**NOTE**

*Psychromonas marina* sp. nov., a novel halophilic, facultatively psychrophilic bacterium isolated from the coast of the Okhotsk Sea

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A facultatively psychrophilic bacterium, strain 4-22ᵀ, was isolated from a cold current off the Monbetsu coast of the Okhotsk Sea in Hokkaido, Japan. The isolate was a rod-shaped facultative anaerobe that reduced nitrate to nitrite and hydrolysed starch, DNA and algicin acid, but not chitin or gelatin. The isolate grew at 0 °C, but not at 26 °C; the optimum growth temperature was 14–16 °C. NaCl was required for growth. The DNA G+C content was 43.5 mol%. The whole-cell fatty acids consisted of significant amounts of an unsaturated fatty acid, C₁₆:₁ < ω₃ and a saturated fatty acid, C₁₆:₀. A polyunsaturated fatty acid, docosahexaenoic acid (C₂₂:₆), was also detected (1.6%). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 4-22ᵀ was closely related to *Psychromonas antarctica* (95.7% similarity). DNA–DNA hybridization revealed a relatedness of 31% between strain 4-22ᵀ and *P. antarctica*. Based on physiological and biochemical characteristics and the phylogenetic position as determined by 16S rRNA gene analysis and DNA–DNA relatedness, it is concluded that the isolate represents a novel species, for which the name *Psychromonas marina* sp. nov. is proposed. The type strain is 4-22ᵀ (= JCM 10501ᵀ = IAM 14899ᵀ = NCIMB 13792ᵀ).

Keywords: *Psychromonas marina* sp. nov., facultative psychrophile, polyunsaturated fatty acids, 16S rRNA phylogeny

The temperature of most of the Earth’s biosphere is below 10 °C. However, the temperature of some environments fluctuates seasonally. Morita (1975) defined psychrophiles as those having an optimum growth temperature of about ≤ 15 °C and a maximum growth temperature of ≤ 20 °C; facultatively psychrophilic or psychrotolerant micro-organisms can grow at 0–5 °C, with a maximum growth temperature above 20 °C. Psychrophilic micro-organisms are considered to live in permanently cold environments, such as polar regions, the deep sea and cold currents in the ocean. Recently, the diversity and evolutionary relationships of psychophilic bacteria in the deep sea (DeLong et al., 1997) and Antarctic sea ice (Bowman et al., 1997) have been studied. Both studies showed that psychrophilic strains belong to the γ-Proteobacteria, such as *Colwellia*, *Shewanella* and several new groups. In the Antarctic sea ice, Gram-positive bacteria such as *Planococcus* and members of the family *Flavobacteriaceae* were isolated. On the other hand, Gosink & Staley (1995) isolated psychrophilic strains belonging to the α-, β- and γ-Proteobacteria and to the family *Flavobacteriaceae* from the Antarctic sea ice. Recently, a novel group of psychophilic bacteria belonging to the γ-Proteobacteria, *Psychromonas antarctica*, was isolated from sediment taken from below the cyanobacterial mat of a high saline pond near Bratina Island on the McMurdo Ice Shelf, Antarctica, by anaerobic enrichment (Mountfort et al., 1998). Several strains belonging to the same lineage as this isolate in the phylogenetic tree based on 16S rRNA gene sequences

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have been reported in several studies (Bowman et al., 1997; Delong et al., 1997; Gosink & Staley, 1995).

In this study, a facultative psychrophile, strain 4-22\textsuperscript{T}, belonging to the same lineage as *P. antarctica* in the 16S rRNA phylogenetic tree, is reported. The strain was isolated from a cold current carrying drifting ice in the Okhotsk Sea as a result of screening for isolates that have the ability to grow at 0 °C but not at 25 °C on marine agar 2216 and the presence of \(\beta\)-galactosidase activity. Phenotypic and chemotaxonomic characteristics were examined, as well as the phylogenetic position of the isolate, and it was found that the strain should be classified as a novel species belonging to the genus *Psychromonas*.

Sea water was collected in February, 1997, from the Monbetsu coast (44° 31' N, 143° 39' E), Hokkaido, Japan, which opens to the Okhotsk Sea. The temperature of the sea-water sample was −1 °C. The sea-water sample was spread directly onto pre-chilled marine agar 2216 (Difco) plates and incubated at 0 °C for 4 weeks. About 300 colonies were randomly selected and subcultured for purification. Strain 4-22\textsuperscript{T}, which could not grow on marine agar 2216 at 25 °C and exhibited \(\beta\)-galactosidase activity, was selected. Cells for chemotaxonomic analysis were harvested after 2 days cultivation with shaking (140 r.p.m.) at 15 °C. In addition to the isolate, *P. antarctica* DSM 10704\textsuperscript{T} was used as the reference strain for DNA–DNA relatedness. The micro-organism was cultivated using marine broth 2216 (Difco) with shaking (100 r.p.m.) at 12 °C for 2 days.

Marine broth or agar 2216 (Difco) was used as the basal medium for aerobic cultivation unless otherwise stated. The culture was incubated at 15 °C for 3 weeks and the experiment was performed at least twice. Morphological, physiological and biochemical tests were performed as described by Barrow & Feltham (1993). Carbohydrate metabolism was tested according to the method of Leifson (1963). The result was checked daily for 1 month after inoculation. For hydrolysis of macromolecular substances, PYB agar (pH 7.5) was used as the basal medium. PYB medium contained (1\textsuperscript{−1} 50% Herbst's artificial sea water): 5 g polypeptone (Nihon Pharmaceutical), 1 g yeast extract (Kyokuto), 15 g agar and 0.5–10% substrate. Herbst's artificial sea water contained the following: (l\textsuperscript{−1} distilled water): 30 g NaCl, 0.7 g KCl, 5.3 g MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 1.3 g CaSO\(_4\) \(\cdot\) 2H\(_2\)O and 10.8 g MgCl\(_2\), 6H\(_2\)O. Alginase activity was determined using a 2-week-old culture on a PYB agar plate containing 0.5% alginic acid by flooding the plates with 70% ethanol. Susceptibility to the vibriostatic compound O/129 (2,4-diamino-6,7-di-iso-propylpiperidine phosphate) was determined after agar plate culture for 1 week using diagnostic discs (10 and 150 µg per disc; Oxoid). Various substrates were tested as sole sources of carbon and energy in USTM medium (pH 7.5) containing 0.2% substrate, 50 mM Tris/HCl, 190 mM NH\(_4\)Cl, 0.33 mM K\(_2\)HPO\(_4\) and 0.1 mM FeSO\(_4\) \(\cdot\) 7H\(_2\)O in 1 l of 50% Herbst's artificial sea water (described above). Media lacking a carbon source were prepared as negative controls to account for any background growth. To compare growth under anaerobic conditions with that under aerobic conditions, marine broth 2216 was used and air was replaced with N\(_2\) as the gas phase for anaerobic conditions.

The optimum growth temperature of strain 4-22\textsuperscript{T} was determined using a temperature gradient incubator (Toyokagaku Sangyo). The temperature gradient range was 1–25 °C and L-shaped tubes containing 10 ml marine broth 2216 were used.

Cells grown aerobically and anaerobically on marine agar 2216 were suspended in physiological saline. Cells were grown anaerobically by the method of Hungate (1950) using black rubber stoppers to seal the tubes. A small drop of the suspension was placed on a carbon-coated copper grid and negatively stained with 1% phosphotungstic acid and observed by TEM (H-800; Hitachi).

Whole-cell fatty acids were extracted from 100 mg freeze-dried cells, which were cultivated on marine broth 2216, and esterified by acid methanolysis. The methyl esters were analysed using a GLC equipped with a flame-ionization detector (model GC 353; GL Sciences) and an SP-2560 column (0.25 mm \(\times\) 100 m, 0.2 µm film; Supelco) at an oven temperature of 140 °C for 15 min, which was then increased to 240 °C at 4 °C min\(^{−1}\). Fatty acids were identified by comparing their retention times with those of fatty acid methyl ester standards purchased from Supelco and GL Sciences, and using GC/MS (model INCOS 50; Finnigan MAT) connected to a GLC (model 3400; Varian).

DNA was prepared from bacterial cells according to the method of Marmur (1961). The G + C content of the DNA was determined according to the method of Tamaoka & Komagata (1984). The levels of DNA relatedness were determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes and microplates.

The almost complete 16S rRNA gene of strain 4-22\textsuperscript{T} was amplified by PCR using *P. fluorescens* DNA polymerase (Stratagene) and a primer set, 5’-CGCTGGCGGCA-GGCTAA-3’ and 5’-AAGGAGGTGATCCAGCGGC-3’, respectively corresponding to nt 34–51 and 1521–1540 in the 16S rRNA sequence of *Escherichia coli* (Brosius et al., 1978). The amplified DNA fragment was cloned into a plasmid vector and the nucleotide sequence was determined using a BigDye Primer Cycle Sequencing kit (Applied Biosystems) with a DNA sequencer (model 377; Applied Biosystems). The search for a sequence similar to the 16S rRNA gene sequence of strain 4-22\textsuperscript{T} was performed with *sequence_match* version 2.7 of the Ribosomal Database Project (Maidak et al., 1996). Multiple alignments of the sequences were performed, nucleotide substitution rates (\(K_{\text{sub}}\) values) were calculated.
and a neighbour-joining phylogenetic tree (Kimura, 1980; Saitou & Nei, 1987) was constructed using the program CLUSTAL W (Thompson et al., 1994) with the determined 1473 bp sequence. The similarity values of the sequences were calculated using the GENETYX computer program (Software Development).

Colonies of strain 4-22T were circular, convex and colourless and had an entire margin after incubation at 15 °C on marine agar. The cells were non-spore-forming, Gram-negative rods, 1.5–2.0 × 0.8–1.2 μm in size. TEM revealed a single polar flagellum, as well as numerous blebs and fimbriae around the cell surface (Fig. 1). The blebs were observed regardless of culture age and presence or absence of oxygen (data not shown). Growth occurred at 0 °C. The optimum growth temperature was 14–16 °C. The upper limits of growth temperature for the strain were 20 °C on solid medium and 25 °C in liquid medium. Strain 4-22T was isolated from a sea-water sample that was obtained from the coast of the Okhotsk Sea in Hokkaido, Japan, when a cold current containing drifting ice from the east coast of Sakhalin, Russia, flowed into the area. It is considered that the sample contained permanently cold sea water suitable for the isolation of psychrophilic micro-organisms. Although the strain was considered to be a cold-adapted bacterium, the isolate was categorized as a facultatively psychrophilic bacterium by Morita’s definition because its upper limit of growth temperature was around 25 °C in liquid medium. However, it is considered that strain 4-22T is quite closely related to psychrophilic micro-organisms.

The physiological and biochemical characteristics of strain 4-22T are given in the species description. Growth under anaerobic conditions was not as good as that under aerobic conditions, as determined turbidimetrically.

GLC analysis of the methyl ester derivatives of the fatty acids of the strain revealed that the major components were C16:0 (43.6%), C16:1 (39.4%), C18:1 (3.1%), C14:1 (3.2%), and iso-C16:0 (2.7%). A detectable amount (1.6%) of the polyunsaturated fatty acid docosahexaenoic acid (C22:6) was also present.

The almost complete 16S rRNA sequence of strain 4-22T, which consists of 1473 nt, had 95.7% similarity to that of P. antarctica. In contrast, its similarity to the 16S rRNA sequences of Colwellia, Pseudoalteromonas, Alteromonas, Moritella, Ferrimonas, Escherichia, Tolamonas, Aeromonas, Photobacterium, Vibrio and Shewanella species was less than 90%. The phylogenetic tree constructed based on the 16S rRNA sequence showed that the isolate is related to the γ-Proteobacteria and most closely related to P. antarctica.
The isolate also revealed high 16S rRNA sequence similarity values to a gas vacuolate strain, IC004 (95-1%) (Gosink & Staley, 1995), and a marine psychrophilic strain, 90-P (95-3%) (Bowman et al., 1997), isolated from Antarctica, as well as with a psychrophilic and barophilic marine bacterium, strain CNPT3 (97-2%), isolated from the deep sea (Delong et al., 1997) (Fig. 2). These strains are all cold-adapted micro-organisms isolated from permanently cold environments. Until now, there have been no examples of bacteria related to the genus Psychromonas described in terms of the 16S rRNA sequence other than cold-adapted micro-organisms isolated from permanently cold environments. These facts suggest that the genus Psychromonas is widely distributed in permanently cold environments.

The genomic G+C content of strain 4-22^T was 43.5 mol%. According to 16s rRNA sequence analysis, strain 4-22^T was closely related to the genus Psychromonas. Only one species belonging to the genus Psychromonas, P. antarctica, has been previously reported. Therefore, the level of DNA relatedness between strain 4-22^T and P. antarctica DSM 10704^T was determined; relatedness was 31%.

Phylogenetic analysis based on the 16S rRNA sequence revealed that strain 4-22^T was closely related to P. antarctica (Mountfort et al., 1998). The similarity in 16S rRNA sequence between the two strains was 95-7%, which may not be sufficiently high to confirm that they are of the same species. However, several of their growth and phenotypic characteristics differed (Table 1). The DNA–DNA relatedness value between strain 4-22^T and P. antarctica was 31%. This value indicated that these two strains do not belong to the same species. On the basis of phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA sequence and relatedness by DNA–DNA hybridization, strain 4-22^T was confirmed as a novel species belonging to the genus Psychromonas, for which the name Psychromonas marina sp. nov. is proposed.

**Description of Psychromonas marina sp. nov.**

*Psychromonas marina* (ma.ri’na. L. adj. marina of the sea, marine).

Cells are rod-shaped (1.5–2.0 × 0.8–1.2 µm), Gram-negative and have a single polar flagellum. Colonies are circular, convex and colourless with an entire margin. The species is positive for catalase and oxidase. It ferments D-glucose, D-fructose, D-maltose, sucrose, D-xyllose, D-mannitol and D-galactose; gas production is not detected during fermentation. Growth is not observed in the absence of NaCl in the culture medium; however, growth occurs in a medium supplemented with 3–5% NaCl, but not in a medium with salinity higher than 7% NaCl. Does not require an organic nitrogen source, amino acids or vitamins for growth. Susceptible to the vibriostatic compound O/129 (10 and 150 µg). Growth occurs between 0 and 25 °C, but not at temperatures higher than 26 °C. The organism is positive for the production of H₂S, the ONPG test and the reduction of NO₃ to NO₂, but negative for indole production. It hydrolyses starch, DNA, alginic acid, tributyrin and Tweens 20, 40, 60 and 80, but not casein, chitin or gelatin. The organism utilizes D-glucose, glycerol, D-fructose, lactose, D-maltose, sucrose, D-xyllose, D-mannitol, N-acetyl glucosamine, D-galactose and D-cellobiose as sole carbon and energy sources for growth, but not L-arabinose, D-mannose, melibiose, raffinose, sorbitol, myo-inositol, L-rhamnose or trehalose. The whole-cell fatty acids consist predominantly of C₁₆:0, C₁₆:1, C₁₈:1, C₁₃:0 and iso-C₁₆:0. The polyunsaturated fatty acid C₂₂:6 is also present (1-6%). The G+C content of the DNA is 43.5 mol%, as determined by HPLC. The type strain is strain 4-22^T (= JCM 10501^T = IAM 14899^T = NCIMB 13792^T).

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**References**


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