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 K5T = DSM 17306T = VKM B-2378T) and Psychrobacter arcticus sp. nov. (type strain 273-4T = DSM 17307T = VKM B-2377T).

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, Topt = 15 °C) to the eu thermophilic psychrophile Psychrobacter okhotakensis (−5 to 35 °C, Topt = 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
permafrost is continuous, approximately 800 m thick and, at depth, remains stable at \(-9 \text{ to } -11^\circ C\) (Gilichinsky et al., 1992; Shi et al., 1997). Isolates 273-4\textsuperscript{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\textsuperscript{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 \(\circ C\) 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 cococci; diploid forms were common. Strains formed non-pigmented, circular, smooth, opaque colonies with a diameter of \(\sim 2\) mm when grown on marine agar (MA) (Bowman, 2005). Cells were approximately 0.5–1.5 \(\mu m\) (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 1:1,000 dilution of inoculum into fresh medium (Bozal et al., 2003). Isolate K5\textsuperscript{T} was able to grow at pH values ranging from 6 to 9.5; while isolates 273-4\textsuperscript{T} and 215-51 only grew at pH 7–9. Isolates K5\textsuperscript{T}, 273-4\textsuperscript{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\textsuperscript{+} was not required for the growth of isolate K5\textsuperscript{T}; however, Na\textsuperscript{+} was required at concentrations of 10 and 5 mM, respectively, for the growth of isolates 273-4\textsuperscript{T} and 215-51. The majority of described *Psychrobacter* species grow in the absence of Na\textsuperscript{+}; however, *P. frigidicola*, *Psychrobacter glacincola* and *Psychrobacter pacificensis* grow weakly in the absence of Na\textsuperscript{+}, 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 Vittek) were used according to the manufacturer’s instructions except that incubation was carried out at 20 \(\circ C\) for 48 and 20 h, respectively, and that strips were inoculated with cells grown on MA for 3 days at 20 \(\circ C\). 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\textsuperscript{T} and 273-4\textsuperscript{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\textsuperscript{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\textsuperscript{T} and 273-4\textsuperscript{T} were \(-10\) to \(30\) \(\circ C\) and \(-10\) to \(28\) \(\circ C\), respectively (growth at \(-10\) and \(28\) \(\circ C\) was established in broth cultures). The growth temperature range of the isolates was compared with those of described *Psychrobacter* species by spotting 10 \(\mu l\) of \(10^{-1}\), \(10^{-2}\), \(10^{-3}\) and \(10^{-4}\) dilutions of 3-day cultures grown at 20 \(\circ C\) onto MA and incubating at 37, 30, 20, \(\sim 4\), 0 and \(\sim 5\) \(\circ C\). 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 \(0\) \(\circ C\), we demonstrated that, similar to our isolates, most species are capable of growth on MA at \(-5\) \(\circ C\) (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\textsuperscript{T} was dominated by the monoenoic PLFAs 18:1\textsuperscript{7c} (58 ± 11 %) and 16:1\textsuperscript{7c} (31 ± 10 %), with 18:0 as a minor component (\(\sim 7\) %). The PLFA profile of isolate 273-4\textsuperscript{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 18:1\textsuperscript{9c} (30–84 %) and 16:1\textsuperscript{7c} (3–53 %) (Bowman, 2005).

Phylogenetic analysis of the 16S rRNA gene sequence confirmed the placement of isolates K5\textsuperscript{T}, 273-4\textsuperscript{T} and 215-51 in the genus *Psychrobacter*. The 16S rRNA gene was amplified by PCR and sequenced as described previously (Reyesenbach et al., 1994; Suzuki & Giovannoni, 1996). Sequence reads were assembled using PHRED 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.
16S rRNA gene trees clearly demonstrated that isolates K5T, 273-4T and 215-51 were members of the genus Psychrobacter (Fig. 1a). The high nucleotide sequence identity of isolates 273-4T and 215-51 (99.8% nucleotide identity) suggests that these isolates may be strains of the same species. Isolates 273-4T and 215-51 formed a cluster with Psychrobacter luti, Psychrobacter okhotskensis and P. fozi, 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, Psychrobacter marincola and Psychrobacter 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, Psychrobacter glacincola and Psychrobacter 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 Xyella fastidiosa RSA 493 (GenBank accession no. NC_002971), Xanthomonas citri pv. citri 306 (NC_003919), Xylella fastidiosa 9a5c (NC_002488), Xylella fastidiosa Temecula1 (NC_004556) and isolate 273-4T (CP000082) were translated in ARB and their corresponding amino acid sequences were translated in ARB and their corresponding amino acid sequences were translated in ARB and their corresponding amino acid sequences.
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 µl reactions containing 50–100 ng genomic DNA, 2·5 mM MgCl₂, 50 pmol each primer [147-FD1 (5'-RWRCGYCCH-GGVATGTAYAT) and 2421-RD (5'-TTCATYTCRCCTA-RVCCYTT)], 1 × PCR buffer (Promega), 200 µM 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'-ATGAATATCTTGGCGTGAAAG), 1288 474R (5'-CGCCAGATATTCATCTCAAG), 704F (5'-TGAGCGTGATTGACTAGCCGTC), 1500R (5'-TCAGGGCTTGAGGCAGAAGAA) and 2040R (5'-GTTTGCGGATGGGATT). 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 (Yamamoto & Harayama, 1996). For example, P. faecalis and P. jeotgali no longer formed a cluster with each other or with P. submarinus and P. marina. Isolates 273-4ᵀ and 215-51

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**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%.
still formed a cluster in gyrB trees, but their closest relatives were now isolate K5T and \textit{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 \textit{gyrB} is a better phylogenetic marker for establishing phylogenetic relationships between \textit{Psychrobacter} species than the 16S rRNA gene.

To examine the DNA relatedness of isolates to each other and to previously described \textit{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 \textit{et al.}, 1989). All three isolates had DNA–DNA relatedness values of 50% or less with other described \textit{Psychrobacter} species (Fig. 2). Isolates 273-4T and 215-51 can be considered to belong to the same species as their DNA was 89% related, while isolate K5T had 64 and 50% relatedness with 273-4T and 215-51, respectively. These data demonstrate that isolate K5T and isolate 273-4T represent distinct species of \textit{Psychrobacter}, as their DNA–DNA relatedness values fall well below the 70% similarity cut-off recommended to define a species (Wayne \textit{et al.}, 1987).

Additional analyses were possible due to the availability of whole genome sequences for isolates K5T and 273-4T. 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 \textit{Escherichia coli} and \textit{Salmonella} spp. genomes is ~80% (Konstantinidis \& Tiedje, 2005). Thus, by this definition, the two isolates can also be considered to represent distinct species.

Isolates K5T, 273-4T and 215-51 belong in the genus \textit{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 \textit{P. glacincola}. Phylogenetic, genotypic, chemotaxonomic and phylogenetic analyses demonstrated that isolates 273-4T and 215-51 were strains of the same species (273-4T was designated the type strain). However, DNA–DNA hybridization studies demonstrated that isolates K5T and 273-4T were members of distinct species with relatedness values to previously described species of \textit{Psychrobacter} well below 70% (Wayne \textit{et al.}, 1987). Thus, isolates K5T and 273-4T form two unique taxa within the genus \textit{Psychrobacter} and it is proposed that the new groups are named \textit{Psychrobacter cryohalolentis} sp. nov. and \textit{Psychrobacter arcticus} cryohalolentis sp. nov., respectively.

\textbf{Description of \textit{Psychrobacter cryohalolentis} sp. nov.}

\textit{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 mm long and 0.5–0.8 mm 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 K5T (=DSM 17306T =VKM B-2378T), was isolated from a cryopeg within permafrost in the Kolyma lowland, Siberia, Russia.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Comparison of DNA–DNA hybridization of isolates with various \textit{Psychrobacter} species. Error bars indicate the standard deviation of values for reciprocal hybridizations in triplicate. Abbreviations: Plo, \textit{P. fozii} LMG 21280T; Pgl, \textit{P. glacincola} ATCC 700754T; Pim, \textit{Psychrobacter immobilis} ATCC 43116T; Plu, \textit{P. luti} LMG 21276T; Pok, \textit{P. okhotskensis} JCM 11840T; Ppr, \textit{Psychrobacter proteolyticus} DSM 13887T; Pur, \textit{P. urativorans} ATCC 15174T.}
\end{figure}
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-Bl-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 their mono- and dihydroxy derivatives). Cells contain Tween 80. Cells contain Tween 80.

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

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References


