

Psychrobacter cryohalolentis sp. nov. and *Psychrobacter arcticus* sp. nov., isolated from Siberian permafrost

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Three Gram-negative, non-motile, non-pigmented, oxidase-positive coccobacilli capable of growth at temperatures from -10 to 30 °C and salinities of 0 to 1.7 M NaCl were isolated from Siberian permafrost and characterized. Both 16S rRNA and *gyrB* gene sequencing studies placed the isolates in the *Gammaproteobacteria* within the genus *Psychrobacter*. However, with higher bootstrap values and reproducible tree topologies, *gyrB* represented a more reliable phylogenetic marker for the taxonomy of *Psychrobacter* species. DNA–DNA hybridization data supported *gyrB* tree topologies and established two relatedness groups within the three isolates; neither of these groups was related at the species level to any previously described *Psychrobacter* species. The two groups of isolates could be differentiated phenotypically from 13 previously described *Psychrobacter* species using API strips. These results support the existence of two novel species of *Psychrobacter*, for which we propose the names *Psychrobacter cryohalolentis* sp. nov. (type strain K5^T = DSM 17306^T = VKM B-2378^T) and *Psychrobacter arcticus* sp. nov. (type strain 273-4^T = DSM 17307^T = VKM B-2377^T).

The genus *Psychrobacter* comprises psychrophilic to psychrotolerant, halotolerant, aerobic, non-motile, Gram-negative coccobacilli and was first described as a genus separate from *Acinetobacter* in 1986 (Bowman, 2005; Juni & Heym, 1986). Known *Psychrobacter* species are capable of reproduction at temperatures ranging from -10 to 37 °C. Examples range from the strict (stenothermal) psychrophile *Psychrobacter frigidicola* (0 to 22 °C, $T_{\text{opt}} = 15$ °C) to the eurythermal psychrophile *Psychrobacter okhotskensis* (-5 to 35 °C, $T_{\text{opt}} = 25$ °C). While *Psychrobacter* species are cold-adapted, other distinguishing characteristics include salt tolerance, natural competence and cellular fatty acid content (Juni & Heym, 1986; Moss *et al.*, 1988). *Psychrobacter* species have been isolated from a variety of low-temperature

marine environments including Antarctic sea ice, ornithogenic soil and sediments, the stomach contents of the Antarctic krill *Euphausia*, seawater (north-western Pacific Ocean, 300 m depth), the deep sea and the internal tissues of a marine ascidian (Bowman *et al.*, 1997; Maruyama *et al.*, 2000; Romanenko *et al.*, 2002; Yumoto *et al.*, 2003); other sources of *Psychrobacter* include pigeon faeces, fish, poultry, dairy products, fermented seafood, clinical sources and an infected lamb (Gonzalez *et al.*, 2000; Juni & Heym, 1986; Kämpfer *et al.*, 2002; Vela *et al.*, 2003; Yoon *et al.*, 2003).

Here, we report the characterization of three *Psychrobacter* isolates from Arctic permafrost (Bakermans *et al.*, 2003; Vishnivetskaya *et al.*, 2000). The taxonomic position of these isolates was established through a polyphasic approach utilizing phenotypic, genotypic, chemotaxonomic and phylogenetic analyses. These analyses led to the description of two novel species.

The three isolates were recovered from permafrost samples within the Kolyma lowland region of Siberia; in this area, the

Abbreviations: ANI, average nucleotide identity; PLFA, phospholipid fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates K5^T, 237-4^T and 215-51 are AY660685, AY444822 and AY444823, and those for the *gyrB* sequences obtained in this study are DQ143914–DQ143928 and CP000082.

permafrost is continuous, approximately 800 m thick and, at depth, remains stable at -9 to -11 °C (Gilichinsky *et al.*, 1992; Shi *et al.*, 1997). Isolates 273-4^T and 215-51 were recovered from borehole 1/97 cores at depths of 12.5 and 13.0 m, respectively, from a 20 000- to 30 000-year-old sandy loam within a 15–20 m late Pleistocene icy complex (Vishnivetskaya *et al.*, 2000). This complex froze as it was deposited and has remained frozen to modern times (Sher, 1974). In this region, the deposition rate of sediments is 1–2 mm per year; therefore, a 0.5 m difference in the depths of these samples implies a difference in time of deposition of 250 to 500 years. Isolate K5^T was recovered from a cryopeg [a highly saline (13 %) lens of water] intersected by borehole 16/99 at a depth of 11 m within a marine layer of the permafrost that was deposited beneath shallow lagoons at temperatures slightly above 0 °C and froze subaerially as the polar ocean regressed 110 000 to 112 000 years ago (Bakermans *et al.*, 2003; Gilichinsky *et al.*, 2003, 2005).

The isolates were non-motile, Gram-negative coccobacilli; diploid forms were common. Strains formed non-pigmented, circular, smooth, opaque colonies with a diameter of ~2 mm when grown on marine agar (MA) (Bowman, 2005). Cells were approximately 0.5×1.5 µm (width by length) in size. The pH range, salt tolerance and sodium requirement of the isolates were tested as described previously, except that the experiments were conducted in broth cultures and verified via three successive transfers of a 1:1000 dilution of inoculum into fresh medium (Bozal *et al.*, 2003). Isolate K5^T was able to grow at pH values ranging from 6 to 9.5; while isolates 273-4^T and 215-51 only grew at pH 7–9. Isolates K5^T, 273-4^T and 215-51 tolerated high concentrations of NaCl, respectively growing in 1.7, 1.3 and 1.3 M NaCl. *Psychrobacter* species typically tolerate high salt concentrations, from 1 to 2.6 M (Bowman, 2005). Na⁺ was not required for the growth of isolate K5^T; however, Na⁺ was required at concentrations of 10 and 5 mM, respectively, for the growth of isolates 273-4^T and 215-51. The majority of described *Psychrobacter* species grow in the absence of Na⁺; however, *P. frigidicola*, *Psychrobacter glacincola* and *Psychrobacter pacificensis* grow weakly in the absence of Na⁺, while *Psychrobacter fozii*, *Psychrobacter marincola* and *Psychrobacter submarinus* do not grow at all (Bowman, 2005).

The physiological and biochemical properties of the isolates and 13 previously described *Psychrobacter* species were determined (Table 1). API strips 20NE and ZYM (bioMérieux Vitek) were used according to the manufacturer's instructions except that incubation was carried out at 20 °C for 48 and 20 h, respectively, and that strips were inoculated with cells grown on MA for 3 days at 20 °C. While all of the isolates and species had similar patterns of response on the API strips, no two patterns were identical. Clustering of API test results did not yield significant differences from trees constructed using the 16S rRNA gene sequence (data not shown). Isolates K5^T and 273-4^T did not have phenotypes identical to any of the described

Psychrobacter species, suggesting that they represent distinct species. The phenotypes of isolates 273-4^T and 215-51 varied from each other only in reduction of nitrate to nitrite, indicating that these two isolates may be members of the same species. Variability in the ability to reduce nitrate within and between species of *Psychrobacter* is common (Bowman, 2005).

The growth temperature ranges of isolates K5^T and 273-4^T were -10 to 30 °C and -10 to 28 °C, respectively (growth at -10 and 28 °C was established in broth cultures). The growth temperature range of the isolates was compared with those of described *Psychrobacter* species by spotting 10 µl of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions of 3-day cultures grown at 20 °C onto MA and incubating at 37, 30, 20, ~4, 0 and -5 °C. Growth was considered possible only if the highest dilution showed growth by colony formation. While *Psychrobacter* species are not routinely tested for growth at temperatures below 0 °C, we demonstrated that, similar to our isolates, most species are capable of growth on MA at -5 °C (Table 1). The low-temperature limit for growth remains to be determined for many of these species.

The phospholipid fatty acid (PLFA) composition of membranes was determined using standard methods (Navarrete *et al.*, 2000; Ponder *et al.*, 2005). The profile of isolate K5^T was dominated by the monoenoic PLFAs 18:1ω7c (58 ± 11 %) and 16:1ω7c (31 ± 10 %), with 18:0 as a minor component (~7 %). The PLFA profile of isolate 273-4^T was dominated by the saturated PLFAs 18:0 (44.2 ± 0.5 %) and 16:0 (28.3 ± 0.5 %); however, in the presence of salt or low temperatures, the PLFA composition shifted to unsaturated PLFAs 18:1 and 16:1 (Ponder *et al.*, 2005). Both isolates had PLFA profiles similar to, but distinct from, all other *Psychrobacter* species, which predominantly contain the monoenoic PLFAs 18:1ω9c (30–84 %) and 16:1ω7c (3–53 %) (Bowman, 2005).

Phylogenetic analysis of the 16S rRNA gene sequence confirmed the placement of isolates K5^T, 273-4^T and 215-51 in the genus *Psychrobacter*. The 16S rRNA gene was amplified by PCR and sequenced as described previously (Reysenbach *et al.*, 1994; Suzuki & Giovannoni, 1996). Sequence reads were assembled using PHREP and CONSED (Gordon, 2004). Consensus 16S rRNA gene sequences and the most similar sequences from GenBank identified by BLAST searches were aligned against the most similar sequences in the Ribosomal Database Project II release 8.0 database (Maidak *et al.*, 2001) using the fast align procedure of the ARB software package (Ludwig *et al.*, 2004). Alignments were corrected manually by taking into account primary and secondary structure considerations and ambiguously aligned regions were removed from the analysis. Phylogenetic trees were constructed using the following methods: Fitch–Margoliash distance method implemented in the FITCH program of the PHYLIP package (Felsenstein, 2004), maximum-likelihood method implemented in the fastDNAmL program (Olsen *et al.*, 1994) and parsimony analysis as implemented in the PAUP 4.0 program.

Table 1. Physiological characteristics of the novel isolates and type strains of *Psychrobacter* species

Reference strains: 1, *P. faecalis* DSM 14664^T; 2, *P. fozii* LMG 21280^T; 3, *P. frigidicola* DSM 12411^T; 4, *P. glacincola* ATCC 700754^T; 5, *P. immobilis* ATCC 43116^T; 6, *P. jeotgali* JCM 11463^T; 7, *P. luti* LMG 21276^T; 8, *P. marincola* DSM 14160^T; 9, *P. okhotskensis* JCM 11840^T; 10, *P. pacificensis* IFO 16270^T; 11, *P. proteolyticus* DSM 13887^T; 12, *P. submarinus* DSM 14161^T; 13, *P. urativorans* ATCC 15174^T. ++, Strongly positive; +, positive; (+), weakly positive; −, negative; ND, no data. All strains were positive for esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and cytochrome oxidase. All strains were negative for trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, N₂ production from nitrates, tryptophanase (indole production), fermentation, arginine dihydrolase, protease (gelatin hydrolysis) and β-galactosidase. All strains were also negative for assimilation of D-glucose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid and phenylacetic acid. All data were obtained in this study.

Test	215-51	273-4 ^T	K5 ^T	1	2	3	4	5	6	7	8	9	10	11	12	13
Presence of:																
Alkaline phosphatase	+	++	+	+	++	−	+	++	+	+	+	++	+	++	++	+
Lipase (C14)	−	−	+	+	−	−	+	+	−	+	−	+	−	−	−	+
Valine arylamidase	+	+	−	+	+	−	+	+	−	−	+	−	+	+	+	−
Cysteine arylamidase	−	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−
Acid phosphatase	+	+	−	−	+	−	−	−	−	−	+	−	++	−	−	+
β-Galactosidase	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
α-Fucosidase	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Urease	−	−	−	−	(+)	−	−	−	+	−	−	−	−	−	−	(+)
β-Glucosidase	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	(+)
Reduction of nitrate to nitrite	−	+	−	+	−	−	+	+	+	+	−	−	−	−	−	−
Assimilation of:																
L-Arabinose	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Malic acid	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+
Trisodium citrate	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
Growth on MA at*:																
−5 °C	ND	+	+	−	+	ND	+	+	−	+	−	+	−	+	−	+
0 °C	ND	+	+	+	+	ND	+	+	+	+	−	+	−	+	−	+
30 °C	ND	−	+	+	+	ND	−	+	+	+	+	+	+	+	+	−
37 °C	ND	−	−	+	−	ND	−	−	−	−	−	−	−	−	−	−

*Growth was tested on MA at 37, 30, 20 ~4, 0 and −5 °C as described in the text. All strains grew at ~4 and 20 °C.

16S rRNA gene trees clearly demonstrated that isolates K5^T, 273-4^T and 215-51 were members of the genus *Psychrobacter* (Fig. 1a). The high nucleotide sequence identity of isolates 273-4^T and 215-51 (99.8% nucleotide identity) suggests that these isolates may be strains of the same species. Isolates 273-4^T and 215-51 formed a cluster with *Psychrobacter luti*, *P. okhotskensis* and *P. fozii*, all species that have been isolated from cold marine waters or sediments (Bozal *et al.*, 2003; Yumoto *et al.*, 2003). Interestingly, many of the species clustered according to growth temperature range, whether by the low-temperature or high-temperature limit. For example, *Psychrobacter faecalis*, *Psychrobacter jeotgali*, *P. marincola* and *P. submarinus* have low-temperature growth limits at 0–4 °C and high-temperature growth limits at 35–37 °C and form a cluster of species in 16S rRNA gene trees (bootstrap values of 87–100%). *Psychrobacter urativorans*, *P. glacincola* and *P. frigidicola* do not grow above 27 °C and cluster together (bootstrap values of 67 and 72%). These associations support the hypothesis that there are definitive genomic and molecular differences between species that

lead to differences in growth temperature ranges. While monophyletic groups could be identified within the 16S rRNA gene tree, the low bootstrap values (25–72%) and the lack of reproducibility in tree topologies when different tree algorithms were used (data not shown) indicated that the resolution of the 16S rRNA gene was not high enough to generate a reliable phylogenetic tree of closely related species (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994).

To define better the phylogenetic relationships between *Psychrobacter* species, analyses were performed with nearly full-length sequences of *gyrB*. To design primers targeting conserved regions of the *gyrB* gene, nucleotide sequences from complete genome sequences of *Coxiella burnetii* RSA 493 (GenBank accession no. NC_002971), *Xanthomonas campestris* pv. *campestris* ATCC 33913^T (NC_003902), *Xanthomonas axonopodis* pv. *citri* 306 (NC_003919), *Xylella fastidiosa* 9a5c (NC_002488), *Xylella fastidiosa* Temecula1 (NC_004556) and isolate 273-4^T (CP000082) were translated in ARB and their corresponding amino acid

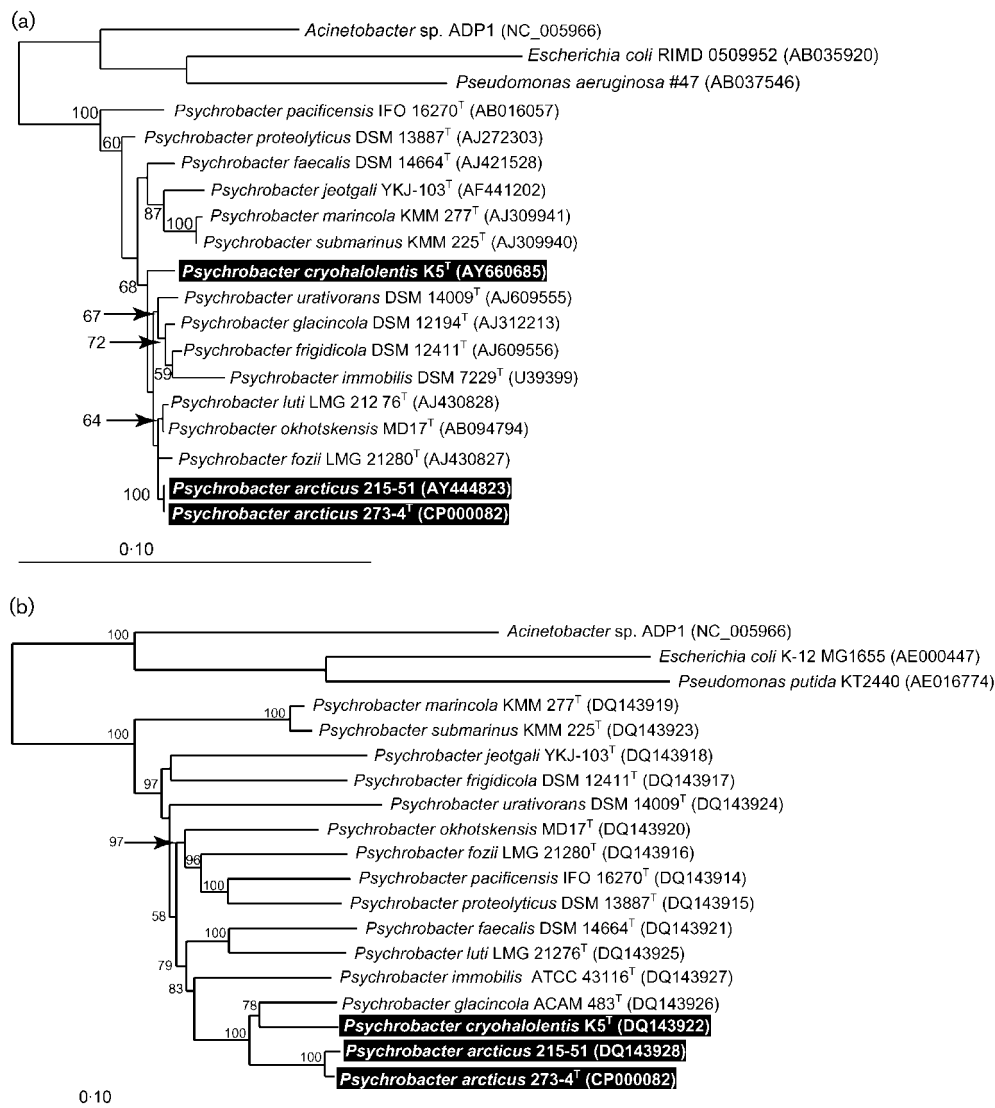


Fig. 1. Fitch–Margoliash phylogenetic trees of 16S rRNA (a) and *gyrB* (b) gene sequences. Isolate sequences are highlighted. Bars, 0.1 base substitutions per nucleotide position. Numbers at nodes represent numbers of 100 bootstrap values that were greater than 50%.

sequences were aligned using the T-COFFEE program (Notredame *et al.*, 2000). Primers were designed by the Probe Design tool of the ARB package and modified by visual inspection. The *gyrB* gene was amplified using PCR in 50 μ l reactions containing 50–100 ng genomic DNA, 2.5 mM MgCl₂, 50 pmol each primer [147-FD1 (5'-RWRCGYCCHGGVATGTAYAT) and 2421-RD (5'-TTCATYTCRCCARVCCYTT)], 1 \times PCR buffer (Promega), 200 μ M each dNTP, 0.2 mg BSA ml⁻¹ (New England Biolabs) and 2.5 U *Taq* polymerase (Promega). The following touchdown thermal profile was used for incubation: 95 °C for 5 min; 10 cycles at 95 °C for 45 s, 55.5 °C (minus 0.5 °C per cycle) for 50 s and 72 °C for 3 min; 28 cycles at 95 °C for 45 s, 54 °C for 50 s and 72 °C for 3 min; and a final extension at 72 °C

for 7 min. The ~2.2 kbp PCR products were concentrated and gel-purified prior to sequencing by the GTSF, MSU, using primers 147-FD1, 2421-RD, 480F (5'-ATGAATATCTGGCGTGAAGG), 474R (5'-CGCCAGATATTCATCTCAAG), 704F (5'-TGAGCGTATTGATAAGCGTC), 1500R (5'-TCAGGCGTTTGACGGAAGAA) and 2040R (5'-GGT-TGCGGTGCATGGATATT). Sequences were assembled and trees constructed as described above.

As anticipated, the *gyrB* gene tree had a different topology from the 16S rRNA gene tree (Fig. 1b) (Yamamoto & Harayama, 1996). For example, *P. faecalis* and *P. jeotgali* no longer formed a cluster with each other or with *P. submarinus* and *P. marincola*. Isolates 273-4^T and 215-51

still formed a cluster in *gyrB* trees, but their closest relatives were now isolate K5^T and *P. glacincola*, relationships that were supported by DNA–DNA hybridization data (see below). The high bootstrap values (48–100 %; 9 of the 13 nodes had bootstrap values greater than 96 %) and reproducibility of tree topologies using three different phylogeny reconstruction methods (data not shown) indicated that *gyrB* is a better phylogenetic marker for establishing phylogenetic relationships between *Psychrobacter* species than the 16S rRNA gene.

To examine the DNA relatedness of isolates to each other and to previously described *Psychrobacter* species on a broader scale, whole genome DNA–DNA hybridizations were performed. Genomic DNA was prepared from cells grown at 20 °C using the Qiagen Genomic DNA kit with 500/G Genomic-tips (Qiagen). DNA fixation, probe preparation and quadruplicate reciprocal hybridization was performed in microplates with a hybridization temperature of 37 °C (Ezaki *et al.*, 1989). All three isolates had DNA–DNA relatedness values of 50 % or less with other described *Psychrobacter* species (Fig. 2). Isolates 273-4^T and 215-51 can be considered to belong to the same species as their DNA was 89 % related, while isolate K5^T had 64 and 50 % relatedness with 273-4^T and 215-51, respectively. These data demonstrate that isolate K5^T and isolate 273-4^T represent distinct species of *Psychrobacter*, as their DNA–DNA relatedness values fall well below the 70 % similarity cut-off recommended to define a species (Wayne *et al.*, 1987).

Additional analyses were possible due to the availability of whole genome sequences for isolates K5^T and 273-4^T. The average nucleotide identity (ANI) of all conserved

genes between the two isolates was calculated to be 88 % (Konstantinidis & Tiedje, 2005). Based on the current standard for species description, DNA–DNA reassociation values of 70 % correspond to an ANI of about 94–95 %; for example, the ANI between *Escherichia coli* and *Salmonella* spp. genomes is ~80 % (Konstantinidis & Tiedje, 2005). Thus, by this definition, the two isolates can also be considered to represent distinct species.

Isolates K5^T, 273-4^T and 215-51 belong in the genus *Psychrobacter* based on physiological and phenotypic features detailed in the original description (Juni & Heym, 1986) and based on phylogenetic analyses. They are halotolerant, psychrotolerant, aerobic, non-motile, Gram-negative, non-pigmented, oxidase-positive coccobacilli that are unable to grow at temperatures above 30 °C. Phylogenetically, these isolates were most closely related to *P. glacincola*. Phenotypic, genotypic, chemotaxonomic and phylogenetic analyses demonstrated that isolates 273-4^T and 215-51 were strains of the same species (273-4^T was designated the type strain). However, DNA–DNA hybridization studies demonstrated that isolates K5^T and 273-4^T were members of distinct species with relatedness values to previously described species of *Psychrobacter* well below 70 % (Wayne *et al.*, 1987). Thus, isolates K5^T and 273-4^T form two unique taxa within the genus *Psychrobacter* and it is proposed that the new groups are named *Psychrobacter cryohalolentis* sp. nov. and *Psychrobacter arcticus* sp. nov., respectively.

Description of *Psychrobacter cryohalolentis* sp. nov.

Psychrobacter cryohalolentis (cry.o.ha.lo.len'tis. Gr. n. *kryos* ice, Gr. n. *hals* salt, L. gen. fem. n. *lentis* from a lens; N.L. gen. fem. n. *cryohalolentis* from a frozen salt lens).

Cells are Gram-negative, non-motile, non-pigmented, non-spore-forming coccobacilli, 0.9–1.3 µm long and 0.5–0.8 µm wide. Growth occurs at –10 to 30 °C. Optimal growth temperature is 22 °C. Colonies on MA are about 2 mm in diameter, smooth, opaque and circular after 5 days at 20 °C. NaCl is not required for growth, but growth occurs in 1.7 M NaCl. Strictly aerobic; oxidase test is positive. Acid is not produced from carbohydrates. Cells are not able to reduce nitrate to nitrite. Urease and tryptophan deaminase are not produced. Positive in the following biochemical tests: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative for hydrolysis of casein. Growth occurs on citrate, lactate, acetate and L-glutamic acid. Cells can reduce Tweens 40 and 80. The main cellular fatty acids are 18:1ω7c and 16:1ω7c. The G+C content of DNA of the type strain is 42.3 mol%. Genome size is ~3.1 Mb.

The type strain, strain K5^T (=DSM 17306^T=VKM B-2378^T), was isolated from a cryopeg within permafrost in the Kolyma lowland, Siberia, Russia.

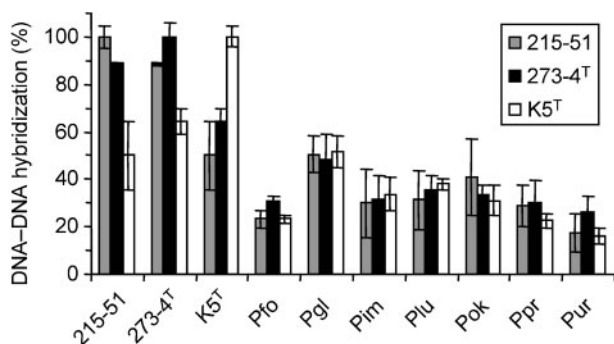


Fig. 2. Comparison of DNA–DNA hybridization of isolates with various *Psychrobacter* species. Error bars indicate the standard deviation of values for reciprocal hybridizations in triplicate. Abbreviations: Pfo, *P. fozii* LMG 21280^T; Pgl, *P. glacincola* ATCC 700754^T; Pim, *Psychrobacter immobilis* ATCC 43116^T; Plu, *P. luti* LMG 21276^T; Pok, *P. okhotskensis* JCM 11840^T; Ppr, *Psychrobacter proteolyticus* DSM 13887^T; Pur, *P. urativorans* ATCC 15174^T.

Description of *Psychrobacter arcticus* sp. nov.

Psychrobacter arcticus (arc'tic.us. L. masc. adj. *arcticus* northern, Arctic).

Cells are Gram-negative, non-motile, non-pigmented, non-spore-forming coccobacilli, 1.62 ± 0.13 μm long and 0.73 ± 0.03 μm wide. Growth occurs at -10 to 28 °C. Optimal growth temperature is 22 °C. Colonies on MA are about 2 mm in diameter, smooth, opaque and circular after 5 days at 20 °C. At least 10 mM NaCl is required for growth. Growth can occur in 1.25 M NaCl. Strictly aerobic; oxidase and catalase tests are positive. Acid is not produced from carbohydrates. Strains variably reduce nitrate to nitrite (type strain is positive). Urease and tryptophan deaminase are not produced. Positive in the following biochemical tests: alkaline phosphatase, esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative for hydrolysis of casein. Growth occurs on lactate, acetate, glutamate, pyruvate, butyrate and leucine. Cells can reduce Tweens 40 and 80. The main cellular fatty acids are 18:0 and 16:0 (or 18:1 and 16:1 at low temperatures or high salt concentrations). The G + C content of DNA is 42.7 mol%. Genome size is 2.6 Mb.

The type strain, strain 273-4^T (=DSM 17307^T =VKM B-2377^T), was isolated from permafrost sediment cores in the Kolyma lowland, Siberia, Russia.

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