Psychrobacter fulvigenes sp. nov., isolated from a marine crustacean from the Sea of Japan

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Two novel *Psychrobacter*-like bacteria, strains KC 40^T and KC 65, were isolated from a marine crustacean specimen collected from the Sea of Japan, and were characterized by using a polyphasic approach. Strains were selected on the basis of their ability to produce black–brown diffusible pigments on commonly used organic media, which appears to be a unique characteristic of recognized members of the genus *Psychrobacter*. Phylogenetic analyses based on both 16S rRNA and *gyrB* gene sequences showed that the novel isolates formed a separate cluster within the genus *Psychrobacter*. Strains KC 40^T and KC 65 shared highest levels of 16S rRNA gene sequence similarity with *Psychrobacter urativorans* DSM 14009^T (98.0 %), *Psychrobacter pulmonis* CCUG 46240^T (97.9 %), *Psychrobacter cibarius* JG-219^T (97.9 %), *Psychrobacter faecalis* Iso-46^T (97.8 %), *Psychrobacter aquimaris* SW-210^T (97.6 %), *Psychrobacter namhaensis* SW-242^T (97.6 %) and *Psychrobacter nivimaris* 88/2-7^T (97.6 %). DNA–DNA hybridization experiments revealed 84 % DNA–DNA relatedness between strains KC 40^T and KC 65 but much lower levels of relatedness (7–35 %) between the novel strains and the type strains of recognized *Psychrobacter* species, confirming their assignment to a single novel species of the genus *Psychrobacter*. The two novel strains could be distinguished from recognized species of the genus *Psychrobacter* based on a combination of physiological and biochemical characteristics. On the basis of phenotypic and molecular properties, strains KC 40^T and KC 65 are considered to represent a novel species of the genus *Psychrobacter*, for which the name *Psychrobacter fulvigenes* sp. nov. is proposed. The type strain is KC 40^T (=KMM 3954^T=NRIC 0746^T=JCM 15525^T).

The genus *Psychrobacter* was created by Juni & Heym (1986) to accommodate Gram-negative coccobacilli that are non-pigmented, oxidase-positive, non-motile, psychrophilic or psychrotolerant, and halotolerant. At the time of writing, the genus *Psychrobacter* comprises 29 recognized species. Members of the genus have been isolated from a wide range of sources, including the gills and skin of fish, poultry, food and clinical sources (Juni & Heym, 1986), terrestrial (Kämpfer *et al.*, 2002; Vela *et al.*, 2003) and marine animals (Denner *et al.*, 2001; Romanenko *et al.*, 2002), surface and deep seawater (Maruyama *et al.*, 2000; Romanenko *et al.*, 2002; Yoon *et al.*, 2005a, b), marine sediments (Romanenko *et al.*, 2004), seafood (Yoon *et al.*, 2003, 2005c; Jung *et al.*, 2005), Antarctic sea ice, seawater, ornithogenic soil, ponds and cyanobacterial mat samples (Bowman *et al.*, 1996, 1997; Bozal *et al.*, 2003; Heuchert *et al.*, 2004; Shivaji *et al.*, 2004, 2005), and Arctic permafrost (Bakermans *et al.*, 2006).

In the present study, the taxonomic position of two novel *Psychrobacter*-like strains, KC 40^T and KC 65, isolated from a marine crustacean is described. Phylogenetic analyses based on 16S rRNA and *gyrB* gene sequences showed that strains KC 40^T and KC 65 belong to the genus *Psychrobacter* and constitute a separate cluster. Phylogenetic analysis and DNA–DNA relatedness data, together with differential phenotypic properties demonstrate that these strains represent a novel species of the genus *Psychrobacter*.

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences of strains KC 40^T and KC 65 are AB438958–AB438959 and those for the *gyrB* gene sequences are AB438960–AB438961, respectively.

Maximum-parsimony phylogenetic trees based on 16S rRNA and *gyrB* gene sequences showing the positions of strains KC 40^T and KC 65 among species of the genus *Psychrobacter*, and a table giving the cellular fatty acid contents of strains KC 40^T and KC 65 are available with the online version of this paper.

Strains KC 40^T and KC 65 were isolated from the crab *Paralithodes camtschatica*, a marine crustacean of the order Decapoda, collected from Peter the Great Bay of the Sea of Japan, Russia, in May 2006. Strains KC 40^T and KC 65 were isolated from the internal liquor and gill tissue homogenate of a specimen of *Paralithodes camtschatica*, respectively. Aliquots of the samples were spread onto trypticase soy agar (TSA) and colonies were picked for further processing. The novel strains grew aerobically on TSA, nutrient agar (NA), R2A agar, marine 2216 agar (MA) and marine 2216 broth (MB; all from Difco) at 28 °C; they grew most abundantly on TSA and NA. They were stored at −80 °C in MB supplemented with 30 % (v/v) glycerol.

Gram staining, oxidase and catalase reactions, H_2S production and hydrolysis of gelatin, casein, chitin, starch, DNA and Tweens 20, 40 and 80 were tested according to the standard methods described by Smibert & Krieg (1994). The oxidation/fermentation medium of Leifson (1963) was used for testing acid production from carbohydrates; a concentration of 1 % (w/v) of each compound was used. Growth at different temperatures and in the presence of various NaCl concentrations was studied as described by Romanenko *et al.* (2002, 2004). In addition, biochemical tests were carried out by using API 20NE, API ID32 GN and API ZYM test kits (bioMérieux) as described by the manufacturer. For fatty acid analyses, strains KC 40^T and KC 65 were cultivated on TSA at 28 °C and on MA at 15 °C for 3 days. Fatty acid methyl esters were obtained by alkaline methanolysis (15 % NaOH/methanol). The resultant fatty acid methyl esters were extracted by hexane and were analysed by using a GLC-MS Hewlett Packard model 6890 GC equipped with an HP 5 MS 5 % phenyl methyl siloxane capillary column (30 m × 250 μm × 0.25 μm) and connected to a Hewlett Packard model 5973 MS. The photobiotin-labelled DNA probe microplate method of Ezaki *et al.* (1989) was used to determine levels of DNA–DNA relatedness between strains KC 40^T and KC 65 and between these novel strains and the type strains of related *Psychrobacter* species (Table 1). The 16S rRNA gene sequences of strains KC 40^T and KC 65 (1532 and 1523 nt, respectively) were determined as described by Shida *et al.* (1997). The sequences obtained were compared with 16S rRNA gene sequences retrieved from the EMBL/GenBank/DDBJ databases by using the FASTA program (Pearson & Lipman, 1988). Phylogenetic analysis of the 16S rRNA gene sequences was performed by using the MEGA 4 software package (Tamura *et al.*, 2007) after multiple alignment of the data by CLUSTAL_X (version 1.83; Thompson *et al.*, 1997). Phylogenetic trees were constructed via the neighbour-joining and maximum-parsimony methods, and distances were calculated according to the Kimura two-parameter model. The robustness of the phylogenetic trees was estimated by bootstrap analysis of 1000 replicates. Sequencing of the *gyrB* gene (2260 nt) was performed for strains KC 40^T and KC 65 after amplifying *gyrB* gene fragments based on the method described by Bakermans *et al.* (2006). Additional primers used for sequencing in this study were 1140F (5′-CATGCACGACAGATTTAATG-3′) and 1624R (5′-AGAAGGTCAACAGCAAGGTA-3′). Partial *gyrB* gene sequences of strains KC 40^T and KC 65 were analysed in the same manner as for the 16S rRNA gene sequences.

Table 1. Levels of DNA–DNA relatedness between strains KC 40^T and KC 65 and the type strains of related species of the genus *Psychrobacter*

Strain	DNA–DNA relatedness (%)	
	KC 40^T	KC 65
KC 40^T	100	
KC 65	84	
P. urativorans DSM 14009^T	19	7
P. faecalis DSM 14664^T	33	16
P. pulmonis CCUG 46240^T	35	25
P. nivimaris DSM 14093^T	22	13
P. alimentarius KCTC 12186^T	10	7
P. aquimaris KCTC 12254^T	16	14
P. namhaensis KCTC 12255^T	21	17
P. cibarius KCTC 12256^T	21	11

Comparison of the nearly complete 16S rRNA gene sequences of strains KC 40^T and KC 65 showed that they were phylogenetically undistinguishable (100 % similarity). Phylogenetic analysis based on 16S rRNA gene sequences showed that the novel strains were affiliated to the genus *Psychrobacter* as an independent lineage adjacent to the cluster containing the type strains of *Psychrobacter faecalis*, *Psychrobacter pulmonis* and *Psychrobacter arenosus*. The novel isolates were found to be located at the same position in the phylogenetic trees generated with the neighbour-joining and maximum-parsimony methods based on 16S rRNA gene sequences (Fig. 1 and Supplementary Fig. S1 in IJSEM Online). Comparative analysis based on *gyrB* gene sequences showed a somewhat different position for strains KC 40^T and KC 65, placing them in a lineage more closely related to the type strain of *Psychrobacter okhotskensis* (Fig. 2 and Supplementary Fig. S2). According to phylogenetic analyses based on 16S rRNA and *gyrB* gene sequences, strains KC 40^T and KC 65 formed an independent cluster in the genus *Psychrobacter*, although their relationships with respect to recognized *Psychrobacter* species as well as the relationships among recognized *Psychrobacter* species were not identical (Figs 1 and 2). Bakermans *et al.* (2006) have reported distinct positions among species of the genus *Psychrobacter* in the phylogenetic tree based on 16S rRNA gene sequences compared with that based on *gyrB* gene sequences. Strains KC 40^T and KC 65 showed levels of *gyrB* gene sequence similarity of 86 % with the type strain of *P. pulmonis*, 84.9–81.2 % with those of *P. okhotskensis*, *P. faecalis*, *Psychrobacter luti* and *Psychrobacter jeotgali*, and of <80.5 % with those of other members of the genus *Psychrobacter*. These values are below the value of 89 % *gyrB* gene sequence similarity corresponding to the recommended 70 % level of DNA–

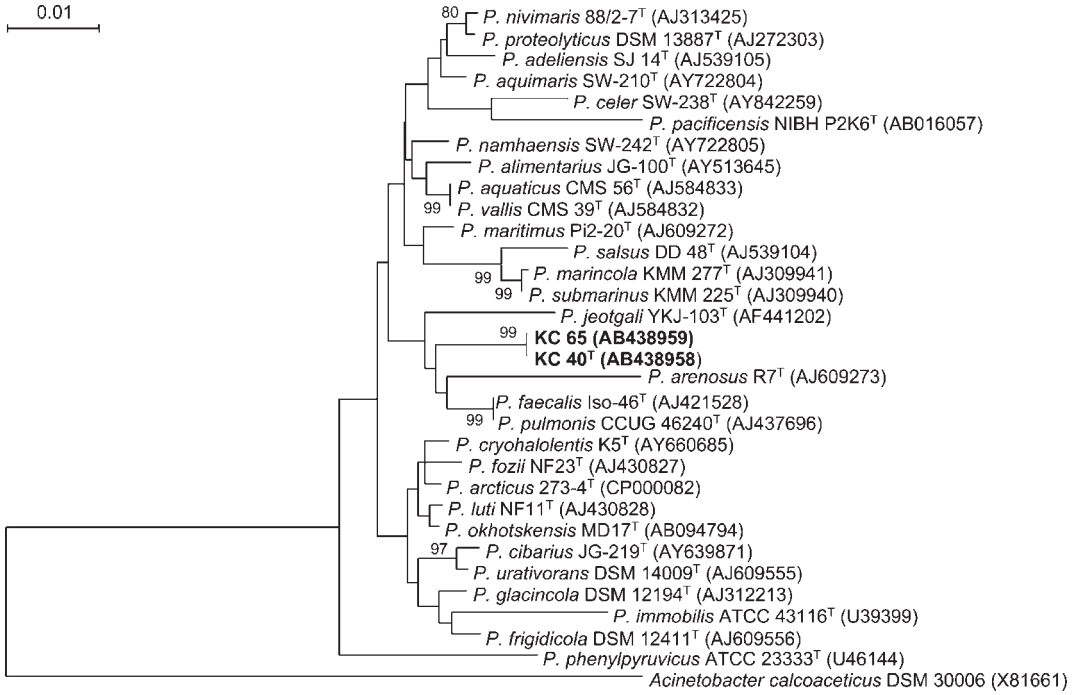

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences available from the GenBank/EMBL/DDBJ databases (accession numbers in parentheses) showing the position of strains KC 40^T and KC 65 among species of the genus *Psychrobacter*. Bootstrap values based on 1000 replications are given as percentages at branch points. Bar, 0.01 substitutions per nucleotide position.

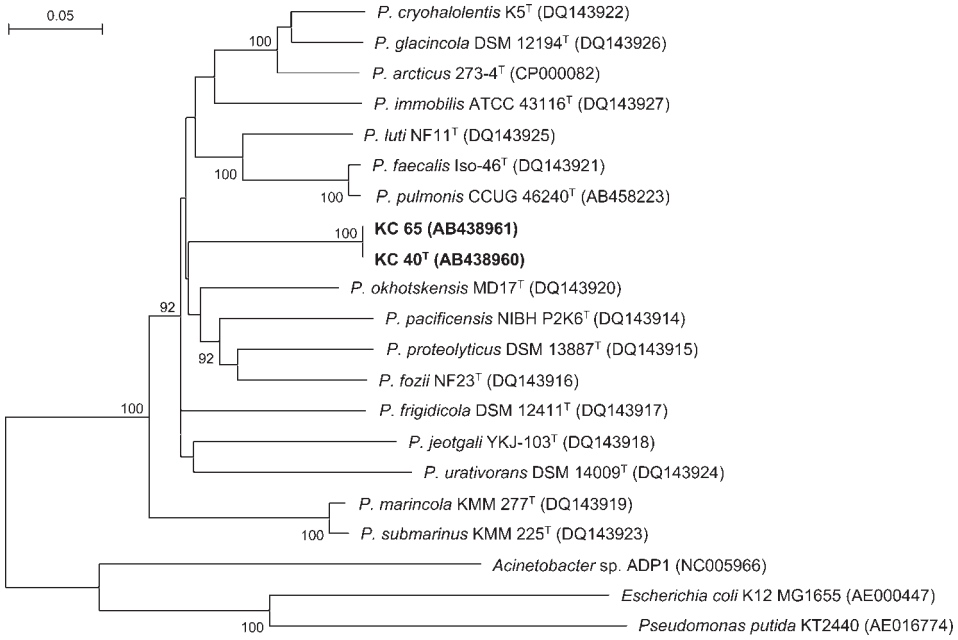

Fig. 2. Neighbour-joining phylogenetic tree based on *gyrB* gene sequences available from the GenBank/EMBL/DDBJ databases (accession numbers in parentheses) showing the position of strains KC 40^T and KC 65 among species of the genus *Psychrobacter*. Bootstrap values based on 1000 replications are given as percentages at branch points. Bar, 0.05 substitutions per nucleotide position.

DNA relatedness accepted for species discrimination (Wayne *et al.*, 1987).

Strains KC 40^T and KC 65 shared highest levels of 16S rRNA gene sequence similarity with *Psychrobacter urativorans* DSM 14009^T (98.0 %), *P. pulmonis* CCUG 46240^T (97.9 %), *Psychrobacter cibarius* JG-219^T (97.9 %), *P. faecalis* Iso-46^T (97.8 %), *Psychrobacter aquimaris* SW-210^T (97.6 %), *Psychrobacter namhaensis* SW-242^T (97.6 %) and *Psychrobacter nivimaris* 88/2-7^T (97.6 %), and showed values of <97.3 % to the type strains of other recognized *Psychrobacter* species. A 16S rRNA gene sequence similarity value of 97.0 % was proposed by Stackebrandt & Goebel (1994) and re-evaluated at 98.7 % by Stackebrandt & Ebers (2006) as a criterion for bacterial species discrimination. Levels of DNA–DNA relatedness between the novel strains and the type strains of closely related *Psychrobacter* species were examined because the highest 16S rRNA gene sequence similarity values (98.0–97.6 %) were close to the recommended cut-off value of 98.7 %. The level of DNA–DNA relatedness between strains KC 40^T and KC 65 was 84 %, indicating that they belong to the same species. Levels of DNA–DNA relatedness between strains KC 40^T and KC 65 and the type strains of closely related *Psychrobacter* species were 7–35 % (Table 1), significantly less than the 70 % cut-off value recommended by Wayne *et al.* (1987) for species delineation, and confirming that strains KC 40^T and KC 65 constitute a separate species. Based on 16S rRNA and *gyrB* gene sequence analyses together with DNA–DNA hybridization results, we concluded that isolates KC 40^T and KC 65 could not be assigned to any recognized species and represent a novel species of the genus *Psychrobacter*.

The fatty acid profiles of strains KC 40^T and KC 65 were similar (see Supplementary Table S1). Strains KC 40^T and KC 65 contained a high amount of $C_{18:1}\omega9c$ regardless of whether they were grown on TSA at 28 °C or on MA at 15 °C (79.7 and 83.2 %, and 85.0 and 78.4 % of the total, respectively). The presence of $C_{18:1}\omega9c$ as a predominant component is in accordance with data previously reported for species of the genus *Psychrobacter* (Bowman *et al.*, 1996, 1997; Denner *et al.*, 2001; Romanenko *et al.*, 2002; Shivaji *et al.*, 2005).

Phenotypically, the novel strains appeared to be characteristic of the genus *Psychrobacter*, being Gram-negative, aerobic, oxidase- and catalase-positive, non-motile, and psychrotolerant. Strains KC 40^T and KC 65 formed non-pigmented colonies, but were able to produce black–brown pigments on and in commonly used commercial media (TSA or TSB, NA or NB, MA or MB, or R2A). The ability to produce black–brown pigments appears to be a unique characteristic amongst recognized members of the genus *Psychrobacter*. Based on the formation of black–brown pigments, the novel isolates could be distinguished clearly from other psychrobacters by plating on commonly used media. The biochemical and physiological properties of strains KC 40^T and KC 65 are detailed in Table 2 and in the species description below. The phylogenetic distinctiveness of strains KC 40^T and KC 65 was supported by a combination of physiological properties, maximal and minimal growth temperatures, salinity range for growth and substrate utilization patterns (Table 2). Although strains KC 40^T and KC 65 clustered phylogenetically to the seawater bacterium *P. okhotskensis* based on *gyrB* gene sequence analysis, they were phenotypically most similar to the terrestrial animal-derived bacteria *P. faecalis* and *P. pulmonis* (being able to grow at 37 °C and to reduce nitrate). Strains KC 40^T and KC 65 were adjacent to the type strains of *P. faecalis* and *P. pulmonis* in the phylogenetic trees based on 16S rRNA gene sequences. Differential characteristics between the novel isolates and *P. okhotskensis* were a negative reaction for indole production and Tween 80 hydrolysis, and the ability to grow at 37 °C and in the presence of 12 % (w/v) NaCl. In addition, strains KC 40^T and KC 65 differed from related species by being able to produce black–brown diffusible pigments and to produce urease (except *P. urativorans* and *P. jeotgali*, which have variable and positive reactions for urease, respectively) (Table 2). Based on phenotypic and biochemical characteristics, as well as data from molecular analyses, strains KC 40^T and KC 65 are considered to represent a novel species of the genus *Psychrobacter*, for which the name *Psychrobacter fulvigenes* sp. nov. is proposed.

Description of *Psychrobacter fulvigenes* sp. nov.

Psychrobacter fulvigenes (ful.vi.ge′nes. L. adj. *fulvus* brown; Gr. v. *gennaio* to produce; M.L. adj. *fulvigenes* brown pigment-producing).

Cells are aerobic, Gram-negative, non-motile, non-pigmented, non-spore-forming and ovoid (1.6–1.9 μm long and 0.7–0.9 μm in diameter). Oxidase- and catalase-positive. Colonies on NA are beige, circular with an entire margin, convex and 2–4 mm in diameter. Produces black–brown pigments when grown on and in standard media, including TSA, TSB, MA, MB, NA, NB and R2A. Black–brown pigment is produced on MA supplemented with 1 % L-tyrosine. Hydrolyses L-tyrosine weakly. Growth occurs in the presence of 0–12 % (w/v) NaCl. The temperature range for growth is −5 to 37 °C with optimum growth at 25–28 °C; no growth occurs at 39–40 °C. Negative for H_2S production. Tween 20 is hydrolysed, but casein, gelatin, Tween 80, starch, chitin and DNA are not. Hydrolysis of Tween 40 is variable; the type strain reaction is positive. Acid is not produced from D-glucose, arabinose, mannose, maltose, rhamnose, galactose, maltose, cellobiose, D-xylose or mannitol. Other physiological properties and biochemical test results are given in Table 2. In API ID32 GN tests, positive for assimilation of malonate, lactic acid, L-fucose, propionic acid, valeric acid and 4-hydroxybenzoic acid, but negative for assimilation of L-rhamnose, *N*-acetylglucosamine, D-ribose, inositol, sucrose, maltose, itaconic acid,

Table 2. Differential phenotypic characteristics between strains KC 40^T and KC 65 and recognized species of the genus *Psychrobacter*

Taxa: 1, strain KC 40^T; 2, strain KC 65; 3, *P. faecalis* (data from Kämpfer *et al.*, 2002); 4, *P. pulmonis* (Vela *et al.*, 2003); 5, *P. arenosus* (Romanenko *et al.*, 2004); 6, *P. urativorans* (Bowman *et al.*, 1996); 7, *P. okhotskensis* (Yumoto *et al.*, 2003); 8, *P. cibarius* (Jung *et al.*, 2005); 9, *P. alimentarius* (Yoon *et al.*, 2005c); 10, *P. jeotgali* (Yoon *et al.*, 2003); 11, *P. luti* (Bozal *et al.*, 2003); 12, *P. marincola*; 13, *P. submarinus* (Romanenko *et al.*, 2002); 14, *P. aquimaris*; 15, *P. namhaensis* (Yoon *et al.*, 2005b). +, Positive; (+), 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; −, negative; ND, not detected.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Growth at/in:															
37 °C	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+
12 % NaCl	+	+	+	−*	−	−	−	−	+	−	−	+	+	+	+
Nitrate reduction	+	+	+	+	+	V(−)	+	+	−	+	+	−	−	−	−
Acid production from sugars	−	−	−	−	+	−	−	−	+	−	−	−	+	+	+
Urease	+	+	−	−	−	V(+)	−	−	−	+	−	−	−	−	−
Enzyme activity in API ZYM tests:†															
Acid phosphatase	−	−	−	−	−	−	−	−	(+)	−	−	−	−	−	−
Alkaline phosphatase	+	+	+	−	+	−	+	V(+)	+	+	+	+	+	+	+
Cystine arylamidase	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−
Esterase (C4)	+	+	(+)	+	+	−	+	−	+	+	+	+	(+)	+	+
Esterase lipase (C8)	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Leucine arylamidase	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
Lipase (C14)	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−
Naphthol-AS-BI-phosphohydrolase	(+)	(+)	+	−	(+)	−	+	+	(+)	V(+)	+	+	(+)	−	−
Valine arylamidase	−	−	−	+	−	−	−	V(+)	−	−	−	−	−	−	−
Utilization of:															
Acetate	+	+	+	ND	+	V(+)	ND	+	+	+	+	−	−	+	+
L-Alanine	+	+	+	ND	−	−	ND	+	ND	ND	−	−	−	ND	ND
L-Arabinose	−	−	(+)	ND	+	−	−	ND	ND	ND	−	−	−	−	−
Caprate	−	−	+	−	−	−	ND	ND	ND	ND	ND	−	−	ND	ND
Citrate	+	+	+	−	+	−	ND	−	+	−	+	−	−	−	−
L-Histidine	+	+	+	ND	−	−	ND	ND	ND	ND	+	−	−	ND	ND
3-Hydroxybutyrate	+	+	+	ND	−	+	+	ND	ND	ND	ND	−	−	ND	ND
L-Malate	+	+	+	−	+	V(+)	+	+	+	V(−)	+	−	−	+	+
L-Proline	+	+	−	ND	−	+	ND	ND	ND	ND	+	−	−	ND	ND
Propionate	+	+	(+)	ND	+	−	ND	ND	ND	ND	−	−	−	ND	ND
L-Serine	+	+	−	ND	−	−	ND	V(+)	ND	ND	ND	−	−	ND	ND

*Data for *P. pulmonis* CCUG 46240^T were obtained in the present study.
†Data for *P. arenosus* R7^T, *P. faecalis* Iso-46^T, *P. marincola* KMM 277^T and *P. submarinus* KMM 225^T were obtained from the present study; data for *P. okhotskensis* MD17^T were taken from Bakermans *et al.* (2006).

suberic acid, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, D-mannitol, D-glucose, salicin, melibiose, D-sorbitol, L-arabinose, capric acid and potassium 2-ketogluconate. In API 20NE tests, negative for indole production, acid production from glucose, hydrolysis of arginine dihydrolase, aesculin and gelatin, and for assimilation of *β*-galactosidase, glucose, arabinose, mannose, *N*-acetylglucosamine, maltose, mannitol, caprate and adipate; positive for nitrate reduction and for the assimilation of urease, gluconate, malate and citrate. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase, weakly positive for naphthol-AS-BI-phosphohydrolase, and negative for lipase (C14), valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase. Test for cystine arylamidase was variable between strains; the reaction of the type strain was positive.

The type strain, KC 40^T (=KMM 3954^T=NRIC 0746^T=JCM 15525^T), was isolated from a specimen of the marine crustacean *Paralithodes camtschatica*, collected from Peter the Great Bay of the Sea of Japan. Strain KC 65, isolated from the same specimen, is a second strain of the species.

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