Psychroflexus aestuariivivens sp. nov., isolated from a tidal flat

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A Gram-stain-negative, aerobic, non-flagellated, non-gliding and ovoid or rod-shaped bacterium, designated DB-3ᵀ, was isolated from a tidal flat on the Yellow Sea in South Korea, and subjected to a taxonomic study using a polyphasic approach. Strain DB-3ᵀ grew optimally at 30 °C, at pH 7.0–8.0 and in the presence of 2.0–3.0 % (w/v) NaCl. Phylogenetic trees based on 16S rRNA gene sequences showed that strain DB-3ᵀ fell within the clade comprising the type strains of species of the genus Psychroflexus. Strain DB-3ᵀ exhibited 16S rRNA gene sequence similarities of 93.2–96.9 % to the type strains of species of the genus Psychroflexus. Strain DB-3ᵀ contained MK-6 as the predominant menaquinone and iso-C₁₅:₀ anteiso-C₁₅:₀ and iso-C₁₇:₀ 3-OH as the major fatty acids. The major or significant amounts of polar lipids detected in strain DB-3ᵀ were phosphatidylethanolamine, an unidentified aminolipid and seven unidentified lipids. The DNA G+C content was 34.7 mol%. Differential phenotypic properties, together with phylogenetic distinctiveness, revealed that strain DB-3ᵀ is separated from recognized species of the genus Psychroflexus. On the basis of the data presented, strain DB-3ᵀ is considered to represent a novel species of the genus Psychroflexus, for which the name Psychroflexus aestuariivivens sp. nov. is proposed. The type strain is DB-3ᵀ (=KCTC 52037ᵀ=NBRC 111757ᵀ).

The genus Psychroflexus was proposed by Bowman et al. (1998) with the description of Psychroflexus torquis (type species) and the reclassification of Flavobacterium gondwanesense as Psychroflexus gondwanesensis. Subsequently, seven further species of the genus Psychroflexus with validly published names, Psychroflexus tropicus (Donachie et al., 2004), Psychroflexus sediminis (Chen et al., 2009), Psychroflexus salinarum (Yoon et al., 2009), Psychroflexus halocasei (Seiler et al., 2012), Psychroflexus salarius (Chun et al., 2014) and Psychroflexus salis and Psychroflexus planctonicus (Zhong et al., 2016), have been described. One additional species of the genus Psychroflexus, ‘Psychroflexus lacisalis’ (Zhang et al., 2010) has been described but the name has not yet been validly published. Phylogenetic analyses based on 16S rRNA gene sequences have shown that the genus Psychroflexus belongs to the family Flavobacteriaceae of the phylum Bacteroidetes (Bowman et al., 1998; Bernardet et al., 2011). Members of the genus Psychroflexus have been isolated from a variety of environments, including Antarctic sea ice, hypersaline or saline lakes, marine solar salters and cheese (Bowman et al., 1998; Donachie et al., 2004; Chen et al., 2009; Yoon et al., 2009; Seiler et al., 2012; Chun et al., 2014; Zhong et al., 2016). During a screening of novel bacteria from a tidal flat at Daebu Island in the Yellow Sea, South Korea, many novel bacterial strains have recently been isolated and characterized taxonomically. One of these isolates, a Psychroflexus-like bacterial strain (designated DB-3ᵀ), is described in this study. The aim of the present work was to determine the exact taxonomic position of strain DB-3ᵀ by using a polyphasic characterization that included the determination of chemotaxonomic and other phenotypic properties, and a detailed phylogenetic investigation based on 16S rRNA gene sequences.
A tidal flat sediment was collected from Daebu Island in the Yellow Sea of South Korea, and used as a source for the isolation of bacterial strains. Strain DB-3 was isolated by the standard dilution plating technique at 25°C on marine agar 2216 (MA; Becton Dickinson) and cultivated routinely at 30°C on MA. P. gondwanensis DSM 5423T, P. tropicus DSM 15496\(^T\), P. sediminis KCTC 22166\(^T\) and P. torquis DSM 21429\(^T\) were used as reference strains for fatty acid and polar lipid analyses and other phenotypic characterizations. The cell morphology, Gram reaction, pH range for growth and anaerobic growth were determined as described by Park et al. (2014). Gliding motility was investigated as described by Bowman (2000). Growth at 4, 10, 15, 20, 25, 30, 37 and 40°C was measured on MA to determine the optimal temperature and temperature range for growth. Growth at various concentrations of NaCl (0, 0.5 and 1.0–17.0%, at increments of 1.0%) was investigated by supplementing appropriate concentrations of NaCl in marine broth 2216 (MB) prepared according to the formula of the BD Difco medium except that NaCl was excluded. The requirement for Mg\(^{2+}\) ions was investigated by using MB, prepared according to the formula of the BD Difco medium and comprising all of the constituents except MgCl\(_2\) and MgSO\(_4\). Catalase and oxidase activities were determined as described by Lánya (1987). Hydrolysis of casein, starch, hypoxanthine, L-tyrosine and xanthine was investigated on MA using the substrate concentrations described by Barrow & Feltham (1993). Hydrolysis of ascinin and Tweens 20, 40, 60 and 80, and nitrate reduction were investigated as described previously (Lánya, 1987) with the modification that artificial seawater was used for the preparation of media. Hydrolysis of gelatin and urea were investigated by using nutrient gelatin and urea agar base media (BD Difco), respectively, with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (l:\(\ell\)) distilled water 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl\(_2\) \cdot 6H\(_2\)O, 5.94 g MgSO\(_4\), 7H\(_2\)O and 1.3 g CaCl\(_2\) \cdot 2H\(_2\)O (Bruns et al., 2001). Production of H\(_2\)S was tested as described previously (Bruns et al., 2001). The presence of flexirubin-type pigments was investigated as described previously (Reichenbach, 1992; Bernardet et al., 2002). Acid production from carbohydrates was tested as described by Leifson (1963). Susceptibility to antibiotics was tested on MA plates using antibiotic discs (Advantec) containing the following (µg per disc unless otherwise stated): ampicillin (10), carbenicillin (100), cephalothin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), novobiocin (5), oleandomycin (15), penicillin G (20 IU), polymyxin B (100 IU), streptomycin (50) and tetracycline (30). Enzyme activities were determined, after incubation for 8 h at optimal temperatures, by using the API ZYM system (bioMérieux); the strip was inoculated with cells suspended in artificial seawater from which CaCl\(_2\) was excluded to avoid the formation of precipitates.

Cell mass of strain DB-3\(^T\) for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown for 2 days in MB at 30°C, and cell mass of P. torquis DSM 21429\(^T\) for polar lipid analysis was obtained from cultures grown for 21 days in MB at 10°C. Chromosomal DNA was extracted and purified as described previously (Yoon et al., 1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR as described previously (Yoon et al., 1998) using two universal primers, 9F (5’-GAGTTTGTATCC TGGCTCAG-3’) and 1512R (5’-ACGGTTACCTTGTTACGACTT-3’). Sequencing of the amplified 16S rRNA gene and phylogenetic analyses were performed as described by Yoon et al. (2003).

Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250×4.6 mm) column. The isoprenoid quinones were eluted by a mixture of methanol/isopropanol (2:1, v/v) using a flow rate of 1 ml min\(^{-1}\) at room temperature and detected by UV absorbance at 270 nm. For cellular fatty acid analyses, cell mass of strain DB-3\(^T\) was harvested from MA plates after cultivation for 2, 3 and 5 days at 30°C. Cell mass of P. gondwanensis DSM 5423\(^T\), P. sediminis KCTC 22166\(^T\) and P. tropicus DSM 15496\(^T\) was harvested from MA plates after cultivation for 3 days at 30°C, and cell mass of P. torquis DSM 21429\(^T\) was harvested from MA plates after cultivation for 30 days at 10°C. Fatty acids were saponified, methylated and extracted using the standard MIDI protocol (Sherlock Microbial Identification System, version 6.2B). The fatty acids were analysed by GC (6890; Hewlett Packard) and identified using the TSBA6 database of the Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin et al. (1984), and separated by two-dimensional TLC using chloroform/methanol/acetate acid/water (40:7.5:6:1.8, by vol) for the first dimension and chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol) for the second dimension as described by Embley & Wait (1994). Individual polar lipids were identified by spraying the plates with 10% ethanolic molybdophosphoric acid and α-naphthol reagents (Minnikin et al., 1984) and with the ninhydrin spray, molybdenenum blue spray and Dragendorff’s reagents (Sigma). The DNA G+C content of strain DB-3\(^T\) was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC equipped with a YMC ODS-A (250×4.6 mm) column. The nucleotides were eluted by a mixture of 0.55 M NH\(_4\)H\(_2\)PO\(_4\) (pH 4.0) and acetonitrile (40:1, v/v), using a flow rate of 1 ml min\(^{-1}\) at room temperature, and were detected by UV absorbance at 270 nm.

Morphological, cultural, physiological and biochemical characteristics of strain DB-3\(^T\) are given in the species description and in Table 1 or Fig. S1 (available in the online Supplementary Material). The almost-complete 16S rRNA gene sequence of strain DB-3\(^T\) comprising 1444 nucleotides was determined in this study. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain DB-3\(^T\) fell within the clade comprising the type strains of

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species of the genus *Psychroflexus*, particularly clustering with the type strains of *P. sediminis, P. tropicus* and *P. gondwanensis* (Fig. 1). The clustering of strain DB-3T and the type strains of species of the genus *Psychroflexus* was also found in the trees reconstructed using the maximum-likelihood and maximum-parsimony algorithms (Figs S2 and S3). Strain DB-3T exhibited the highest 16S rRNA gene sequence similarity of 96.9% to *P. sediminis* YIM-C238T and sequence similarities of 93.2–96.5% to the type strains of the other species of the genus *Psychroflexus*.

The predominant isoprenoid quinone detected in strain DB-3T was menaquinone-6 (MK-6), which is the same as that shown in the genus *Psychroflexus* (Bowman et al., 1998). The cellular fatty acid profiles of strain DB-3T and the type strains of *P. gondwanensis, P. sediminis, P. tropicus* and *P. tropicus* are compared in Table 2. The fatty acid profiles of strain DB-3T from the three different growth phases were found to be similar (Table 2). The major fatty acids (>10% of the total fatty acids in three growth phases) detected in strain DB-3T were iso-C15:0, anteiso-C15:0 and iso-C17:0 3-OH. The fatty acid profile of strain DB-3T was similar to those of the type strains of the phylogenetically related species of the genus *Psychroflexus* (Table 2). Nevertheless, there were noteworthy differences in the proportions of some fatty acids between strain DB-3T and the reference strains, particularly iso-C15:0, iso-C15:1 G, anteiso-C15:1 A, iso-C16:0 3-OH and iso-C17:0 3-OH (Table 2). The fatty acid profiles of the type strains of *P. gondwanensis, P. sediminis, P. tropicus* and *P. tropicus* obtained from this study were similar to those analysed in the studies of Donachie et al. (2004) and Chen et al. (2009). The major or significant amounts of polar lipids detected in strain DB-3T were phosphatidylethanolamine, an unidentified aminolipid and seven unidentified lipids (Fig. S4). The polar lipid profile of strain DB-3T was similar to that of *P. tropicus* LMG 21429T in that phosphatidylethanolamine, one unidentified aminolipid and six unidentified lipids are present as major components (Fig. S4). The DNA G+C content of strain DB-3T was 34.7 mol%, a value in the range reported for species of the genus *Psychroflexus* (Table 1).

The results obtained from the phylogenetic and chemotaxonomic analyses are sufficient to assign strain DB-3T as a
member of the genus *Psychroflexus* (Figs 1, S2, S3 and S4, Table 2). Strain DB-3 was distinguished from the type strains of *P. gondwanensis, P. sediminis, P. torquis* and *P. tropicus* by differences in several phenotypic characteristics, including optimal growth temperature, hydrolysis of some substrates, acid production from some substrates, susceptibility to some antibiotics and activity of some enzymes (Table 1). These differences, in combination with the phylogenetic distinctiveness of strain DB-3, suggest that the novel strain is separated from other species of the genus *Psychroflexus* (Stackebrandt & Goebel, 1994). On the basis of genetic distinctiveness of strain DB-3 (Table 1). These differences, in combination with the phylogenetic distinctiveness of strain DB-3, suggest that the novel strain is separated from other species of the genus *Psychroflexus* (Stackebrandt & Goebel, 1994). On the basis of genetic distinctiveness of strain DB-3 (Table 1). These differences, in combination with the phylogenetic distinctiveness of strain DB-3, suggest that the novel strain is separated from other species of the genus 

*Description of *Psychroflexus aestuariivivens* sp. nov.*

*Psychroflexus aestuariivivens* (aes.tu.a.ri.i.vi’vens. L. neut. n. aestuarium -i tidal flat; L. part. vivens living; N.L. part. adj. aestuariivivens living in a tidal flat).

Cells are Gram-stain-negative, non-flagellated, non-gliding and ovoid or rod-shaped, approximately 0.2–0.5 µm in width and 0.3–>10.0 µm in length; a few cells greater than 10 µm in length are observed. Colonies on MA are circular, convex, smooth, glistening, strong orange in colour and 0.5–1.0 mm in diameter after incubation for 3 days at 30 °C. The optimal temperature for growth is 30 °C; growth occurs at 4 and 37 °C, but not at 40 °C. The optimal pH for growth is 7.0–8.0; growth occurs at pH 6.0, but not at pH 5.5. Growth occurs with 0.5–15.0 % (w/v) NaCl (optimum 2.0–3.0 %). Mg²⁺ ions are not required for growth. *H₂S* is not produced. Aesculin, casein, gelatin, starch, Tweens 20, 40, 60 and 80 and L-tyrosine are hydrolysed, but hypoxanthine, urea and xanthine are not. Acid is produced from D-glucose and maltose, but not from L-arabinose, cellobiose, D-fructose, D-galactose, lactose, D-mannose, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, sucrose, trehalose, D-xylene, myo-inositol, D-mannitol and D-sorbitol. In assays with the API ZYM system, activities of alkaline phosphatase, esterase (C₄), esterase lipase (C₈), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but activities of lipase (C₁₄), cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucoroniidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase,

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain *Psychroflexus* DB-3, the type strains of other species of the genus *Psychroflexus* and representatives of some other related taxa. Only bootstrap values (expressed as percentages of 1000 replications) >70% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Capnocytophaga ochracea* ATCC 27872 (GenBank accession no. U41350) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.](http://ijs.microbiologyresearch.org)
**Table 2.** Cellular fatty acid contents (%) of strain DB-3<sup>T</sup> and the type strains of phylogenetically related species of the genus *Psychroflexus*.

Strains: 1, DB-3<sup>T</sup> (2 days); 2, DB-3<sup>T</sup> (3 days); 3, DB-3<sup>T</sup> (5 days); 4, *P. gondwanensis* DSM 5423<sup>T</sup>; 5, *P. sediminis* KCTC 22166<sup>T</sup>; 6, *P. torquis* LMG 21429<sup>T</sup>; 7, *P. tropicus* DSM 15496<sup>T</sup>. All data were obtained from this study. Fatty acids that represented <1.0 % in all columns were omitted. Fatty acids that represented >10.0 % are indicated in bold. TR, Traces (<1.0 %); --, not detected.

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*Double bond position indicated by a capital letter is unknown.
†Summed features are groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 3 contained C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>.

α-mannosidase and α-fucosidase are absent. Susceptible to ampicillin, carbenicillin, cefalotin, chloramphenicol, lincomycin, oleandomycin, penicillin G and tetracycline, but not to gentamicin, kanamycin, neomycin, novobiocin, polymyxin B or streptomycin. The predominant menaquinone is MK-6. The major fatty acids (>10 % of the total fatty acids) are iso-C<sub>15:0</sub>, anteiso-C<sub>15:0</sub> and iso-C<sub>17:0</sub> 3-OH. Phosphatidylethanolamine, an unidentified aminolipid and seven unidentified lipids are present as major or significant amounts of polar lipids.

The type strain, DB-3<sup>T</sup> (=KCTC 52037<sup>T</sup>=NBRC 111757<sup>T</sup>), was isolated from a tidal flat at Daebu Island in the Yellow Sea, South Korea. The DNA G+C content of the type strain is 34.7 mol%.

**Acknowledgements**

This work was supported by the project on survey of indigenous species of Korea of the National Institute of Biological Resources (NIBR)

under the Ministry of Environment (MOE) and the Program for Collection, Management and Utilization of Biological Resources (grant NRF-2013M3A9A5075953) from the Ministry of Science, ICT & Future Planning (MSIP) of the Republic of Korea.

**References**


sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of Flavobacterium gondwanense (Dobson et al. 1993) as Psychroflexus gondwanense gen. nov., comb. nov. Microbiology 144, 1601–1609.


