Psychrobacter maritimus sp. nov. and *Psychrobacter arenosus* sp. nov., isolated from coastal sea ice and sediments of the Sea of Japan

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Four strains of Gram-negative, aerobic, psychrotolerant, non-motile, non-pigmented bacteria were isolated from coastal sea-ice and sediment samples. These strains displayed the general chemotaxonomic and phenotypic features of members of the genus *Psychrobacter*. 16S rRNA gene sequencing positioned the three isolates KMM 3646^T, KMM 3643 and KMM 3645 and isolate KMM 3659^T in two distinct lineages within the genus *Psychrobacter*, displaying less than 98.5% sequence similarity to the type strains of other *Psychrobacter* species. Genomic distinctness was supported by phenotypic differences in growth temperatures, salinity range for growth and metabolic properties. Based on a combination of phenotypic and biochemical characteristics and phylogenetic position, it is proposed that the members of these two distinct lineages represent two novel species, for which the names *Psychrobacter maritimus* sp. nov. (type strain Pi2-20^T = KMM 3646^T = DSM 15387^T) and *Psychrobacter arenosus* sp. nov. (type strain R7^T = KMM 3659^T = DSM 15389^T) are proposed.

Gram-negative, aerobic, non-motile, psychrophilic or psychrotolerant, non-pigmented bacteria belonging to the genus *Psychrobacter* (Juni & Heym, 1986) have been reported to be components of the population of psychrophiles associated with Antarctic and marine environments (Bowman *et al.*, 1996, 1997a). Several *Psychrobacter* species originate from these ecosystems, such as *Psychrobacter urativorans*, *Psychrobacter frigidicola*, *Psychrobacter glacincola* (Bowman *et al.*, 1996, 1997b), *Psychrobacter pacificensis* (Maruyama *et al.*, 2000), *Psychrobacter marincola* and *Psychrobacter submarinus* (Romanenko *et al.*, 2002) and *Psychrobacter fozii* and *Psychrobacter luti* (Bozal *et al.*, 2003). In the course of studies on the microbial community able to colonize short-term coastal sea ice and marine sediments

of the Sea of Japan, four *Psychrobacter*-like strains were isolated and taxonomically characterized by a polyphasic approach, including 16S rRNA gene sequence, DNA–DNA hybridization, fatty acid, morphological and biochemical analyses.

Three strains were isolated from coastal sea-ice samples obtained from columns of short-term sea-ice at depths of 0.8–1.0 m in Amursky Bay, Sea of Japan, Russia, in March 2002. The sea-ice samples were collected in sterile flasks with a small amount of sterile sea water and placed at 15 °C for 2 days to be melted carefully. An aliquot of melting sea ice was spread on marine 2216 agar (MA; Difco) plates and these were incubated for 7 days at 15 °C. The isolated strains were stored at –80 °C in liquid nutrient medium supplemented with 20% (v/v) glycerol. The sea-ice strains were designated KMM 3646^T (= Pi2-20^T = DSM 15387^T), KMM 3643 (= Pi2-4) and KMM 3645 (= Pi2-25). Strain KMM 3659^T (= R7^T = DSM 15389^T) was recovered from a marine sediment sample from the Sea of Japan, Peter the Great Bay, Russia, in June 2002. *Psychrobacter immobilis* CIP 102557^T, *P. urativorans* CIP 105100^T, *P. frigidicola* CIP 105101^T, *P. glacincola* CIP 105313^T, *Psychrobacter*

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KMM 3646^T and KMM 3659^T are AJ609272 and AJ609273, respectively.

SEMs of cells of strains KMM 3646^T, KMM 3645 and KMM 3659^T are available as supplementary material in IJSEM Online.

proteolyticus DSM 13887^T and *Psychrobacter faecalis* DSM 14664^T were used as reference strains in DNA–DNA hybridization studies. The strains were routinely grown on MA, marine broth (MB; Difco) and tryptic soy agar (TSA; Serva); the latter was supplemented with 1.5 % (w/v) NaCl. Gram-reaction, oxidase, catalase, production of caseinase, DNase, gelatinase and lipase (Tween 80) were tested according to Smbert & Krieg (1994). Cell morphology was examined by SEM of cells grown in MB after 30 h incubation. Oxidation/fermentation medium of Leifson (1963) was used for testing acid production from carbohydrates with 1 % (w/v) of each compound. Growth at different temperatures and pH values and determination of the salinity range for growth using various sodium ion concentrations were examined as described previously by Romanenko *et al.* (2002). In addition, biochemical tests were carried out using API 20NE test kits (bioMérieux) as described by the manufacturer, except that strains were suspended in 1.5 % (w/v) NaCl solution. API test results were read after 24 h and 2, 5 and 7 days incubation at 28 °C. The isolates were also physiologically characterized using the Biolog GN MicroPlate method. Strains were grown for 24 h at 28 °C on TSA and the microtitre plates were inoculated with cells suspended in 1.5 % (w/v) NaCl. Results were read automatically with a spectrophotometer after incubation at 28 °C for 24 and 48 h and for up to 5 days. Fatty acid methyl esters were analysed using the standard procedure of the Microbial Identification system (Microbial ID) and compared to the fatty acid database. For fatty acid analysis, strains were grown on TSA supplemented with up to 2.0 % NaCl for 2 days incubation at 28 °C. DNA base composition was determined according to Marmur & Doty (1962), modified as described by Owen *et al.* (1969). DNA–DNA relatedness was measured using the hybridization method described by De Ley *et al.* (1970).

Genomic DNA extraction, PCR-mediated amplification of 16S rRNA genes and sequencing of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced directly using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. An Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence reaction products. 16S rRNA gene sequences of the strains studied were aligned manually with nucleotide sequences obtained from GenBank/EMBL. The algorithm of Jukes & Cantor (1969) was applied to transform sequence dissimilarities into evolutionary distances. Phylogenetic dendrograms were reconstructed using the method of DeSoete (1983) and the neighbour-joining method (Felsenstein, 1993).

Phenotypically, the isolates matched the description of the genus *Psychrobacter*, being Gram-negative, small, ovoid or spherical cells, aerobic, oxidase- and catalase-positive, non-pigmented, non-motile and psychrotolerant. Cells occurred in pairs or chains; some cellular pilus-like structures were observed (see figure available as supplementary material in

IJSEM Online). Biochemical and physiological properties of the strains studied are displayed in Table 1 and in the species descriptions.

The fatty acid profiles (Table 2) are characterized by the occurrence of 18:1 ω 9c, 17:1 ω 8c and 16:1 ω 7c/i15:0 2-OH (up to 80 % total fatty acids) as dominant components, which is in accordance with those previously reported for *Psychrobacter* species (Bowman *et al.*, 1996, 1997a, b; Denner *et al.*, 2001; Romanenko *et al.*, 2002), although some quantitative and qualitative differences are observed. Unlike members of the other *Psychrobacter* species, strains KMM 3646^T, KMM 3645 and KMM 3643 contained a significant amount of i17:0 (7.2–9.0 %) and smaller amounts of i11:0 (1.27–1.40 %), but did not contain 12:0 (Table 2). The DNA G + C contents of the novel strains ranged from 44.6 to 45.0 mol%, which are also characteristic for members of *Psychrobacter* (Table 1).

Comparative 16S rRNA gene sequence analysis confirmed the phenotypic affiliation as it also grouped the novel strains within the genus *Psychrobacter* (94.0–98.5 % sequence similarity) as two independent groups (Fig. 1). The first group comprised sea-ice isolates KMM 3646^T, KMM 3645 and KMM 3643, which were almost phylogenetically undistinguishable from one another (99.9 % sequence similarity). These organisms shared between 94.0 and 98.5 % sequence similarity with the type strains of other *Psychrobacter* species. The phylogenetic position of KMM 3659^T was determined; this strain did not display more than 96 % sequence similarity with other members of the genus (Fig. 1).

DNA–DNA hybridization experiments were done to clarify the intraspecific relationship of the metabolically similar and phylogenetically highly related strains KMM 3646^T, KMM 3645 and KMM 3643. Values between 83 and 85 % indeed confirmed that these three strains were members of the same species (Wayne *et al.*, 1987). As advised by Stackebrandt & Goebel (1994), DNA–DNA reassociation experiments were not performed with strain KMM 3659^T because 16S rRNA gene sequence similarities between this strain and the type strains of *Psychrobacter* species were consistently lower than 97.5 %. The single DNA–DNA relatedness value of 33 % for strain KMM 3659^T and the type strain of *P. faecalis* confirmed the suggestion by Stackebrandt & Goebel (1994). However, it has recently been shown that species of certain genera, e.g. *Psychrobacter* (Romanenko *et al.*, 2002), *Pseudoalteromonas* (Romanenko *et al.*, 2003) and the Gram-positive genus *Amycolatopsis* (Wink *et al.*, 2003), show less than 70 % DNA–DNA reassociation values even at about 99 % 16S rRNA gene sequence similarity. Hence, the correlation value between genomic and gene similarities for the delineation of species can be raised provided these values have been determined for at least some species of a genus. In the case of *Psychrobacter*, a DNA–DNA hybridization value of 15 % had been determined for the type strains of *P. submarinus* and *P. marincola*, which share 99.9 % 16S rRNA gene sequence

Table 1. Phenotypic characteristics for differentiation of novel isolates and *Psychrobacter* species

Strains/species: 1, KMM 3646^T, KMM 3643 and KMM 3645; 2, KMM 3659^T; 3, *P. immobilis*; 4, *P. urativorans*; 5, *P. frigidicola*; 6, *P. phenylpyruvicus*; 7, *P. glacincola*; 8, *P. proteolyticus*; 9, *P. faecalis*; 10, *P. marincola*; 11, *P. submarinus*; 12, *P. pacificensis*; 13, *P. fozi*; 14, *P. luti*. Data from this study and from Bowman *et al.* (1996, 1997b), Denner *et al.* (2001), Kämpfer *et al.* (2002), Romanenko *et al.* (2002), Maruyama *et al.* (2000) and Bozal *et al.* (2003). All strains were negative for arginine dihydrolase, ONPG test, H₂S production and hydrolysis of DNA and starch. Acid was not produced from sucrose, maltose, D-mannitol or glycerol. +, Positive reaction; –, negative reaction; (+), weak positive reaction; v+, variable reaction between strains, the type strain reaction is positive; v–, variable reaction between strains, the type strain reaction is negative; ND, not detected.

Feature	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth at (°C):														
30	+	+	+	v+	–	+	–	+	+	+	+	+	+	+
35	+	+	v+	–	–	+	–	–	+	+	+	+	–	–
37	+	+	v+	–	–	+	–	–	+	–	–	+	–	–
Tolerance to 12% NaCl	–	–	v+	–	–	–	+	+	+	+	+	–	+	–
Nitrate reduction	+	+	v+	v–	–	–	v+	–	+	–	–	–	–	+
Acid from sugars	–	+	+	–	–	–	–	+	–	–	+	+	–	–
Proteinase	–	–	–	–	–	–	–	+	–	–	–	–	–	+
Utilization of:														
L-Arabinose	–	+	–	–	–	–	–	–	(+)	–	–	–	–	–
Malate	v–	+	+	v+	+	+	–	–	+	–	–	+	+	+
Citrate	–	+	v–	–	–	+	–	+	+	–	–	–	–	+
Caprate	–	–	–	–	–	–	–	+	+	–	–	–	ND	ND
Pyruvate	–	–	+	+	–	+	+	+	+	–	–	ND	+	ND
3-Hydroxybutyrate	–	–	+	+	–	+	v+	+	+	–	–	v–	ND	ND
Acetate	+	+	+	v+	+	+	+	+	+	–	–	v–	+	+
Propionate	–	+	v+	–	–	+	+	+	(+)	–	–	–	+	–
L-Phenylalanine	v+	–	+	v+	+	+	–	–	–	–	–	ND	+	+
L-Proline	–	–	+	+	+	+	+	ND	–	–	–	+	+	+
L-Alanine	–	–	+	–	–	+	v+	+	+	–	–	v–	+	–
L-Histidine	–	–	+	–	–	–	v+	+	+	–	–	+	+	+
G + C content (mol%)	44.6–44.9	45.0	44–47	44–46	41–42	43	43–44	43.6	44.1*	50.7	46.8	44–45	44–46	45

*Data for *P. faecalis* DSM 14664^T obtained from this study.

Table 2. Cellular fatty acid composition (%) of the novel *Psychrobacter* strains

Strains: 1, KMM 3646^T; 2, KMM 3643; 3, KMM 3645; 4, KMM 3659^T. Values of <2% were not considered when recorded for all strains.

Fatty acid	1	2	3	4
10:0	2.90	2.81	2.78	2.85
12:0	0	0	0	2.64
12:0 3-OH	2.70	2.89	2.64	3.43
14:0 3-OH	0	1.66	1.41	0
Summed feature*	15.52	13.75	14.45	6.62
16:0	1.23	2.95	2.82	0.76
i17:0	8.99	7.22	7.23	1.88
17:1 ω 8c	11.13	3.72	3.42	13.93
18:1 ω 9c	52.12	57.53	59.22	61.31
18:0	1.28	2.54	3.49	2.01
18:3 ω 6c (6,9,12)	0	1.40	0	0

Summed feature: 16:1 ω 7c and/or i15:0 2-OH.

similarity. As the sequence similarities between strain KMM 3646^T and type strains of *Psychrobacter* species were lower than 98.5% and these strains also differed in phenotypic and other properties, extensive determination of DNA–DNA relatedness was not carried out. The values determined between strain KMM 3643 and selected type strains, e.g. *P. immobilis*, *P. proteolyticus* and *P. urativorans*, revealed low similarities (means of 19, 20 and 15%, respectively).

The novel sea-ice isolate KMM 3646^T (together with strains KMM 3645 and KMM 3643) and the sediment strain KMM 3659^T can be distinguished from each other as well as from type strains of *Psychrobacter* species with validly published names by a combination of physiological and biochemical properties, growth temperatures, salinity range for growth, utilization patterns (Table 1) and phylogenetic distances. Based on this evidence, it is proposed that the novel marine isolates be assigned to the genus *Psychrobacter* as the novel species *Psychrobacter maritimus* sp. nov. and *Psychrobacter arenosus* sp. nov.

Fig. 1. 16S rRNA gene sequence dendrogram showing the position of strains KMM 3646^T and KMM 3659^T among *Psychrobacter* species. Numbers at branching points refer to bootstrap values (only values above 60% are shown). Bar, 2 inferred nucleotide substitutions per 100 nucleotides.

Description of *Psychrobacter maritimus* sp. nov.

Psychrobacter maritimus (ma.ri'ti.mus. L. masc. adj. *maritimus* maritime, marine).

Aerobic, Gram-negative, non-motile, non-pigmented, non-spore-forming, ovoid (1.5–1.6 µm long and 1.2–1.3 µm in diameter) or spherical (0.9–0.8 µm in diameter) cells. Oxidase- and catalase-positive. Sodium ions are not required for growth; growth occurs in 0–10% (w/v) NaCl, but not in 12 or 15% NaCl. Psychrotolerant. Grows at 4–37 °C, with an optimum growth temperature of 25–28 °C. Does not grow at 39–40 °C. Grows at pH 5.0–10.0, with optimum growth at pH 6.0–8.5. Acid is not formed from carbohydrates. Metabolic reactions are indicated in Table 1. The type strain is positive for L-leucine, L-ornithine and urease, but negative for L-serine. Negative for arginine dihydrolase, ONPG test, indole production, hydrolysis of aesculin and gelatin and utilization of glucose, maltose, mannitol, gluconate and phenylacetate, according to the API substrate panel reactions. Some strains produce urease and utilize adipate, mannose (weakly), α-ketoglutaric acid and L-asparagine. According to Biolog GN tests, positive or weakly positive for hydrolysis of Tween 40 and Tween 80 and utilization of α-ketovaleric acid and L-glutamic acid. The following substrates are not used as sole carbon sources: α-cyclodextrin, glycogen, dextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, *m*-inositol, lactose, maltose, D-mannitol, D-melibiose, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, *cis*-aconitic acid, formic acid, D-galacturonic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric

acid, DL-lactic acid, malonic acid, D-saccharic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanyl-glycine, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-threonine, γ-aminobutyric acid, D-serine, inosine, uridine, thymidine, putrescine, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Cellular fatty acid composition is given in Table 2. The G + C content of the DNA is 44.6–44.9 mol% (determined by the thermal denaturation method).

Isolated from a coastal sea-ice sample, Amursky Bay, the Sea of Japan, Russia. The type strain is strain KMM 3646^T (= DSM 15387^T = Pi2-20^T).

Description of *Psychrobacter arenosus* sp. nov.

Psychrobacter arenosus (a.re.no'sus. L. masc. adj. *arenosus* sandy, dwelling in marine sediment sand).

Aerobic, Gram-negative, non-motile, non-pigmented, non-spore-forming, ovoid cells (1.4–1.7 µm long and 0.6–0.8 µm in diameter). Oxidase- and catalase-positive. Sodium ions are not required for growth; growth occurs in 0–10% (w/v) NaCl, but not in 12% NaCl. Psychrotolerant. Grows at 4–37 °C, with an optimum growth temperature of 25–28 °C. Does not grow at 39 or 40 °C. Grows at pH 5.0–10.0, with optimum growth at pH 6.0–9.0. Acid is formed from D-glucose, rhamnose, galactose, lactose and arabinose. In addition to biochemical characteristics listed in the Table 1, the type strain is positive for L-leucine, but negative for L-ornithine, urease, L-serine, arginine dihydrolase, ONPG test, indole production, hydrolysis of aesculin and gelatin and utilization of glucose, arabinose, mannose, maltose, mannitol, gluconate and phenylacetate in the API tests. According to Biolog GN tests, the type strain is positive for hydrolysis of Tween 40 and Tween 80 and utilization of L-arabinose, L-asparagine and L-glutamic acid. The following substrates are not used as sole carbon sources: α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, *m*-inositol, lactose, maltose, D-mannitol, D-melibiose, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, *cis*-aconitic acid, formic acid, D-galacturonic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, DL-lactic acid, malonic acid, D-saccharic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanyl-glycine, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-threonine, γ-aminobutyric acid, D-serine, inosine, uridine, thymidine, putrescine, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Cellular fatty acid composition is displayed in Table 2. The DNA G + C content of the type strain is 45.0 mol% (determined by the thermal denaturation method).

Isolated from a marine sediment sand sample from the Sea of Japan, Russia. The type strain is KMM 3659^T (=DSM 15389^T=R7^T).

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