). The bottom layers of some Antarctic Dry Valley and Vestfold Hills surface lakes with a permanent ice cover have temperatures below 0 °C and salinity up to 150–200 g/l [3,4], but they are not impermeable to microbial penetration from the outside.

At the same time, the Quaternary transgression and regressions of the Polar Ocean in the high Arctic against a cold climatic background favored the formation of overcooled water brine lenses (cryopegs) in the marine sediments. Cryopegs are defined as a layer of unfrozen ground that is perennially cryotic (forming part of the permafrost), in which freezing is prevented by freezing-point depression due to the dissolved-solids content of the pore water. An isolated cryopeg is entirely

* Corresponding author. Tel.: +7 096 773 3604; fax: +7 096 733 0595.
E-mail address: gilichin@online.stack.net (D. Gilichinsky).

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surrounded by perennially frozen ground [5]. One type of cryopegs, a marine cryopeg, is found in coastal or subsea perennially frozen ground and defined as lenses of ''cryosaline water'' or ''overcooled brine'' [6]. They are embedded in permanently frozen Holocene and Pleistocene strata [7,8] and are the only hydrological systems on the Earth with permanent subzero temperatures, high salinity (60–300 g/l) and isolation from external factors throughout their geologic history. The first descriptions of microbial populations in this habitat were published, mostly as a proxy for astrobiology [9–12]. This paper reviews the aforementioned papers and, together with recently received data, describes the biodiversity of viable microorganisms in cryopegs.

1.1. Description of site and objectives

Several isolated lenses of water brines were exposed by boreholes in the tundra zone near the East Siberian Sea coast. This is a continuous permafrost area with temperatures ranging −9 to −11 °C and a calculated thickness of up to 800 m. The cryopegs are confined to a 20-m-thick sodium-chloride marine horizon, sandwiched between terrigenous non-aligned layers at depths of 40–50 m below the surface (Fig. 1). This horizon was named as kon'kovskaya suite and dated to the end of the mid Pleistocene: 100–120,000 years ago [13] (an age on the same order as Lake Vostok). This suite marks the border of the Polar Ocean before sea level decreased and regressed to the North in the late Pleistocene. The foraminifer complex (*Trochammina ochracea*, *Proteonella difflugiformis*, *Patellina corrugata*, *Cibicides* sp., *Elphidius* sp., *Furcenkoisa gracilis*, *Cassidulina subacuta*) shows that finely dispersed sands and sandy loams with woody detritus, beetle shells, diatoms (*Melosira*), specu-

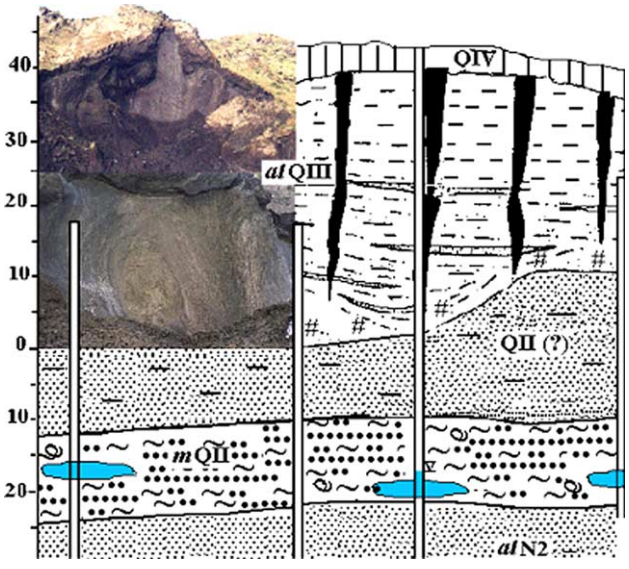

Fig. 1. Geological cross-section: cryopegs and embedding sediments.

las and skeletal fragments of tree-fungus were deposited in littoral lagoons at shallow depths and temperatures slightly above 0 °C [14]. Deposition of sediments was accompanied by active methane formation [15].

After regression of the Polar Ocean, the water/methane-saturated bottom sediments of the continental shelf were exposed to polar climate for thousands of years and froze [16]. Because of the pressure caused by freezing, the initial cryogenesis was accompanied by migration and release of water as the freezing front penetrated downward. Then, the process of cryometamorphism was accompanied by a freezing out of salts in the water to form lenses of overcooled brines. Within the marine horizon, these cryopegs (0.5–1.5 m thick, 3–5 m wide) occur at different depths. Some lenses represent non-artesian water, and some exist under low pressure with a hydrostatic head 2–3 m higher than the enclosed sediments. Differential salinity and distribution of low-resistance deposits as isolated circuits confirm the lenticular nature and isolated bedding of cryopegs.

Terrigenous deposits later covered the marine horizon and were built up under harsh climate conditions, as is evident from their pollen patterns. Hydrochemical analyses of the cryopegs that formed in the marine sediments allowed reconstruction of the temperature of their formation [17]. As shown by calculations, based on the brine salinity and chloride content using the program FREZCHEM2 [18], the cryometamorphism of sediments and water took place at mean annual ground temperatures that were 5–7 °C lower than present day (−9 to −11 °C) [19], which correlates with temperatures characteristic of this time by most researchers [20].

The section is crowned by late Pleistocene, 26,000–43,000 years old, 15–20-m-thick unit of alluvial Icy complex that was frozen upon deposition. The presence of ice veins in this overlying layer (Fig. 1) indicates that neither the Icy complex nor the brine-containing marine layer has ever been thawed. At past and present ground temperatures, fine-grained sediments are firmly bound together by ice. Due to the high ice content (up to 60%), pores are completely filled with ice, excluding any migration within the stratum and below the seasonally thawing layer, which is less than 0.5 m; therefore, infiltration into the cryopegs is precluded. Although unfrozen water films in the Icy complex loams vary 3–5%, the thermal diffusion of cells within these films is impossible. The cells are adsorbed on the surface of mineral particles, and the size of cells found in brines (0.3–1.5–7 μm) is not comparable with the thickness of these films, which, according to Andersen [21], are only 10^{-3} μm. Thus, the microorganisms in cryopegs could not have penetrated into the brine from the overlying sediments, but have been inherited from the marine sediments and were present in situ.

2. Materials and methods

The permafrost cores were aseptically collected in 1999 from boreholes by dry rotary drilling without any solutions or chemicals. The strict protocol for the drilling, sampling, storage, transportation, and handling were designed to ensure recovery of uncontaminated material [22]. When the drill entered brine, water was collected with a sterile bottle placed inside a core device. Nevertheless, due the drilling procedure, this water was contaminated with some debris particles from the overlying layers (the presence of HCO_3^- ion is the direct evidence of this) with their attached microorganisms, i.e., the recovered brine contained not only indigenous, but also exogenous microflora. Therefore, the first goal was the identification of cryopeg aborigines. The contribution of contaminating bacteria from the overlying soils in cryopeg was estimated by the incorporation of ^{14}C from glucose into the cryopeg microbial biomass [9]. The metabolic activity measured in the autoclaved brine inoculated with the drilling contaminants was extremely low in comparison with the metabolic activity of native brine. Consequently, the exogenous microflora that contaminated the brine due to drilling had minimal metabolic activity. The obtained results have shown that at least the part of microbial community in cryopegs is a native-born and formed the background for all study.

2.1. Enumeration and isolation

The brine was stored in a refrigerator at −10 °C. The number of bacteria was determined by direct counting of cells stained with propidium iodide on membrane filters [23]. The stained cells were counted using a Lumam fluorescence microscope with excitation and emission filters. Diversity and enumeration of viable aerobic bacteria were obtained by direct plating on R2A and starch ammonium agar at 4, 10 or 22 °C. Isolation of bacterial and mycelial pure cultures were described earlier [9–12]. Anaerobic acetogens, methanogenes, heterotrophs and sulfate reducers were detected by most probable number (MPN) method following Hungate technique [24] and saturated cultivation on a liquid nutrient medium [12].

For yeast isolation, the brine were inoculated on malt agar (MA) and 1/3 MA, with sodium pyruvate added in some cases. For the definition of amount and isolation of fungal strains, water for inoculation was concentrated by filtration through sterilized filter. Then filters were cut and transferred to a tube with sterilized water. For better resupsension of cells, the tube was shaken with a vortexer. Inoculation was conducted on organic malt extract agar (MEA) and synthetic Czapek media with different concentrations of sucrose. For isolating halotolerant fungi, NaCl was used at concentrations of 1%, 5% and 20%. In both, fungi and yeast cases, lactic acid was added to media to suppress the undesirable growth of bacterial cells. The inoculated plates were incubated at 4 and 26 °C and the grown colonies were examined and enumerated after 1 month. To ensure sterility of air in the microbiological box, open Petri dishes with agar medium were exposed to the air and then were incubated at 4 and 26 °C. To ensure sterility of filters, they were inoculated on agar plates and incubated at the same conditions. In the control on dishes, the fungi were not found out.

2.2. Phenotypic tests

Phenotypic tests to determine the key taxonomical characteristics of bacteria: Gram's stain, the presence of oxidase and catalase, and glucose fermentation were determined by standard methods [25]. DNA was isolated by Marmur [26] method and analyzed for nucleotide composition by the method of thermal denaturation using a Pye Unicam SP1800 spectrophotometer.

Bacterial growth was measured by optical density at 600 nm using Specol-221 spectrophotometer. Actively growing liquid culture was 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). Effects of freezing-thawing were tested by placement of actively growing cultures in a freezer at −40 °C for 1 month. In both cases, the growth was monitored during incubation under optimal conditions for each strain.

The genera of isolated yeasts and micromycetes were identified based on their cultural, physiological, morphological and biochemical characteristics using the respective manuals [27–30]. The influence of temperature on growth of *Geomyces* strains was tested on MEA after 7 days incubation at a range of 2–30 °C. To test the effect of NaCl on the growth of *Geomyces* strains they were inoculated on Czapek agar (without dilution and diluted in 10 times) with increasing concentration of NaCl (0%, 10% and 20%) and incubated at 4 and 26 °C. The colony's diameter was measured after 1 month. Spore germination was observed microscopically (160×).

2.3. Microscopy

Bacterial cell morphology was examined using ultrathin sections. Bacterial cells were pre-fixed with 1.5% glutaraldehyde in cacodylate buffer (pH 7.2) at 4 °C, washed thrice in the same buffer, and refixed in a 1% solution of OsO_4 in the buffer at 20 °C. The sections were dehydrated in a series of alcohol solutions of increasing concentration, embedded in Epon 812 epoxy resin, mounted on a grid, and contrasted in a 3% solution of uranyl acetate in 70% ethanol and then with lead citrate at 20 °C. Ultrathin sections were examined in a JEM100 electron microscope.

2.4. DNA sequence analyses

The phylogenetic relationship between strains 2pS, 1pS, and the reference strains was studied using 16S rRNA gene sequences by distance matrix, parsimony, and maximum likelihood analyses. The 16S rRNA gene was amplified by PCR using prokaryotic 16S rRNA primers 27f and 1492r. The PCR product was purified by Wizard PCR Preps DNA Purification Systems. The sequencing reactions were performed using a CEQ Dye Terminator Cycle Sequencing kit and automatic DNA sequencer CEQ2000 XL according to protocols provided by manufacturer. The nucleotide sequences of strain 2pS and 14D1 have been deposited in the GenBank database under respective Accession Nos. AF5177557 and AY117755. The isolates 14D1, 1pS and 2pS have been deposited in Russian Collection of Microorganisms (VKM B-2271, VKM B-2269 and VKM B-2270, respectively).

The samples of yeast for PCR were prepared as described by Danilevich and Grishin [31]. For the amplification of LSU rRNA gene fragments primers 5.8SR and LR3 [32] were used. PCR was performed with Taq DNA polymerase (Fermentas, Lithuania) at Perkin–Elmer Cetus 480 thermocycler for 35 cycles (45 s at 95 °C, 45 s at 55 °C and 1 min at 72 °C) with final incubation at 72 °C for 5 min. The amplified DNA fragment was purified by electrophoresis in 1% agarose in TAE buffer. The resulting fragment was sequenced with LR3 primer and CycleReader DNA Sequencing Kit (Fermentas, Lithuania) according to manufacturer's instructions. Yeast species' diversity was identified by comparing rRNA gene sequences (D2 fragment of 26S rRNA gene) with the GenBank database using BLAST program (http://ncbi.nlm.nih.gov/BLAST/) [33].

3. Results

3.1. Biodiversity of viable bacteria

The total number of microorganisms found in cryopegs, 10^7 cells/ml, is close to that of common aquatic habitats [34] and frozen sediments [35]. The population of culturable aerobic bacteria in the brine, where 80–95% of cells were cocci and coccibacilli, varied from hundreds to hundreds of thousand cells/ml of water. The numbers of anaerobic microbes varied from tens (acetogens) to 2×10^6 cells/ml (halophilic sulfate reducers). The number of heterotrophs was maximal (2×10^2 cells/ml) in a mineral medium with glucose as a sole carbon source incubated at 5 °C, whereas their number at 18 °C was an order of magnitude lower (2×10^1 cells/ml). The largest number of methanogens (10^2 cells/ml) was detected in a medium with acetate. Halophilic acetogens, heterotrophs, and methanogens were not detected. When brine waters were incubated with heterotrophic media under a N_2 atmosphere at 5 °C, spore-forming rod-shaped cells were the dominant forms of the psychrophilic community present.

In Bakermans et al. [10], 46 bacteria were isolated from cryopegs by cultivation on R2A at 4, 10, or 22 °C and represented 17 distinct patterns or phylotypes (as determined by RFLP analysis of the 16 S rRNA genes). Sequence analysis of the 16S rRNA genes showed that the 17 phylotypes were most closely related (>95% sequence similarity) to the following 9 genera: *Psychrobacter*, *Arthrobacter*, *Frigoribacterium*, *Subtercola*, *Microbacterium*, *Rhodococcus*, *Erwinia*, *Paenibacillus*, and *Bacillus* [10]. Most isolates were related to common soil organisms; while eight isolates were related to known psychrotolerant strains of bacteria often found in Polar water environments. For example, species of *Psychrobacter*, *Frigoribacterium*, *Paenibacillus*, and *Rhodococcus* have been isolated from Arctic and Antarctic seawater [36–38]. Counts made from plates used for the isolation indicated that isolate 5, a species of *Psychrobacter*, was the most abundant isolatable species at a concentration of $\sim 2 \times 10^3$ cells/ml.

3.2. Aerobic cocci and coccobacilli

Pure cultures of the aerobic strains were isolated following cultivation on solid media with acetate in aerobic and microaerophilic conditions at temperatures of 18–22 °C (Table 1). Cells of strains were represented by gram-negative non-motile cocci 1–1.5 μm (strain 2pS) and coccobacilli 1–1.5 × 2–3 μm (strain 1pS). As shown by thin section, cells of strains 1pS and 2pS (Fig. 2) had a densely packed cytoplasm and black granules similar to polyphosphate. The G+C contents of the DNA of strains 1pS and 2pS were similar (Table 1). According the DNA–DNA hybridization analyses the strains 1pS and 2pS were close (a reassociation level was 89%), suggesting that they belong to the same species. 16S rRNA sequencing (1478 nucleotides) of strain 2pS was conducted. As was shown by phylogenetic analysis (Fig. 3) the strain was closely related (97% similarity) to *Psychrobacter glacincola*, a halotolerant psychrophilic bacterium isolated from Antarctic sea ice [39]. New strains grow at temperatures −5 to 35 °C with optimal growth at 16–18 °C. They grow on mono- and dicarboxylic acids under aerobic and microaerophilic conditions, as well as at subzero temperatures. Based on phylogenetic properties, we consider strains 1pS and 2pS as a new species of the genus *Psychrobacter*.

3.3. Anaerobic sporeforming bacterium

A pure culture of a polymorphic, Gram-positive, motile, rod-shaped bacilli (1.5 × 7 μm) with a central endospore was obtained by successive transfer of single

Table 1
Characteristics of new *Psychrobacter* strains from Arctic cryopegs

Characteristic	Strains		
	1pS	2pS	5
Morphology	Coccobacilli	Cocci	Coccobacilli
Gram stain	G^-	G^-	G^-
Motility	–	–	–
Anaerobic growth	–	–	–
Catalase	+	+	nd
Oxidase	+	+	+
T (°C)			
Optimum	18–20	16–18	22–24
Range	2 to 35	−5 to 35	−10 to 30
Growth with 3% NaCl	+	+	+
Substrates	Acetate, lactate	Acetate, lactate	Citrate, glutamate
Major fatty acids	$C_{18:0}$, $C_{18:1c9}$, $C_{18:1}$OH	$C_{18:0}$, $C_{18:1c9}$, $C_{18:1}$OH	$C_{18:1\omega7c}$, $C_{16:1\omega7c}$
Growth factors	–	–	nd
G+C (mol%)	46.4	46.0	44

nd, Not determined.

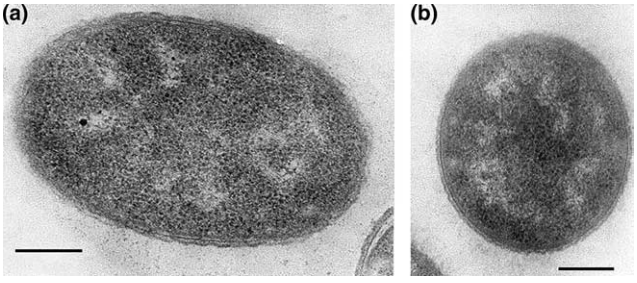

Fig. 2. Ultrathin sections of *Psychrobacter* strain cells: (a) 1pS; (b) 2pS. Scale bar is 5 μm.

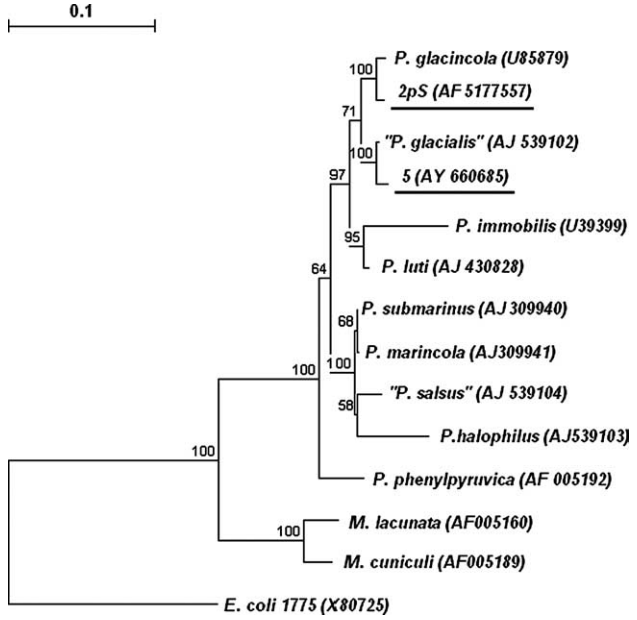

Fig. 3. Positions of the new *Psychrobacter* sp. strains among closely related species based on 16S rRNA phylogenetic analysis. The accession numbers of strains are shown in parentheses. Tree was constructed with neighbor-joined method by using the Jukes–Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

colonies to glucose-containing liquid medium and named 14D1. Ultrathin section of strain 14D1 showed that cells have a large number of reserve substances, probably polysaccharides and lipids (Fig. 4). This strain grew at temperatures ranging −5 to 18 °C with optimal growth temperature at 5 °C indicating that the strain is an obligate psychrophile. The bacterium did not contain catalase and oxidase, or ferment di- and polysaccharides under strongly anaerobic conditions. The G+C content of the DNA was 31.4 mol% and phylogenetic analysis showed association with members of the genus *Clostridium*. The nucleotide sequence of the 16S rRNA gene of strain 14D1 (1460 nucleotides) is closely related to that of a species of *Clostridium* isolated from Antarctic bacterial mats [40], and was 98% similar to *Clostridium frigoris*, *Clostridium bowmanii*, *Clostridium lacusfryxellense*, and *Clostridium estertheticum* (Fig. 5). The new strain differs from other species of the genus by growth substrate, fermentation products, and fatty acid content of the cell membrane. We consider this strain as new specie of the genus *Clostridium* [41].

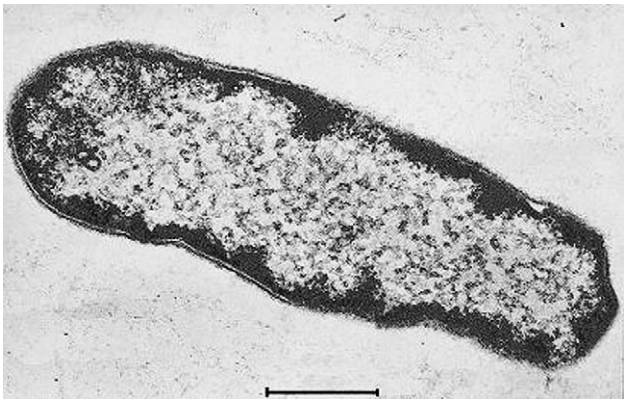

Fig. 4. Ultrathin sections of *Clostridium* sp. cell.

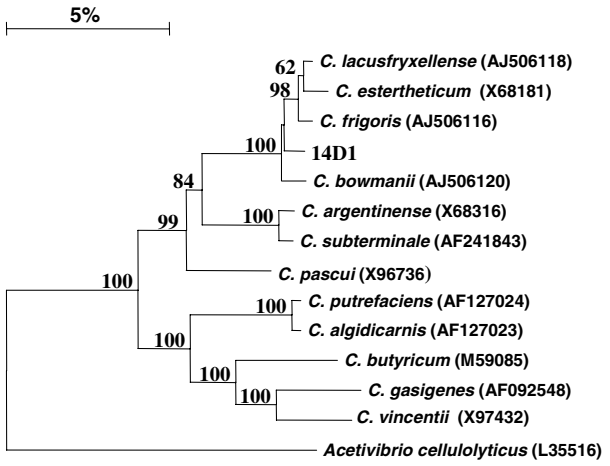

Fig. 5. Position of the strain 14D1 among closely related species of the genus *Clostridium*, based on 16S rRNA phylogenetic analysis. The accession numbers of type strains are shown in parentheses. Tree was constructed with the neighbor-joined method by using the Jukes–Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

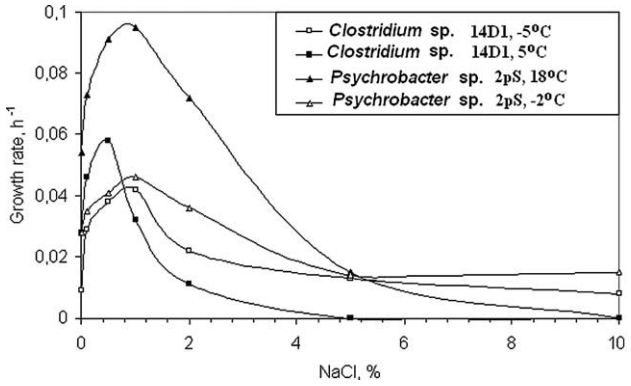

Fig. 6. NaCl effect on the cryopeg's isolates growth.

3.4. Physiological features of isolated bacteria

In consideration of the characteristics of the habitat, we examined relevant bacterial characteristics; such as: growth temperature, tolerance to salinity in the culture medium, and correlation between substrate choice and growth temperature. Strains 14D1, 2pS, and 5 were able to reproduce at a low rate at −2 and −5 °C. Strain 14D1 can be considered a true psychrophile, while the *Psychrobacter* strains were psychrotrophs more than psychrophiles because of their growth above 20 °C. After being frozen at −40 °C for 1 month, both psychrotrophic strains 1pS and 2pS and the psychrophilic strain 14D1 have been growing at the optimal conditions without a lag-period.

Both the optimal and the acceptable growth concentrations of NaCl increased as the growth temperature decreased from positive to negative temperatures. The effect of NaCl on the growth of strain 14D1 was tested at the optimal growth temperature (5 °C) and at temperature close to the natural temperature of the habitat (−5 °C) [9]. The effect of NaCl concentration on *Psychrobacter* sp. growth was studied on strain 2pS at 18 and −2°C. The temperature decrease was accompanied by an increase of the salt concentration (Fig. 6), which did not inhibit microbial growth. Interestingly, the salinity optimum of strain 14D1 shifted at the lower cultivation temperature.

Since cryopegs are characterized by low organic carbon content (0.046%) [9], we studied the dependence of the growth rate on the concentration of the carbon source glucose. Increasing the concentration of glucose from 0.01% to 0.025% at the optimal growth temperature of 5 °C caused the growth rate of strain 14D1 to double; while further increases in glucose concentration led to decreases in growth rate [12]. The ability of isolates to utilize a variety of organic compounds as sole carbon and energy sources was tested at three temperatures, including non-optimal temperatures [12]. *Clostridium* strain 14D1 used L-glutamate only at −2 °C, did not use xylan and cellobiose at 18 °C, but did grow on these substrates at 5 and −2 °C. *Psychrobacter* strain did not use D-glucose, sucrose, trehalose, L-glutamate and L-alanine substrates at the optimal growth temperature (18 °C) but grew on these compounds at minimal growth temperatures. Traditionally, the ability of bacteria to utilize various substrates as carbon and energy sources is determined under optimal growth conditions. When cultivation temperature approached that of the habitat, the spectrum of utilized substrates expanded, enhancing bacterial chances to survive in this econiche.

The lipid content of cell membranes is an important index of adaptation to growth at both sub-zero temperatures and high salinities. Analysis of cell membrane lipids of strains 1pS, 2pS and 14D1 detected the presence of fatty acids, aldehydes, hydroxy-acids with even number of atoms from C_{14} to C_{18} [12]. The lipids of strain 14D1 are characterized by the prevalence of monounsaturated hexadecanoic acid $C_{16:1}$ with the double bond positioned at the 9th carbon (37.0%) and myristamic acid $C_{14:0}$ (32.6%). In the cell membranes of both strains of *Psychrobacter*, we have found significant quantities of hydroxy-acids with various lengths of carbon chains (30.8–34.0%). Membranes with a higher content of unsaturated fatty acids function better under low temperature conditions [42].

3.5. Mycelial fungi

Viable mycelial fungi were isolated from cryopegs at incubation temperatures of 4 and 26 °C. Their number was small, from 1.4 to 400 CFU/ml of water. The forty fungal strains isolated represented 12 different taxa, mostly Anamorfic fungi (Table 2). *Alternaria alternata*,

Table 2
Biodiversity of viable fungi in Arctic cryopegs

Taxa	Media				Ratio of colonies diameter at different salinity and temperature	
	Hole 14/99		Hole 15/99		Control/test[a]	Control/test[b]
	MA	Cz	MA	Cz		
Alternaria alternata (Fries: Fries) von Keissler		Y		Y	1.07	3.22
Aureobasidium pullulans (de Bary) Arnaud var. *pullulans*			Y		1.03	6.67
Cladosporium herbarum (Persoon: Fries) Link	Y	Y	Y		1.20	0.87
Geomyces pannorum (Link) Sigler et Carmichael var. *pannorum*	Y		Y	Y	1.05	0.48
Geomyces vinaceus Dal Vesco				Y	0.97	0.41
Basidiomycetes spp.	Y		Y		nd	nd
Penicillium aurantiogriseum Dierckx	Y				0.97	2.24
Penicillium verrucosum Dierckx			Y		0.91	1.06
Penicillium minioluteum Dierckx	Y		Y		1.01	4.0
Ulocladium botrytis Preuss			Y		1.08	nd
Valsa sordida Nitschke	Y				nd	nd
Verticillium sp.			Y		1.04	2.02

nd, Not determined.
[a] Average diameter of fungal colonies on media without NaCl to the same on media with 1% NaCl.
[b] Average diameter of fungal colonies incubated at 25 °C to the same at 10 °C.

Aureobasidium pullulans, *Cladosporium herbarum*, *Geomyces pannorum* var. *pannorum*, *Geomyces vinaceus*, *Penicillium aurantiogriseum*, *Penicillium minioluteum*, *Penicillium verrucosum*, *Ulocladium botrytis*, *Valsa sordida*, *Verticillium* sp. were identified [11]. All isolated organisms were capable of developing on nutrient media with 1% NaCl; a decrease in growth rate on media with salt, in comparison with control media (without salt), was not observed. For species with low growth rates, *C. herbarum* and *P. verrucosum*, distinctions between development at 10 and 25 °C were not observed; however, the representatives of genera *Alternaria*, *Aureobasidium*, *Verticillium* and other species of *Penicillium* exhibited sharply reduced growth rates at 10 °C.

Micromycetes of the genus *Geomyces* (*G. pannorum* var. *pannorum* and *G. vinaceus*) were the most common isolates (75% of all isolates) from cryopegs. Only strains of *G. pannorum* var. *pannorum* were isolated on nutrient media with 5–20% salt. The tolerance of these fungi to low temperature and high amounts of NaCl was studied. Ten strains of *G. pannorum* from various habitats were studied: four were isolated from cryopegs and two from permafrost of the same drill holes. As a control, two stains from soils of central Russia and two strains from non-saline Antarctic permafrost were studied. The concentration of NaCl tolerated differed among the various strains of micromycetes. All tested strains had lower growth rates at 26 °C than at 4 °C (by the end of one month the average ratio of colony diameters 26/4 °C was 0.77 for rich medium and 0.71 for diluted medium), and high tolerance to NaCl. Microcolonies or germ tubes formed on all types of media with 10% salt at 4 °C (Fig. 7). At 26 °C, salt-tolerance was established only on rich media – the average ratio of colony diameters 26/4 °C for salt media decreased to 0.51. It was found that one of the cryopeg strains formed well-advanced germ tubes on rich medium with 20% salt at low temperature. The control strains were not tolerant

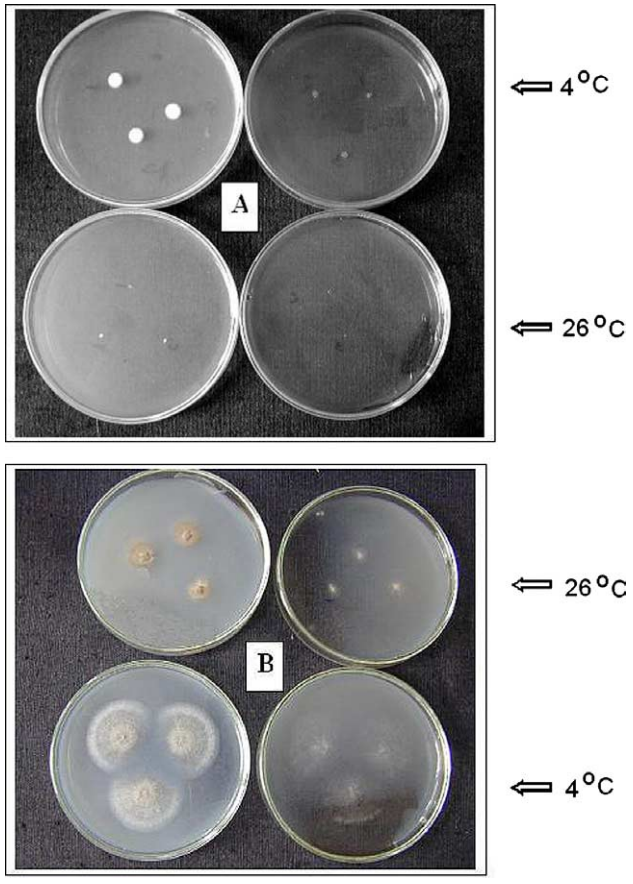

Fig. 7. Growth of the strain *Geomyces pannorum* var. *pannorum* on the media with 10% (A) and without salt (B). The growth on rich media is submitted in the left column, on pure media – right column.

to salt and their spores did not germinate when NaCl was added to media. Controls had higher growth rates at 26 °C than at 4 °C (the average ratio of colony diameters was 1.64–1.77 for rich medium). The strains from non-saline permafrost also did not form germ tubes on media with NaCl.

3.6. Yeast

Viable yeast were isolated from 3 of 4 cryopeg samples represented mesophilic organisms and had maximum growth temperatures of 28 °C, except for the ascomycetes isolate *Pichia guilliermondii*, which was characterized by a maximum growth temperature of 42 °C. At room temperature, colonies formed within 3–7 days and their numbers ranged from 0.3×10^1 to 0.4×10^3 CFU/ml. Four yeast isolates were found and were related to the species *Cryptococcus victoriae* (isolate 01/00), *Cryptococcus laurentii* (01/03-01), *Debaryomyces hansenii* (04/00) and *P. guilliermondii* (03/00).

4. Discussion

Over 75% of the Earth's biosphere is permanently cold and representatives of *Eukarya*, *Bacteria* and *Archea* can live at temperatures just below 5 °C [43,44]. There are two known classes of microorganisms capable of growing at temperatures below 5 °C. The first class, adapted to stable cold conditions, is primarily represented by psychrophilic bacteria isolated from sea water and marine sediments. The second class is represented by microorganisms that inhabit environments with unstable cold conditions. A previous study failed to isolate psychrophilic bacteria from permafrost [9]. However, the bacteria isolated from cryopegs were both psychrophilic and psychrotrophic. Psychrophiles are typically isolated from cold water environments rather than frozen soils [45]; this effect may explain the isolation of psychrophilic bacteria from cryopegs rather than permafrost. According to the definition of Morita [46], the isolated *Clostridium* sp. strain 14D1 is an obligate psychrophile. Based on phenotypic properties, it is closely related to the psychrophilic *C. lacusfryxellense*, which was isolated from an Antarctic microbial mat [40]. It has the lowest known minimal growth temperature (−43 °C) as predicted by the method of Ratkowsky et al. [47]. Almost all known representatives of the genus *Psychrobacter* are marine inhabitants [48,49]. Based on their phenotypic and genetic characteristics, the isolated *Psychrobacters* are closely related to *P. glacincola*, halotolerant psychrophiles found in marine ice of Antarctica [39]. *Psychrobacter* sp. 1pS and 2pS are psychrotrophic bacteria with psychrophilic properties that are able to slowly develop at −2 and −5 °C. Indeed, *Psychrobacter* sp. 5, isolated from a cryopeg, was similarly tolerant to low-temperature conditions and found to be psychrotrophic, but not psychrophilic [10]. For example, *Psychrobacter* sp. 5 reproduces at temperatures of −10 to 30 °C, has its maximum growth rate at 22, and remains active at −20 °C [10,50]. However, similar to true psychrophiles, *Psychrobacter* sp. 5 maximized growth yield at low temperatures and did so by streamlining growth processes at its critical (4 °C) temperature [10,51]. Slow growth at low temperatures is typical of permafrost microorganisms; for example, a strain of *Psychrobacter* that was isolated from the overlying late Pleistocene sediments has the same temperature range for growth as *Psychrobacter* strains isolated from cryopegs [52].

As incubation temperature decreased, the incorporation of ^{14}C from glucose into the cryopeg microbial biomass decreased, but remained significant (8.500 cpm) even at −15 °C [9]. Therefore, subzero temperatures themselves do not exclude biochemical reactions and the observed consumption of labeled glucose provides reason to conclude that microorganisms are metabolically active in cryopeg. This strategy does not accept that cells can multiply in situ. Measuring the kinetics of resazurin reduction in several cryopeg isolates showed their ability to reduce resazurin at −10 °C but not reproduce [10]. These facts indicated that survival was possible without reproductive growth. At the same time, several isolates could also reproduce at −10 °C with generation times ranging from 39 to 257 days [10].

A novel habitat for psychrophiles was recently described at depths near 3600 m, close to the surface of the large subglacial Lake Vostok [53]; here liquid veins exist due to the presence of dissolved ions and have been proposed to support at least one cell/cm^3. Microorganisms live within a great range of salinities from essentially distilled water to saturated salt solutions; halophilic bacteria remain viable at 80 °C in the presence of 25% NaCl and extreme halophiles require NaCl concentrations above 15.6% (w/v) for growth [54,55]. Hypersaline waters are defined as having salt concentrations greater than that of sea water (3.5% w/v). The salinity of cryopegs is 15–20% and a characteristic property of the microbial community of cryopegs is the presence of halophilic microorganisms that have never been isolated from permafrost sediments. The salt tolerance of these organisms is often associated with cold tolerance. Indeed, the lipid content of cell membranes is an important index of adaptation to growth at both subzero temperatures and high salinities, and in strains 1pS and 2pS, lipids were high in hydroxylated fatty acids, which indicated their adaptation to high salinity [56].

Traditionally, the metabolism and physiology of bacteria – including their ability to utilize certain substrates as carbon and energy sources – is investigated at optimal growth conditions. When cultivation temperatures approached that of the habitat, the spectrum of substrates

utilized by the isolates expanded, enhancing the ability of bacteria to survive. These data corroborate results obtained earlier for deep-sea bacteria of the genera *Alteromonas*, *Bacillus*, and *Vibrio* [57]. Based on changes in the ratios of metabolic products, the metabolism of strain 14D1 also changed as cultivation temperature decreased. Presumably, at low temperatures most of the energy is spent on maintenance and population growth. Additional adaptation to in situ conditions was evidenced by the ability of strain 14D1 to grow more rapidly on lower concentrations of glucose, indicating that strain 14D1 is adapted to the low nutrient content of the natural ecosystem [58]. Spore-formation was a response of the new bacterium *Clostridium* sp. 14D1 to the absence of nutrients in the medium. Finally, the isolates may be members of the same trophic chain: anaerobic strain 14D1 produces lactate and butyrate, while the aerobic strain 2pS utilizes these substrates as carbon and energy sources.

The long-term survival of both aerobic and anaerobic bacteria in permafrost sediments, based on the detection of several metabolic reactions, suggests that these microbes can cope with various stresses which include: DNA damage resulting from background soil radiation, cell membrane damage, and other stresses to vital functions that maintain cell viability [59,60]. Among fungi isolated from cryopegs, *Geomyces* strains were the most numerous. One of the first isolates of *Geomyces* from the extreme Antarctic habitats was made more than 40 years ago and named *Crysosporium verrucosum* [61]. Subsequently, *C. verrucosum* was renamed *G. pannorum* var. *pannorum*; strains of *G. pannorum* show marked growth at temperatures below 0 °C after 1 month [62]. As reported by Finotti et al. [63], a strain of *G. vinaceus* that was isolated from Antarctica has an optimum growth temperature optimum near 4 °C. Tubaki [61] considered that *G. pannorum* could be included with fungi that are adapted to cold conditions and possibly can survive also in permafrost. According Tubaki for survival in extreme conditions, for Antarctic and Arctic fungi the slow grow that low temperatures (near 0 °C) could be more significant than fine growth at optimum (20 °C) temperature. *Geomyces* strains isolated from cryopegs had their optimum at 10 °C, but could form germ tubes at 2 °C, whereas, the optimum temperature for extension rate of *G. pannorum* var. *pannorum* ranged 20–25 °C [29]. In our experiments, the decrease of temperature promotes higher activity growth rates of colonies. The increased contents of organic substances promote more active germination of conidia in the given extreme conditions.

Geomyces strains grew and germinated on media with 10–20% NaCl. The *Geomyces* strains isolated from cryopegs are not true halophilic micromycetes; however, they are better adapted to the conditions of cryopegs (high salinity and low temperature) than other fungi. As to the other species isolated in this study, it is necessary to note that most of them were isolated earlier during study of Arctic and Antarctic permafrost [64]. Fungi are also known to grow at NaCl concentrations near 27% [65]. Recently, osmophilic micromycetes of the genus *Wallemia* and halophilic black yeast have been isolated from salterns [66,67]. Obligatory halophilic fungi from the order *Thraustochytriales–Thraustochytrium pachydermum* and *Schizochytrium aggregatum* were discovered in winter pools that are created by the application of crystalline salt (of various origins) to roads and sidewalks in order to melt ice [68]. Among the microorganisms cultivated from a Vestfold Hills hypersaline lake were one species of extremely halophilic archaea and one halophilic alga. Several other species were present, as indicated by molecular analysis, but could not be isolated by the methods used [69]. Thus, application of updated methods for the isolation and cultivation of mycelial fungi could expand the list of fungal species isolated from extreme habitats.

Most of the yeast isolates belonged to the *Basidiomycetes* class. This is not surprising, because *Basidiomycetes* are adapted to harsh conditions and have been isolated from cold regions [70–72]. The ascomycetes isolate *P. guilliermondii* is a cosmopolitan organism and its discovery was not unusual. *C. victoriae* and *C. laurentii* are known as inhabitants of Antarctica and other regions with harsh climates [73]. The isolate 04/00, *D. hansenii*, is of special interest because a characteristic feature of its physiology is resistance to NaCl; it is known to tolerate salinities ranging 0–24% [74]. In addition, the presence of salt improves the performance of *D. hansenii* under stress conditions: low temperature and low pH [75,76].

5. Conclusion

Survival of microorganisms in a low-temperature, high-salt aquatic environment on a geological time scale indicates unknown microbial adaptation. At the same time, the presented results corroborate the available data on the presence of viable organisms in permafrost and saline ecosystems, and expand our knowledge in this area.

The isolated bacteria not only were adapted to subzero temperatures – that is, they survived freezing, grew at subzero temperatures, and expanded the spectrum of consumed substrates as temperature decreased – but they were also tolerant to the high salt concentrations. What is more, the microorganisms detected in cryopegs (some of them novel species) are halophilic and psychrophilic organisms at the same time (sulfate reducers, for example) that have never been isolated from natural

habitats. In the cold saline conditions of cryopegs, special communities were formed.

The fungi representative of these communities are also capable of developing at the low temperatures and high concentrations of salt. The *Geomyces* strains were typical members of this community. The presence of *G. pannorum* var. *pannorum*, and its growth temperature range, allows one to speculate that the *Geomyces* isolates are capable of not only surviving at high salinity and low temperature, but also of actively living in cryopegs.

The study of physiological characteristics of microorganisms at subzero temperatures is complicated due to the low rate of metabolic processes. Nevertheless, active adaptation to low temperatures of already studied bacteria gives hope that fully active and reproducing bacteria can be discovered in saline habitats at subzero temperatures.

Communities such as those found in cryopegs might be found in Lake Vostok, since both ecosystems became isolated in the mid Pleistocene [2] and under overcooled conditions. However, Lake Vostok may have less salt and fewer energy resources. At Lake Vostok, concern over contamination of the lake by entry of the drill and the associated well fluids into the water has presently halted drilling until a means is devised to sample Lake Vostok waters while preventing contamination. In the meantime, studies of the microbiota and ecosystem of the cryopegs, which are less costly and with less concern for contamination, could advance knowledge on such systems.

From the astrobiological perspective, water brines provide the only opportunity for liquid water within the Martian subsurface permafrost [77]. Arctic cryopegs, preserved dozens to hundreds of thousands of years, represent a terrestrial model of such exobiological niches with their unique halotolerant, aerobic and anaerobic, psychrophilic community – a plausible proxy for Martian microbial life. Bacteria isolated from cryopegs in permafrost may be a useful model for studies of the adaptation strategies of biological objects to subzero temperatures.

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