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EXPERIMENTAL ARTICLES

Novel Halotolerant Bacterium from Cryopeg in Permafrost: Description of *Psychrobacter muriicola* sp. nov.

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Abstract—A novel halotolerant psychrotrophic gram-negative bacterium, strain 2pS, was isolated from lenses of water brine in Arctic permafrost (cryopeg). The optimal growth of the new strain was observed at 16–18°C; the maximal and minimal growth temperatures were 37°C and –2°C, respectively. The pH growth range was 5.8 to 8.5 (optimum 6.5–7.5) and the range of medium salinity was 0 to 100 g/l (optimum 3–8 g/l NaCl). The strain 2pS did not produce acid from carbohydrates and utilized acetate, yeast extract, pyruvate, glutarate, fumarate, caproate, heptanoate, butyrate, malate, DL-lactate, citrate, L-proline, L-tyrosine, butanol, and dulcitol as the sole carbon and energy sources. The major fatty acids of the cell wall at optimal growth temperature were $C_{18:1}\omega7$ and $C_{18:1}\omega9$. The G+C DNA base content was 46.0 mol.%. Phylogenetic analysis of the 16S rRNA gene sequences showed that the studied strain was the closest (97% similarity) to *Psychrobacter nivimaris* DSM 16093^T, a halotolerant psychrotrophic bacterium isolated from the Arctic sea's ice. Genotypic and phenotypic differences of the new bacterium from closely related species lead to the conclusion that strain 2pS belongs to a novel species of the genus *Psychrobacter: Psychrobacter muriicola* sp. nov.

Key words: cryopegs, *Psychrobacter muriicola*, salinity, negative temperature.

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The genus *Psychrobacter* is comprised of strictly aerobic chemoorganotrophic, mainly psychrotolerant and halotolerant, nonmotile gram-negative cocci and coccobacilli. The first species in this genus, *Psychrobacter immobilis*, was originally described in 1986 [1] based on the group of strains morphologically resembling *Moraxella* and *Acinetobacter.* These strains were isolated from seawater, fish scales and gills, and were grouped on the basis of phenotypic characteristics and mutual competence to genetic transformation. More recently, especially in the past five years, many new species belonging to this genus have been described, mainly from marine and cold habitats [2]. At present, the genus includes about 30 species combined on the basis of phylogenetic analysis and their common physiological and biochemical characteristics. Based on the 16S rDNA data analysis, the genus *Psychrobacter* was referred to as the family *Moraxellaceae* within the class γ-*Proteobacteria* [2]. The genome of bacterium *Psychrobacter arcticus* 273-4 isolated from permafrost rocks in the Kolyma lowland was sequenced and analyzed in 2006 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=9633). This is a great achievement, because its comparison with closely related mesophilic species contributes to the understanding of evolution of adaptation to cold [3]. Isolation of two strains of halotolerant and psychrotrophic bacteria from Arctic cryopegs has been reported previously [4, 5].

The goal of this work was to study the physiological, biochemical, and phenotypic properties of strain 2pS in order to define its taxonomic position and to investigate the effect of high salinity and negative temperature on fatty acid composition of the cell wall of the novel bacterium.

MATERIALS AND METHODS

Research objects. Strain 2pS isolated from cryopeg water samples with mineralization of 170 g/l was an object of research in the present work. Comparative analysis was performed with strains *P. immobilis* DSM 7229^T and *P. nivimaris* DSM 16093^T from the German Collection of Microorganisms and Cell Cultures.

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Media composition and cultivation conditions. Strains 2pS, *P. immobilis* DSM 7229^T, and *P. nivimaris* 16093^T were cultivated in a liquid medium containing the following (g/l): Na_2HPO_4, 11.2; KH_2PO_4, 4.0; NH_4Cl, 2.0; NaCl, 4.0; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.005; sodium acetate, 10.0; and trace elements solution SL-10, 10 ml [6].

The effects of temperature, pH and salinity on strain growth were studied in the mineral medium with 20 mM acetate. Sterile solutions of 25% (by volume) HCl and 10% (wt/vol) $NaHCO_3$ were used to obtain pH values from 5.3 to 8.5. The effect of temperature on the growth rate was studied at pH 6.9. The effect of salinity was studied in medium containing 0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 6.0% NaCl. Basal medium was used for testing the substrate range; sugars and other substrates were introduced in the amounts of 0.2% and 0.1%, respectively.

Anaerobic growth of the strain was tested in Hungate test tubes on anaerobically [7] prepared mineral medium with 20 mM acetate.

The API ZYM commercial kit for enzymatic express tests (BioMerieux, France) was used to determine enzymatic activities of the strain 2pS. The tests were performed in accordance with the manufacturer's instructions.

Microscopic studies. Cell morphology of the cultures was studied under a phase-contrast light microscope Lumam I-2 (LOMO, Russia) at magnification of 90 × 15 and under a JEM 100 Japan electron microscope, using negatively stained cell preparations and ultrathin sections.

For negative staining of the cells, diluted cell suspension was treated with 3% phosphotungstic acid solution, pH 5.0, or 1% ammonium molybdate, pH 7.0. The time of staining was 3–5 min at room temperature.

Ultrathin sections were studied by electron microscopy as described in [8]. Ultrathin sections were obtained on a LKB-3 ultratome and stained with 1% uranyl acetate solution.

Gram staining was performed according to the standard procedure.

DNA isolation from biomass was performed by the method of Marmur [9]. Nucleotide composition was studied by the method of DNA thermal denaturation in a Pye Unicam SP1800 spectrophotometer (Great Britain). DNA–DNA hybridization was determined by the reassociation method [10].

Determination of the 16S rRNA gene nucleotide sequence. DNA was isolated as described previously [11]. The 16S rRNA genes were amplified with universal primers 27f and 1492r on a GeneAmp PCR System 2700 (Applied Biosystems). The sequencing of the amplified 16S rDNA fragment was performed in a CEQ2000XL automatic DNA sequencer (Beckman Coulter) with the Dye Terminator Cycle Sequencing kit (Beckman Coulter) according to the manufacturer's protocol.

Phylogenetic analysis. The nucleotide sequences of the 16S rRNA genes were aligned using ClustalX [12]. The rootless phylogenetic tree was constructed using the algorithms realized in TREECON [13].

Fatty acid analysis. Lipids were extracted from the cell biomass (3–5 mg of dry cells) by acid methanolysis in the mixture of 0.4 ml of 1.2 N HCl and methanol at 80°C for 1 h. Methyl ethers of fatty acids and other lipid components were extracted twice with 200 µl of hexane. The extract was dried, treated with 20 µl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide at 80°C for 15 min for the formation of trimethylsilyl ethers of hydroxy acids. Reaction mixture sample 2 µl, was analyzed in a Sherlock (MIDI Inc., Delaware, United States) in the automatic mode. The substances that had been ambiguously determined by retention time on the Sherlock were identified in the gas chromatographer–mass spectrometer AG-5973 system (Agilent Technologies, United States). The separation was performed in a capillary column (25 m × 0.25 mm) covered with chemically bound methyl silicone immobile phase HP-5ms Hewlett-Packard (with the 0.2-µm layer thickness).

Chromatography was performed in the mode of temperature programming from 130 to 320°C at a rate of 5°C/min. The data were processed using the standard software.

RESULTS AND DISCUSSION

Isolation. Inoculation of cryopeg water on agarized R2A medium allowed us to isolate two strains of aerobic bacteria: 1pS and 2pS [4]. The colonies on solid medium were white, convex, with smooth edges, 2–3 mm in diameter. The level of DNA–DNA hybridization between strains 1pS and 2pS was 89%; hence, they may be attributed to the same species. Later on, strain 2pS was studied in detail.

Morphology and ultrastructure. The cells of strain 2pS were nonmotile coccobacilli, 0.5–1.0 µm in width and 0.5–2.0 µm in length (Fig. 1a, b), negatively stained by Gram. The gram-negative type of cell wall structure was confirmed by examination of ultrathin sections (Fig. 1c).

Physiological and biochemical characteristics. According to Morita classification [14], the isolate was psychrotrophic as it grew in the temperature range from 5 to 37°C with the optimum at 16–18°C (Fig. 2a). Investigation of its capacity for growth at negative temperatures showed that strain 2pS could grow at –2°C with the doubling time of 50 h.

Addition of NaCl to the cultivation medium stimulated the growth of strain 2pS. Optimal growth was observed at 3–8 g/l NaCl. The isolate was tolerant to the NaCl content in the cultivation medium up to 60 g/l (Fig. 2b) at the optimal growth temperature. Our previ-

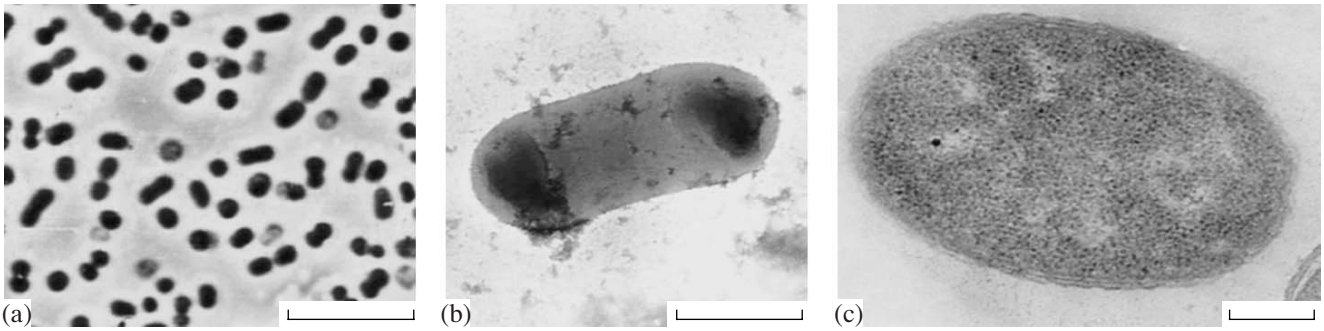

Fig. 1. Micrographs of 2pS cells: a, phase contrast, scale bar 5 μm; b, negative staining, scale bar 1 μm; c, ultrathin section, scale bar 0.5 μm.

ous studies of the physiological peculiarities of strain 2pS showed that this inhabitant of cryopegs could grow with up to 100 g/l NaCl in the medium at –2°C [15].

The spectrum of utilized substrates. After 48 h of incubation, growth was observed on yeast extract, a number of organic acids (acetate, pyruvate, glutarate, fumarate, caproate, heptanoate, butyrate, malate, DL-lactate, citrate), some amino acids (L-proline and L-tyrosine) and alcohols (butanol and dulcitol). Under optimal conditions, growth was not observed on histidine, asparagine, L-glutamine, L-phenylalanine, L-valine, L-cysteine, L-leucine, glycine, DL-methionine, DL-tryptophan, DL-serine, succinate, lactate, formate, sorbitol, inositol, ethanol, propanol, and glycerol. No growth factors were required. The strains under study did not utilize xylose, agarose, galactose, maltose, glucose, arabinose, mannose, sucrose, ribose, rhamnose, fructose, D-raffinose, and melibiose. Thus, strain 2pS neither oxidized carbohydrates nor formed acid from them.

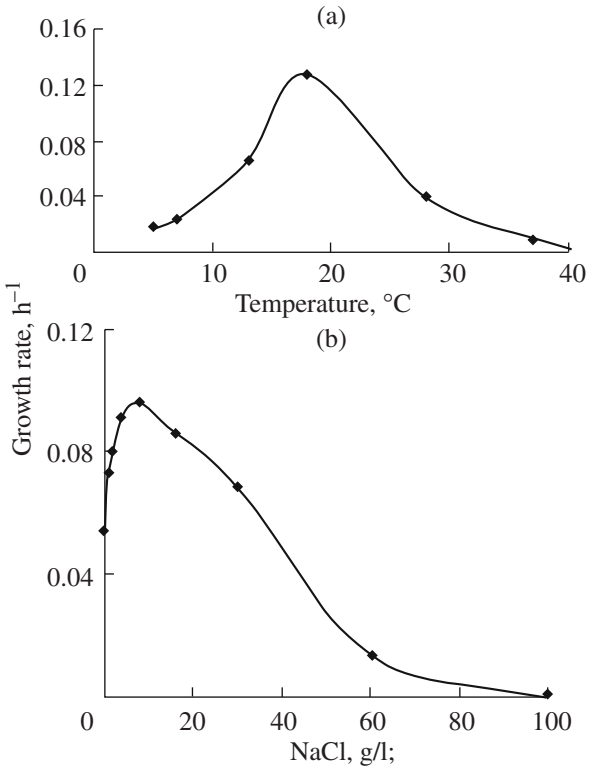

Fig. 2. The effect of temperature (a) and salinity (b) on the growth rate of strain 2pS.

Strain 2pS was oxidase and catalase positive but could grow under anaerobic conditions on acetate. The tests for phenylalanine deaminase, nitrate reductase and urease, alkaline and acid phosphatase, leucine arylamidase, esterase (C_4), lipase (C_8), and naphthol-AS-BI-phosphohydrolase were positive. The tests for arginine deaminase, lysine decarboxylase, ornithine decarboxylase, starch hydrolysis, indole and H_2S production, lipase (C_{14}), valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, β-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, *N*-acetyl-β-galactosidase, β-manosidase, and β-fucosidase were negative.

Fatty acid composition of 2pS membrane lipids at different cultivation temperatures and salinities of the medium. The novel bacterium was isolated from brines in permafrost, permanently existing at a negative temperature. We investigated the changes in the fatty acid profile of 2pS cells grown at different temperatures (–2, 20, and 28°C) and NaCl concentrations in the medium.

The results of this research are presented in Table 1. As a whole, fatty acid composition of the membrane lipid complexes of strain 2pS was characterized by the high content of unsaturated fatty acids: ω7-*cis*-hexadecene, ω8-heptadecene, ω9-octadecene, and ω7-octadecene acids making a total of 79.8% at the optimal cultivation temperature and medium salinity. The experiments showed that cultivation at a temperature below (–2°C) or above (28°C) the optimal value did not result in significant shifts in the ratio for totals of saturated and unsaturated fatty acids (Table 1). Cultivation at a higher salinity and negative temperature resulted in an almost 10% increase in the portion of unsaturated fatty acids. The content of short-chain fatty acids (decane and hydroxy-dodecane) did not change by much. An

Table 1. The effect of cultivation conditions on the cell wall's lipid composition of strain 2pS

Compound, %	Salinity, g/l					
	5.0			50.0		
	Temperature, °C					
	–2	18–20	28	–2	18–20	28
9:0	0.26	0.32	0.66			0.72
10:0	3.94	3.61	4.54	2.59	4.74	4.29
*i*11	0.06	0.12	0.17			0.27
*ai*11:0				0.17		
11:0		0.16	0.36	0.23	0.23	0.40
12:0	0.11	0.13	0.15	0.11	0.15	0.10
3h12	0.72	0.96	1.03	0.51	0.84	1.10
14:0	0.15	0.21	0.14		0.23	
3h13			0.16			
15:1ω6				0.16		
15:0	0.13	0.21	0.18			
16:1	0.57	0.24	0.20	0.46		0.27
16:1ω7c	9.48	6.54	4.49	7.89	8.94	4.37
16:1ω7t	3.64	1.47	0.46	0.97	0.76	0.62
16:0	1.49	2.28	1.86	0.74	2.22	1.60
3hi15	0.44	0.36	0.22	0.15		0.35
*i*17	0.69	0.72	0.85	1.01	0.46	1.27
17:1ω8	4.88	5.27	9.43	8.76	5.50	9.20
17:1ω6	2.31	1.81	1.38	1.70	0.61	1.72
17:0	0.49	0.66	1.32	0.24	0.38	0.67
17:0 10 Me				0.25		
18:1ω9	42.04	45.76	56.98	53.00	57.30	49.61
18:1ω7	18.13	15.94	7.98	12.78	11.46	11.75
18:0	2.45	3.02	4.89	1.55	3.36	2.87
18:1ω11 Me	1.30	2.78	1.57	2.71		3.79
3hi17	0.50	1.56		0.80		2.29
18:0 10 Me				0.27		
*i*19	0.14	0.25	0.42	0.18		
20:1ω9t				0.58		
20:0	5.99	5.63	0.53	2.22	2.83	2.72
Saturated fatty acids, % of the total	16.9	18.2	16.6	9.1	15.2	16.0
Unsaturated fatty acids, % of the total	82.4	79.8	82.5	88.3	84.6	81.3

Note: "hi", hydroxi; *i*, iso; *ai*, antiiso.

interesting result of the experiment was the appearance of some acids untypical for *Psychrobacter* bacteria at –2°C and high salinity (50 g/l): $C_{15:1}$ω6, $C_{17:0}$ 10 Me, $C_{18:0}$ 10 Me, $C_{20:1}$ω9t, and *ai*$C_{11:0}$.

Genotypic characteristics and taxonomic position. The DNA G+C base content of strain 2pS was 46.0 mol/%.

Phylogenetic analysis of the nearly complete sequence of the 16S rRNA gene of strain 2pS (1478 nucleotides) showed that this strain was closest (97% of similarity) to *P. nivimaris* (AJ313425), a halotolerant psychrotrophic bacterium isolated from Antarctic sea ice [16]. The levels of DNA–DNA hybridization for strain 2pS with *P. nivimaris* DSM 16093^T and with the

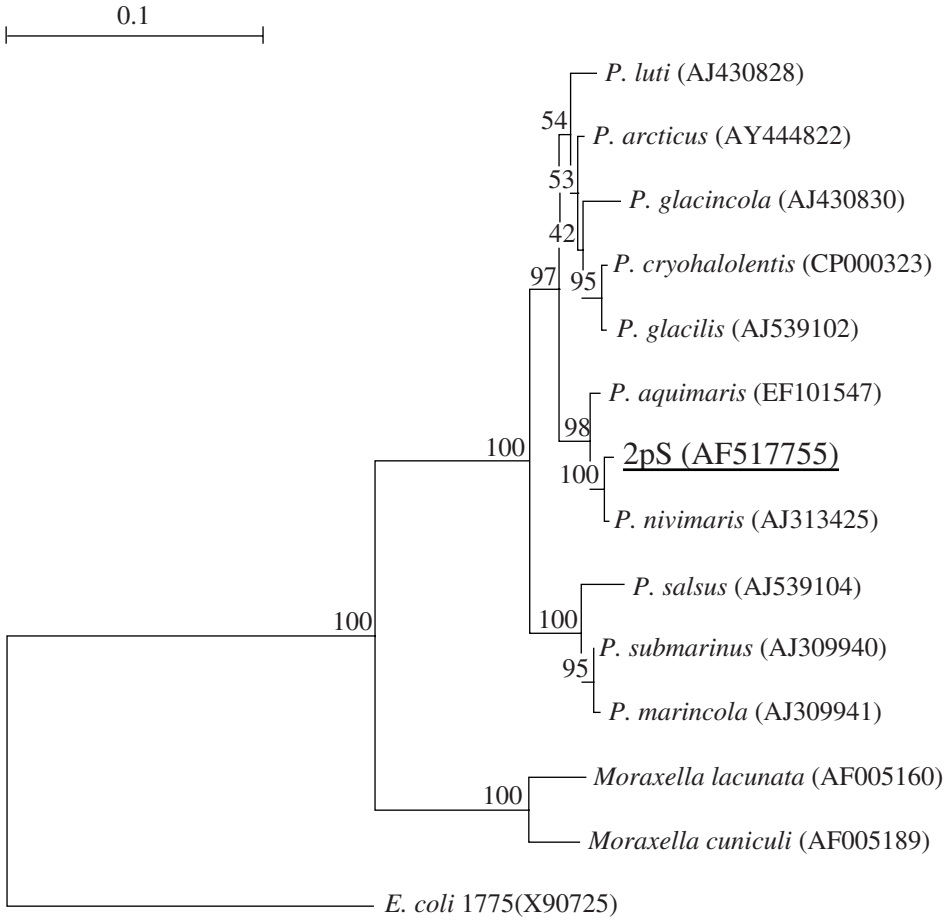

Fig. 3. Phylogenetic tree based on comparative analysis of the 16S rRNA gene sequences, showing the position of the Arctic isolate 2pS. Bootstrap analysis data are indicated in the branching points. Scale: 0.1 of substitution per one nucleotide.

type strain of the genus, *P. immobilis* DSM 7229^T, were 25% and 57%, respectively.

Proposal for a new species of the genus *Psychrobacter.* About 30 *Psychrobacter* species have been described since the first species of this new genus, *P. immobilis*, was described in 1983. Many of them were isolated from marine habitats with permanently low temperatures. Two novel species, *P. cryohalolentis* and *P. arcticus* isolated from Siberian permafrost, were described in 2006 [17].

The analysis of the morphological, physiological, and biochemical characteristics of the described bacterium suggests its affiliation with the genus *Psychrobacter.* Like most representatives of this genus, strain 2pS does not produce pigments, is catalase and oxidase positive, psychrotrophic, and moderately halophilic. It does not produce indole and H_2S, does not hydrolyze starch and gelatin, does not contain lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase, but hydrolyzes Tween 80. The presence of an ample quantity of oleic acid in the lipid profile is also a typical feature of the genus *Psychrobacter.*

The psychrotrophic Arctic isolate 2pS proved to be phylogenetically closest to *P. nivimaris* (Fig. 3). However, the level of DNA–DNA hybridization for the isolated strain and *P. nivimaris* was only 25%, which demonstrates the affiliation of these bacteria with different species.

Physiological and biochemical characteristics of strain 2pS are presented in Table 2. Published descriptions of phylogenetically close species of the genus *Psychrobacter* isolated from cold habitats were used for comparison. Strain 2pS possesses a number of features that vary within the genus *Psychrobacter*. The temperature optimum of this strain is close to the optima for *P. glacincola*, *P. luti* and *P. frigidicola* (16–18 and 15–16°C, respectively), whereas in other species it is higher (22–30°C). Strain 2pS, similar to *P. glacincola*, *P. luti*, *P. frigidicola*, *P. cryohalolentis*, *P. arcticus*, and *P. aquaticus* and in contrast to *P. nivimaris* and *P. aquimaris*, does not ferment glucose and other carbohydrates. The common feature of the novel bacterium and *P. aquimaris* is the ability for anaerobic growth.

Based on the analysis of genetic and phenotypic characteristics of the isolated bacterium, we propose a

Table 2. Phenotypic characteristics of strain 2pS and other species in the genus *Psychrobacter* isolated from the Arctic and Antarctic

Characteristic	1	2	3	4	5	6	7	8
Growth temperature:								
Range	–2–35	5–35	–10–30	–10–28	0–22	0–20	4–25	4–34
Optimum	16–18	10–15	22	22	13–15	15	14–16	25–30
Tolerance to 100 g/l of NaCl	+*	+	+	+	+	+	–	+
Acid production from carbohydrates[a]	–	+	–	–	–	–	–	+
Reduction of nitrates	–	n/t	v	v	–	+	–	–
Hydrolysis:								
Gelatin	–	–	–	–	n/t	n/t	n/t	n/t
Tween 80	+	+	–	+	+	+	+	+
Enzymatic activities:								
Acid phosphatase	+	n/t	–	+	–	–	–	–
Alkaline phosphatase	+	n/t	+	+	+	+	–	+
Urease	–	n/t	–	+	–	–	–	–
Esterase (C_4)	+	n/t	+	–	n/t	+	+	+
Lipase (C_{14})	+	n/t	+	–.	+	+	–	+
Leucine arylamidase	+	n/t	+	+	+	+	+	+
Utilized substrates:								
Citrate	+	–	+	–		+	–	–
Acetate	+	+	+	+	+	+	+	+
Glycerol	–	–					–	–
L-malate	+	–	–	–		+	+	+
L-histidine	–	+	+	+	+	–	–	–
L-proline	+	+	+	+	+	–	+	–
L-alanine	–	+	+	+	–	–	–	–
L-hydroxyproline	–	–	v	+	+	–	–	–
G+C content, mol.%	46.0	42.0	42.3	42.7	44.0	45.0	41–42	43.2

Species/Strains: 1 – 2pS^T VKM B-2270; 2 – *P. nivimaris* DSM 16093^T [16]; 3 – *P. cryohalolentis* DSM 17306^T [17]; 4 – *P. arcticus* DSM 17307^T [18]; 5 – *P. glacincola* DSM 12194^T [19]; 6 – *P. luti* LGM 21276^T [20]; 7 – *P. frigidicola* DSM 12411^T [19]; 8 – *P. aquimaris* DSM 16329^T [21].

* At cultivation temperature –2°C

[a] All taxa were oxidase- and catalase-positive, did not produce indole and H_2S, did not hydrolyze starch, did not contain lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase

[b] Abbreviations: +, positive reaction; –, negative reaction; n/t, not tested; v, variable. The data on enzymatic activities for *P. nivimaris* are absent.

novel species of this genus: "*Psychrobacter muriicola*" sp. nov., with the type strain 2pS.

Description of the novel species "*Psychrobacter muriicola*" sp. nov. [*mu.ri.i.co'la*. L. n. muria, brine; L. suf. -cola (from L. n. incola), inhabitant; N.L. n. *muriicola*, inhabitant of brine].

The cells are gram-negative cocci or coccobacilli, non-spore-forming, single, in pairs, or short chains, 1–1.5 μm in diameter, 0.5–1.0 μm in width, 0.5–2.0 μm in length, nonmotile. They form white flat colonies, 2–3 mm in diameter. The organism is chemoorganotrophic, oxidase and catalase positive, facultatively aerobic. Optimal growth occurs at 16–18°C; the maximal and minimal growth temperatures are 37°C and –2°C, respectively. The pH range for growth is 5.8 to 8.5 (optimum 6.5–7.5); the range of salinity is 0 to 100 g/l with the optimum at 3–8 g/l of NaCl. Acid is not produced from carbohydrates. The tests for phenylalanine deaminase, nitrate reductase and urease are positive. The tests for arginine deaminase, lysine decarbox-

ydase, ornithine decarboxylase, starch hydrolysis, indole and H_2S production are negative. Tests for alkaline and acid phosphatases, leucine arylamidase, esterase (C_4), esterase lipase (C_8), naphthol-AS-BI-phosphohydrolase, as well as lipase (C_{14}), valine arylamidase, cystine arylamidase, trypsine, α-chemotrypsine, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are positive.

Yeast extract, acetate, pyruvate, glutarate, fumarate, caproate, heptanoate, butyrate, malate, DL-lactate, citrate, L-proline, L-tyrosine, butanol, and dulcitol are the sole sources of carbon and energy. Under the optimal conditions, growth was not observed on histidine, asparagine, L-glutamine, L-phenylalanine, L-valine, L-cysteine, L-leucine, glycine, DL-methionine, DL-tryptophan, DL-serine, succinate, lactate, formate, sorbitol, inositol, ethanol, propanol, glycerol, xylose, agarose, galactose, maltose, glucose, arabinose, mannose, sucrose, ribose, rhamnose, fructose, D-raffinose, and melibiose. Major fatty acids of the cell wall at the optimal growth temperature were $C_{18:1}\omega7$ and $C_{18:1}\omega9$. The DNA G+C content is 46.0 mol.%.

The organism was isolated from low-temperature water brines in the permafrost of the Arctic, Kolyma lowland, North-East Siberia. Type strain 2pS was deposited at the All-Russian Collection of Microorganisms, designated as VKM B-2270^T, and the Ukrainian Collection of Microorganisms, designated as IMB B-7124^T.

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