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Novel psychrophilic anaerobic spore-forming bacterium from the overcooled water brine in permafrost: description *Clostridium algoriphilum* sp. nov.

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Abstract A gram-positive, motile, strict anaerobic spore-forming bacterium was isolated from the overcooled brine in the permafrost. The optimal temperature for isolate growth was 5–6°C at pH 6.8–7.2. The bacterium was growing on the medium rich in saccharides and disaccharides. Out of polysaccharides tested, only xylan sustained the growth. Fermentation of the hexoses led to the formation of acetate, butyrate, lactate, H_2,CO_2 and some formate and ethanol. Cell wall peptidoglycan contained *meso*-diaminopimelic acid. The major fatty acids of the cell wall were $C_{14:0}$ and $C_{16:1c9}$. The content of G-C pairs in DNA was 31.4 mol%. As phylogenetic analysis has shown, it is closely linked to the members of cluster 1 of *Clostridium*. It differs from the other species of the genus by the substrates necessary for the growth, products forming as a result of the fermentation and content of the fatty acids in the cell wall. Thus, it was suggested to describe this strain as a new species named *Clostridium algoriphilum*. Type strain 14D1 was deposited into the Russian Collection of the Microorganisms VKM B-2271^T and German Collection of the Microorganisms DSM 16153^T.

Keywords *Clostridium algoriphilum* · Water brines · Adaptation · Psychrophile · Permafrost

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Introduction

Most of the Earth has temperature that either often or constantly is below 5°C. This explains the increased interest in the study of the activity of different microorganisms viable under subzero temperatures. Previous researches have shown that naturally cold econiches are inhabited by psychrophilic and psychrotolerant representatives of Eucarya, Bacteria and Archea (Cavicchioli and Tomas 2000). Over the last 5 years, there has been an increased number of scientific publications concerned with the microorganisms adapted to the cold (Scherer and Neuhaus 2003). An increased interest in the cold adaptation involves studies of cold shock response as well as description of microorganisms mainly from permanently cold habitats. Known anaerobic true psychrophilic microorganisms were isolated from various Antarctic ecosystems (Mountfort et al. 1997; Spring et al. 2003), tundra soils (Simankova et al. 2000), marine sediments (Knoblauch et al. 1999) and frozen products stored in vacuum packs (Collins et al 1992).

In the Arctic, the water systems of marine origin (cryopegs) were found in the 100–120,000 years old permafrost at the depth of several tens of meters in the

form of sodium-chloride water brines with the constant temperature of (−9)–(−11)°C and mineralization of 170–300 g/l (Gilichinsky et al. 2003). Microbiological analysis of the Arctic cryopegs has shown that these isolated econiches were the habitats for microorganisms with different types of metabolism (Gilichinsky et al. 2003). The objective of the present study was to isolate and describe psychrophilic anaerobic bacterium from the samples of low-temperature brines found in the permafrost of Kolyma Lowland.

Materials and methods

Source of isolate

Cryopeg water samples were obtained from the Yakutskoe Lake area during the field expedition of 1999 in the tundra of Kolyma Lowland (69°50′N 159°30′E.). The drilling system and protocol for the subsequent handling of permafrost cores were designed to ensure the recovery of uncontaminated material (Gilichinsky et al. 1989; Shi et al. 1997). Until the laboratory analysis, water was kept frozen (−20°C).

Media and cultivation conditions

Anaerobic techniques of Hungate (Hungate, 1969) were used for the new strain isolation. To obtain an enrichment culture, a medium of the following composition was used (g/l): KH_2PO_4- 0.7; K_2HPO_4-0.7; NH_4Cl-0.5; $MgSO_4{\times}7H_2O$-0.1; NaCl–1.0; Na ascorbate-1.0; glucose-2.0; pepton-2.0. 10 ml of medium and 1 ml of cryopeg water were put into the Hungate tubes, incubated in the dark at 5°C for three months until bacterial turbidity ($OD_{600} = 0.6$). Pure strain 14D1 was maintained and cultivated in liquid medium containing (g/l): KH_2PO_4- 0.7; K_2HPO_4-0.7; NH_4Cl-0.5; $MgSO_4{\times}7H_2O$-0.1; NaCl-5.0; Na ascorbate-1.0; glucose–2.0. Colonies of the strain 14D1 were grown in the same medium with the addition of 20 g/l Difco agar (USA) on Petri dishes placed in the Oxoid anaerostats. Both enrichment and pure cultures were grown in 15-ml Hungate tubes in 10 ml of the medium under nitrogen (100%) atmosphere. Syringes were used to perform all manipulations.

Growth measurements

Growth was measured by optical density at 600 nm using "Specol-221" (Germany) spectrophotometer. Cell doubling time (t_d) was defined as the time of optical density doubling in the exponential phase of growth.

Resistance to heat and freezing, and spore formation

Actively growing liquid culture was tested for resistance to heat by incubation at 20, 30, 37°C (for 24 h) and at 50, 60, 70 and 80°C (for 10 min in the water bath). Following the treatments, growth was monitored during incubation under optimal growth conditions.

Bacterium viability at subzero temperature was tested by placing the actively growing liquid culture in a freezer at −40°C for 2 months. Following the temperature increase of up to 5°C during 30 min, the culture was transferred to fresh media and incubated under optimal growth conditions.

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

Light microscopy

Cell morphology was examined under light microscope Opton ICM 405 (Germany) using phase-contrast at a magnification of 100×3.5.

Electron microscopy

Ultrathin sections Bacterial cells were pre-fixed with 1.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4°C for 1 h, washed thrice in the same buffer and refixed in a 1% solution of OsO_4 in the buffer at 20°C for 4 h. 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 (Reynolds 1963) at 20°C for 4–5 min.

Electron-microscopic cryofractography Bacterial cells were frozen in liquid propane cooled by a surrounding vessel of liquid nitrogen, using attachments to a JEE-4X set-up for vapour-phase deposition and fractured when a vacuum of $3{\times}10^{-4}$ Pa and temperature of −100°C were reached. The surface of the fracture was contrasted with a platinum/carbon mixture at an angle of 45° and fixed by deposition of a carbon layer at an angle of 90°. To detect flagella, cells were negatively stained by 0.1% solution of phosphotungstic acid (Serva) pH 7.0. Ultrathin sections, negatively stained samples and replicas were examined in the JEM100 electron microscope (JEOL, Japan) at the accelerating voltage of 80 kV.

Effects of pH, temperature and NaCl concentration

Effect of pH on the growth was measured at 6°C pH of the basic medium was obtained by adding sterile 6 M solutions of HCl or NaOH. Temperature optimum was measured at pH 6.8. Effect of NaCl was examined in the basic medium containing 0, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0% (w/v) NaCl. Incubation times were from 1 to 2 weeks for optimal conditions to 2 months for utmost values.

Utilization of substrates

The ability of the strain to utilize various substrates was examined using basic medium and by adding sterile solutions of the substrates with their end concentrations of 2 and 5 g/l. Cultures were incubated for 3 weeks; their growth was determined by bacterial density and confirmed by two additional transferrings.

Phenotypic tests

Phenotypic tests, to determine key taxonomic characteristics such as Gram-staining, oxidase and catalase presence and glucose fermentation were performed following standard protocols (Smibert and Krieg 1994).

Quantitative analysis of substrates and products in cultures media

Alcohols were analyzed using a Pye-Unicam 304 gas chromatograph equipped with a glass column (1 m × 2 mm ID) packed with Porapak QS, 80–100 mesh (Fluka). The temperatures of the column, injector and flame-ionization detector were 90, 150 and 180°C, respectively. The carrier gas was nitrogen at the flow rate of 20 ml/min. Fatty acids were analyzed using the same chromatograph equipped with a glass column (2 m × 2 mm ID) 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°C to 175°C at the rate of 6°C/min. The injector and detector were kept at 150 and 180°C, respectively. The carrier gas was CO_2. H_2 and CO_2 were analyzed by gas chromatography according to Shtarkman et al. 1995. Lactate was determined with lactate dehydrogenase by the colorimetric method (Hohorst 1970).

Lipid analysis of bacterial fatty acids

Lipids were extracted from cell biomass that was dried in a stream of helium at 80°C and then vacuumized. A total of 0.4 ml of 1 N solution of HCl in methanol was added to 30 mg of dry biomass, and the mixture was heated at 80°C for three 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 HP-5973 GC-MS system (Hewlett-Packard). Separation was carried out on a fused quartz capillary column (25×0.25 mm) with the immobile phase HP-5 ms Hewlett-Packard (layer thickness of 0.2 μm). Chromatography was conducted in the temperature programming mode from 120°C to 280°C with a rate of 5 degrees per minute. Temperature of the injector and interface was 280°C. Data processing was carried out using standard programs of the GC-MS system.

DNA base composition and DNA–DNA hybridization

Isolation of DNA from the biomass was the carried out according to Marmur method (Marmur 1961). Nucleotide content was assessed by thermal denaturation of DNA using a spectrophotometer Pye Unicam SP1800 (Great Britain). DNA–DNA hybridization was determined by reassociation (DeLey et al. 1970).

16S rRNA analysis

Genomic DNA was extracted by the method previously described (Sambrook et al. 1989). The 16S rRNA gene was amplified by PCR using prokariotic 16 S rDNA primers 27F (5′-AGAGTTTGATCCTGGCTCAG) and 1492r (5′-TACGGYTACCTTGTTACGATT).The PCR product was purified by Wizard PCR Preps DNA Purificaton Systems (Promega, USA). The sequencing reactions were performed using a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, USA) and automatic DNA sequencer CEQ2000 XL (Beckman Coulter, USA) according to the protocols provided by the manufacturer. The NCBI and RDP databases were used for phylogenetic analysis. The nucleotide sequences of strain 14D1 have been deposited in the GenBank database under the accession number AY117755.

Results

Enrichment culture and isolation of pure culture

Preliminary microbiological analysis of the Arctic cryopegs showed that brines contained 2×10^2 cells/ml anaerobes-fermented glucose and peptone at 5°C (Gilichinsky et al. 2003). The enrichment culture of the obligate anaerobic bacteria was obtained by supplement cryopeg water samples on the mineral salt medium containing glucose and peptone. Following the inoculation, cells were incubated at 5°C for three months. After the initial incubation, a culture aliquot with visible turbidity was transferred into a sterile medium of the same content and serial tenfold dilutions were made. The culture from the maximal dilution (−5) was plated on the medium containing agar in the Petri dishes that were placed in the anaerostats. The colonies appearing on agar 4–6 weeks after the inoculation were convex, cream coloured and 1–2 mm in diameter. Resulting from the serial transferred of the single colonies from the solid to liquid medium, a pure culture of psychrophilic anaerobic microorganisms with fermenting type of metabolism called strain 14D1 was isolated.

Cell morphology

Cells of strain 14D1 were motile straight rods, 1.2–1.5 by 3–7 μm, with rounded ends and often occurring in pairs or in short chains (Fig. 1a). Gram staining was positive. Motility of the bacilli was provided by peritrichially arranged flagella (Fig. 1b). As shown by the analysis of the ultrathin sections of the strain cells in an electron microscope, the cell wall of the isolate did not contain an external lipoprotein membrane. The thickness of the cell wall ranged from 13 nm to 17 nm. The cytoplasmic membrane was recognized under the cell wall. The cytoplasm was filled with polysaccharide granules.

Spherical endospores (Fig. 1c) were formed centrally without cell wall bulging. Ultrathin sections and cryochips of sporulating cells (Fig. 1c–e) showed that the spores of strain 14D1 have a structure typical for the bacteria of *Bacillus* and *Clostridium* genera. A matured spore contained cortex and typical spore coats (Fig. 1c, e).

Effects of temperature, pH and NaCl concentration on the growth

Figure 2a displays the results of temperature effects on the growth rate of strain 14D1. Strain 14D1 grew at the temperature range from −5 to 20°C. Optimal growth temperature was 5°C. Below −5°C, bacterial growth was not tested; above 20°C, it was not observed suggesting that strain 14D1 is an obligate psychrophile. The strain grew in the range of pH from 4.5 to 8.5 with optimal pH of 6.5–7.2 (Fig. 2b).

The effect of NaCl on the growth of 14D1 was tested at optimal growth temperature (5°C) and optimal concentration of NaCl was 0.5% (w/v); at the concentration of 5.0% (w/v), the isolate growth ceased (Fig. 2c).

Use of carbon sources and energy

We used two substrate concentrations (2 and 5 g/l) for testing the substrate utilization by new bacterium and obtained the same results. At optimal growth conditions, strain 14D1 utilized xylose, inositol, galactose, maltose, glucose, arabinose, mannose, sucrose, ribose, mannitol, fructose, raffinose, melobiose, lactose, inulin, cellobiose, trehalose, xylan, peptone, yeast extract, fumarate, malate, trypticase, betaine, and choline.

Cultivation of the strain 14D1 on glucose at optimal growth temperature (5°C) has shown that among non-gaseous metabolic products butyrate and lactate dominated in 1:1 ratio. In addition, small amounts of acetate, formate and ethanol were produced. In the gas phase, H_2 (10%) and CO_2 (12%) accumulated after the incu-

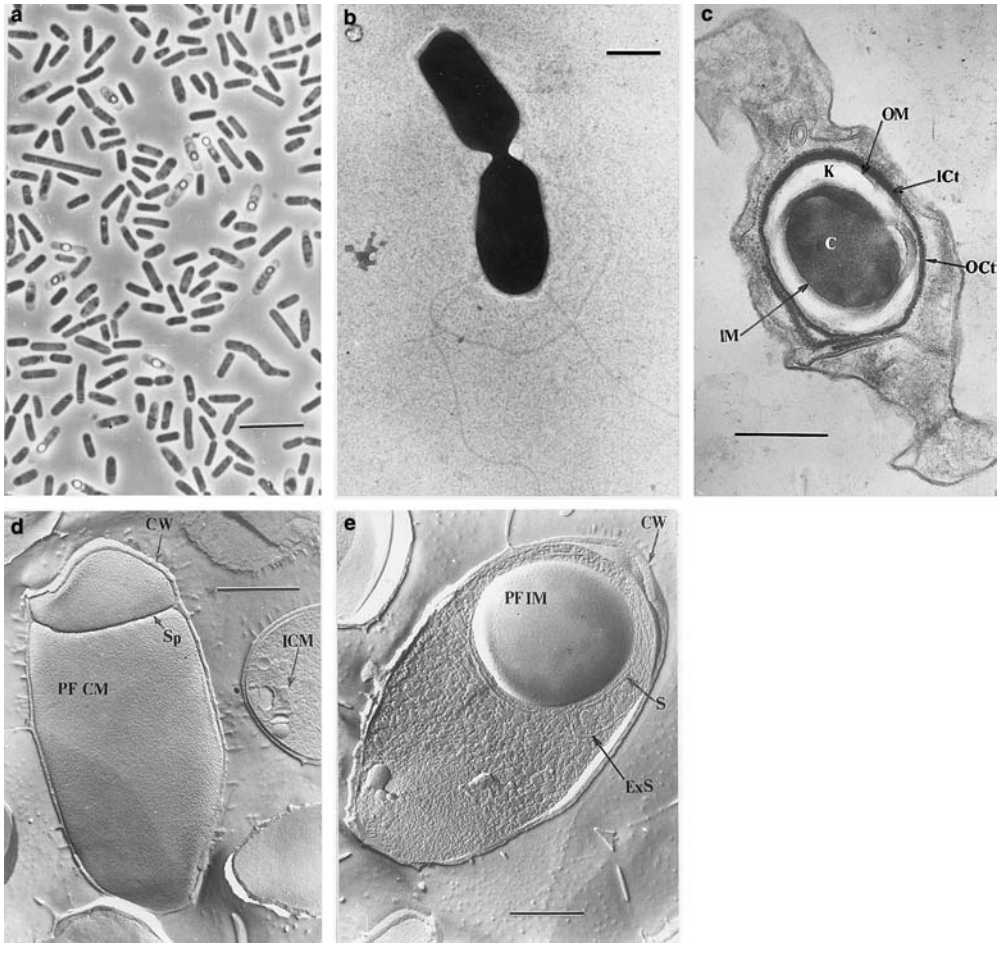

Fig. 1 Micrographs of cells of strain 14D1^T **a** Phase contrast, *bar* 10 μm. **b** Negatively stained cells, *bar* 1 μm. **c** Thin sections of spore, *bar* 1 μm. **d** Cryophotography of sporulating cells surface, *bar* 1 μm. **e** Cross-sectional chip of cell, *bar* 1 μm. *CW* cell wall, *PS* prospore, *C* core, *IM* inner membrane of a spore, *OM* outer membrane of a spore, *K* cortex, *ICt* inner coat of a spore, *Oct* outer coat of a spore, *S* spore, *ExS* exosporium, *PF CM* protoplasmic face of the cytoplasmic membrane, *Sp* septa, *ICM* intracytoplasmic membrane, *PF IM* protoplasmic face of inner membrane of a spore

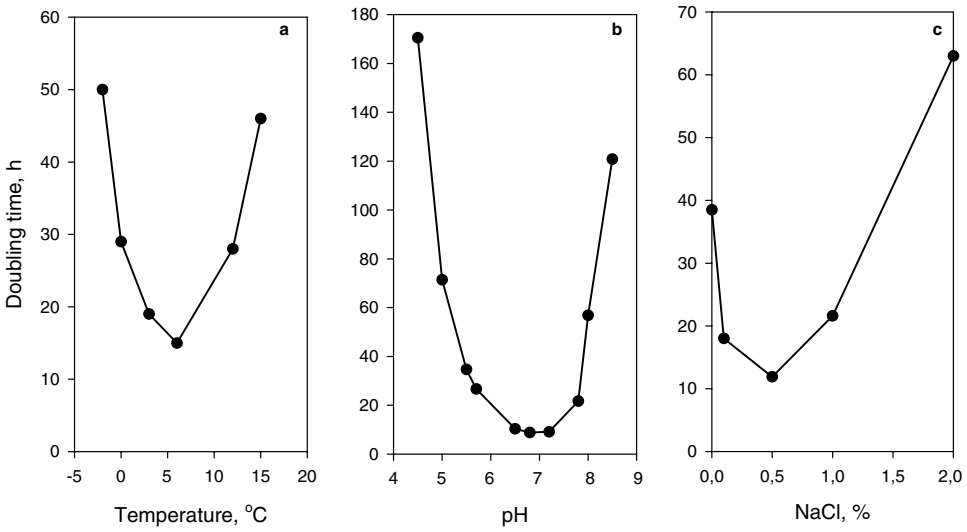

Fig. 2 Temperature (**a**), pH (**b**) and NaCl (**c**) effect on the doubling time of strain 14D1^T

Table 1 Cellular fatty acid composition of the new isolate and other psychrophilic *Clostridium* spp.

Compound	*Clostridium algoriphilum* DSM 16153^T	*Clostridium frigoris* DSM 14204^T	*Clostridium lacusfryxellense* DSM 14205^T	*Clostridium bowmanii* DSM 14206^T	*Clostridium psychrophilum* DSM 14207^T	*Clostridium estertheticum* DSM 8809^T
C $_{12:0}$	0.5[a]		0.2	0.6	0.4	0.8
C $_{14:1\Delta9}$	1.4		0.2	0.3	0.7	
C $_{14:1\Delta11}$	0.7					
C $_{14:0}$	**32.6**	**17.2**	**17.8**	**19.7**	**19.5**	**28.3**
C $_{14:1\Delta9\ dma}$	0.4					
C $_{14:1\Delta11\ dma}$	0.2					
C $_{16:1c7}$		4.7	**8.8**	**6.5**	3.0	3.7
C $_{16:1\Delta9}$	**37.0**	**26.5**	**30.7**	**25.4**	**25.7**	**13.5**
C $_{16:1\Delta11}$	**5.1**	**8.7**	**5.6**	**8.9**	**10.0**	**7.7**
C $_{16:0}$	**6.6**	**11.1**	**5.7**	**12.7**	**9.2**	**8.4**
C $_{16:1\Delta7\ dma}$	**12.5**	1.3	2.9	2.7	1.3	0.9
C $_{16:1\Delta9\ dma}$		**21.1**	**21.5**	**16.3**	**17.3**	**22.8**
C $_{16:0\ dma}$			0.3	0.5	0.3	
C $_{17cyc}$	0.6				1.3	**5.0**
C $_{17cycdma}$					1.0	3.2
C $_{18:1\Delta9}$	0.6		0.7	0.6	1.7	0.4
C $_{18:1\Delta11}$	0.3					
C $_{18:0}$	1.5	1.1	0.3	0.3	1.2	
C $_{18:1\Delta9dma}$			0.4	0.1	0.9	0.3
References		Spring et al. (2003)	Spring et al. (2003)	Spring et al. (2003)	Spring et al. (2003)	Spring et al. (2003)

dma dimethylacetal, *cyc* cyclopropane, *c* cis
[a] Values are percentages of total fatty acids. Major fatty acids (>5%) are in boldface type

bation. 14D1 did not grow on H_2+CO_2, starch, cellulose, agarose, sorbitol, rhamnose, dulcitol, methanol, ethanol, butanol, formate, acetate, propionate, pyruvate, glycine, valine, cysteine, valerate, caproate, geptanoate, citrate and monomethylamine.

Cell wall composition

Peptidoglycan of the cell wall contained *meso*-diaminopimelic acid. This kind of mureine structure is typical for bacteria belonging to clostridial cluster 1. The fatty acid composition of the cell wall was also identified. Comparison of the fatty acid profile (Table 1) of the new strain with that of the representatives of other psychrophilic species of the genus *Clostridium* revealed domination of monounsaturated hexadecane acid $C_{16:1}$ with double bond at C_9 (37%) and myristic acid $C_{14:0}$ (32.6%) in the lipid complex of 14D1. As may be seen from Table 1, the former also dominates in the fatty acid profile of Antarctic clostridia (25.4–30.7%), while the latter in *C. estertheticum* (28.4%).

Resistance to heating and freezing

Actively growing culture 14D1 (including those with spore present) survived exposure to 20°C for 24 h, but not to 24, 30 or 37°C for same period. Cells of strain 14D1 did not survive exposure 50, 60, 70 and 80°C for 10 min. Following freezing at −40°C for two months,

Table 2 Differential characteristics of new isolate and psychrophilic species of *Clostridium* genus

Characteristics	*Clostridium algoriphilum* DSM 16153^T	*Clostridium frigoris* DSM 14204^T	*Clostridium lacusfryxellense* DSM 14205^T	*Clostridium bowmanii* DSM 14206^T	*Clostridium psychrophilum* DSM 14207^T	*Clostridium vincentii* DSM 10228^T	*Clostridium estertheticum* DSM 8809^T
Spore shape and location	Spherical central	Spherical, terminal	Spherical, terminal to subterminal	Spherical, terminal to subterminal	Ellipsoidal, terminal to subterminal	Ellipsoidal, terminal	Ellipsoidal, terminal to central
Temperature optimum	5–6°C	5–7°C	8–12°C	12–16°C	4°C	12°C	6–8°C
pH optimum	6.5–7.2	6.8–7.2	6.5–7.1	6.6–7.2	6.5–7.0	6.5	6.5–7.2
Utilization[a] of							
Arabinose	+	+	–	–	+	ND	+
Cellobiose	+	+	+	–	+	+	+
Galactose	+	+	+	+	–	+	+
Inositol	+	+	+	–	–	ND	+
Lactose	+	+	+	–	–	+	–
Maltose	+	+	–	+	+	+	+
Mannitol	+	–	+	–	–	ND	+
Mannose	+	+	–	+	+	+	+
Melizitoze	+	–	+	–	–	ND	–
Melibiose	+	+	+	–	–	ND	+
Raffinose	+	+	+	–	–	ND	+
Rhamnose	–	+	–	–	–	–	+
Ribose	+	+	+	+	–	ND	–
Salicin	ND	+	+	+	–	ND	+
Starch	–	+	+	–	–	–	+
Trehalose	+	+	+	+	+	ND	–
Xylan	+	ND	ND	ND	ND	+	ND
Hydrolysis of starch	–	–	–	–	–	ND	+
Non-gaseous fermentation products[b]	B, L, a, f, 2	B, f, l, a, 2	B, F, l, a, 2	B,A, f, l, 2, 4	L, 2, 4, b	A, B, F	B,A, f, l, 2, 4
GC, mol%	31.4	31.9	32.1	32.0	31.8	33.0	33.9
References		Spring et al. (2003)	Spring et al. (2003)	Spring et al. (2003)	Spring et al. (2003)	Mountfort et al. (1997)	Collins et al. (1992)

[a] Substrate utilization was determined by measuring the increase in OD_{600}: + substrate used, – not used. All strains were positive for the utilization of glucose, fructose, sucrose, xylose, inulin and negative for hydrolysis of gelatine

[b] Fermentation products produced only in small amounts (< 1 mM) were not considered. Capital letters indicate concentration above 10 mM. Abbreviations: *a* acetic acid, *b* butyric acid, *f* formic acid, *l* lactic acid, *2* ethanol, *4* 1-butanol

strain 14D1 was growing at optimal conditions without a lag period.

Spore formation of strain 14D1

Spore formation was not observed during the period of the exponential growth. At the same time, old cultures did contain single spores. Acidation of the medium down to pH 3.5–4.0, freezing-thawing and heating at 50, 60, 70 or 80°C did not promote spore formation. Partial spore formation was observed on a poor medium. Placement of the isolate vegetative cells into cryopeg water without adding the nutrients stimulated spore formation. Following this procedure, a spore was formed virtually in every cell but when left for a long time at optimal temperature (5°C), some spores germinated.

DNA nucleotide composition

The content of GC pairs in DNA was 31.4 mol%, and the data fit well within those from the group of the so far described psychrophilic clostridia (Table 2). DNA–DNA hybridization of the isolate with *C. frigoris* DSM 14204, *C. lacusfryxellense* DSM 14205, *C. bowmanii* DSM 14206^T, *C. psychrophilum* DSM 14207^T and *C. estertheticum* DSM 8809^T showed 38, 27, 42, 39 and 47% homology, correspondingly.

Phylogenetic analysis

16S rRNA sequencing (1460 nucleotides) of strain 14D1^T was conducted. The results were compared with psychrophilic clostridia and some other spore forming reference species available in GenBank. The phylogenetic dendrogram found in Fig. 3 shows that novel Arctic clostridial isolate clusters with Antarctic species and *Clostridium estertheticum* DSM 8809^T. The obtained branching is confirmed by high values of branching probability. The closest neighbors of the new strain are *C.psychrophilum* DSM 14207^T, *C. frigoris* DSM 14204^T, *C. lacusfryxellense* DSM 14205^T, *C. bowmanii* DSM 14206^T and *C.estertheticum* DSM 8809^T, with 98.8–

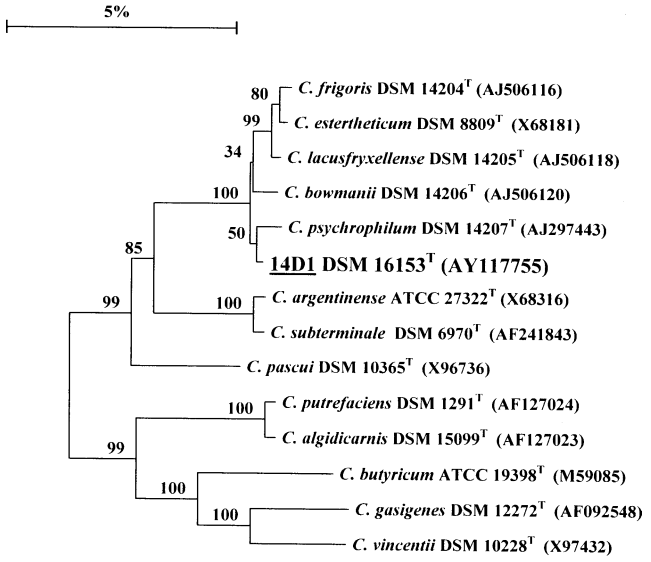

Fig. 3 Position of the strain 14D1^T among closely related species of the genus *Clostridium*, based on 16S rRNA phylogenetic analysis. The accession numbers of type strains are shown in parentheses. The tree was built with the neighbor-joined method by using the Jukes-Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. The sequence of *Acetivibrio cellulolyticus* (L35516) was used as an outgroup (not shown). Bar 5%, estimated substitutions per nucleotide position

99.1% resemblance. On the other hand, Antarctic psychrophilic isolate *C. vincentii* has a smaller resemblance of 90%.

Discussion

General phenotypical properties (cell wall of Gram-positive type, endospore formation, obligate anaerobiosis, inability to dissimilated sulfate reduction) of strain 14D1 were typical to the species of genus *Clostridium*. Until today, the genus *Clostridium* represents one of the largest genera of the prokaryotes. It currently includes more than 170 species of bacteria having a wide range of metabolic properties and GC content from 22 to 55 mol% (Garrity et al. 2004, Hippe et al. 2003, Collins et al. 1994). Among the validly described species of clostridia, only *C. frigoris*, *C. lacusfryxellense*, *C. bowmanii*, *C.psychrophilum*, *C. estertheticum* and *C. vincentii* are true psychrophiles with optimal temperature for growth from 4°C to 15°C. By contrast, several species which were isolated from cold habitats (*C. arcticum*, *C. algidicarnis*, *C. frigidicarnis* or *C. gasigenes*) or normal habitats (*C.akagii*, *C. fimetarium* or *C. putrefaciens*) are only psychrotrophic, with temperature optima around or above 20°C.

A novel psychrophilic microorganism was isolated from the closed, ancient, permanently overcooled aquatic system of marine origin found in Arctic permafrost at the depth of several tens of meters. The bacterium isolated not only adapted to subzero temperatures, but it survived freezing, grew at subzero temperatures, expanded spectrum of consumed substrates at temperature decrease and was also tolerant to high salt content (Shcherbakova et al. 2004). Table 2 displays properties of strain 14D1 and other obligatory psychrophilic clostridia. The cells morphology of 14D1^T strain looks like *C. psychrophilum* DSM 14207^T and *C. estertheticum* DSM 8809^T. However *C. psychrophilum* had a strong tendency to form filamentous cells on agar plates, which reached a length of more than 30 μm (Spring et al. 2003), and it was not observed for strain 14D1^T. The new bacterium resembles *C. frigoris* DSM 14204^T and *C. estertheticum* DSM 8809^T in its optimal temperature. All presented clostridia are close in their optimal growth pH and GC content. Nevertheless, strain 14D1 and its phylogenetically close clostridia have significant differences in the fatty acid profile of the cell wall (Table 1), range of the utilizable substrates and products from the hexoses. Thus, its phenotypical and genotypical properties allow for the classification of strain 14D1 as a new species of genus *Clostridium* - *C. algoriphilum* sp. nov.

Description of *Clostridium algoriphilum* sp. nov.

Clostridium algoriphilum [al.go.ri'phi.lum. L. masc. n. algor -oris, cold, coldness; N.L. neut. adj. philum (from Gr. neut. adj. philon), loving; N.L. neut. adj. algoriphilum, cold-loving].

Cells of strain 14D1 are Gram-positive rods with rounded ends (1.2–1.5 by 3–7 μm), occurring in singles or pairs. Cells are motile with peritrichially arranged flagella. Endospores are spherical to slightly ellipsoidal and locate at a central position. Colonies are round, convex, cream coloured and 1–2 mm in diameter.

Optimal growth temperature is 6°C, and upper growth limit is 20°C. pH growth range is 5.0–8.0 with optimal pH at 6.8–7.2. The bacterium is an obligatory anaerobe. When growing on glucose at optimal conditions, its doubling time is 12 h. The strain utilizes xylose, inositol, sorbitol, galactose, maltose, glucose, arabinose, mannose, sucrose, ribose, mannitol, fructose, raffinose, melobiose, lactose, inulin, cellobiose, trehalose, xylan, peptone, yeast extract, fumarate, malate, trypticase, betaine and choline. The strain does not hydrolyze gelatin and starch. Fermentation of sugars leads to the formation of butyrate, formate, lactate, acetate, ethanol, H_2 and CO_2.

The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major fatty acids of the cell wall are $C_{14:0}$ and $C_{16:1c9}$. The GC-content of DNA is 31.4 mol.%.

It was isolated from overcooled water brines in Arctic permafrost of Kolyma Lowland.

Type strain 14D1^T was deposited into the Russian Collection of the Microorganisms VKM B-2271^T and German Collection of the Microorganisms DSM 16153^T.

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