# *Pseudoalteromonas neustonica* sp. nov., isolated from the sea surface microlayer of the Ross Sea (Antarctica), and emended description of the genus *Pseudoalteromonas*

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A Gram-stain-negative, facultatively anaerobic, rod-shaped and motile strain, designated PAMC 28425$^T$, was isolated from a sea surface microlayer sample from the Ross Sea, Antarctica. Analysis of the 16S rRNA gene sequence of strain PAMC 28425$^T$ showed an affiliation with the genus *Pseudoalteromonas*. Phylogenetic analyses revealed that strain PAMC 28425$^T$ formed a clade with *Pseudoalteromonas prydzensis* MB8-11$^T$ and *Pseudoalteromonas mariniglutinosa* KMM 3635$^T$ with 16S rRNA gene sequence similarities of 98.3–98.6 %. Genomic relatedness analyses based on the average nucleotide identity and the genome-to-genome distance showed that strain PAMC 28425$^T$ is clearly distinguished from the phylogenetically close relatives. Cells of strain PAMC 28425$^T$ grew optimally at 25 °C and pH 7.5–8.5 in the presence of 1.0–3.0 % (w/v) sea salts. The major cellular fatty acids (>10 %) were $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, $C_{16:0}$, and $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$. The genomic DNA G+C content was 39.7 mol%. On the basis of the phylogenetic, genomic, chemotaxonomic and phenotypic data presented, we propose the name *Pseudoalteromonas neustonica* sp. nov. with the type strain PAMC 28425$^T$ (=KCCM 43187$^T$=JCM 31286$^T$).

The genus *Pseudoalteromonas* was established by recombining 11 species of the genus *Alteromonas* and 1 species of the genus *Pseudomonas* into 12 species of the genus *Pseudoalteromonas* based on a wealth of phylogenetic analyses of nearly complete 16S rRNA gene sequences (Gauthier *et al.*, 1995). The genus *Pseudoalteromonas* is classified in the family *Alteromonadaceae*, and members of this genus are Gram-stain negative, aerobic, motile and have $C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:1}\omega8c$ and $C_{18:1}\omega7c$ as major fatty acids and genomic G+C contents of 38–48 mol% (Bowman & McMeekin, 2005). At the time of writing, the genus *Pseudoalteromonas* comprises 41 species and 2 subspecies with validly published names (List of Prokaryotic Names with Standing in Nomenclature, http://www.bacterio.net/pseudoalteromonas.html; Park *et al.*, 2016; Ying *et al.*, 2016). Most species of the genus *Pseudoalteromonas* have been isolated from various marine habitats including seawater, sediment, marine invertebrates, algae and sea ice (Bowman & McMeekin, 2005). With cultivation-independent molecular techniques, pseudoalteromonads are also known as a ubiquitous marine group extending from the sea surface microlayer (SSM) to deep-sea sediment, and from equatorial to polar regions (Cui *et al.*, 2008; Cunliffe & Murrell, 2009; Wietz *et al.*, 2010).

Here, we isolated a bacterial strain, which is the first representative of the genus *Pseudoalteromonas* isolated from Antarctic SSM to our knowledge, and performed a polyphasic taxonomic analysis to determine the taxonomic position of the strain.

A SSM sample was taken using a customized SSM sampler employing a type of rotating drum (Harvey 1966) covered with polycarbonate from the Terra Nova Bay (75.29° S

Abbreviations: ANI, average nucleotide identity; GGDC, genome-to-genome distance calculation; SSM, sea surface microlayer.

The GenBank/EMBL/DBBJ accession number for the 16S rRNA gene sequence and the draft genome sequence of strain PAMC 28425$^T$ are KU716039 and BDDS01000000, respectively.

Two supplementary tables and one supplementary figure are available with the online Supplementary Material.

164.80° E) in the Ross Sea during the *Araon* expedition in December 2014. The thickness of the SSM sample obtained by the rotating drum sampler was about 100 μm, based on preliminary performance tests in a laboratory (data not shown). The SSM sample was amended with autoclaved glycerol (final concentation 20 %, v/v) and preserved at −80 °C until use. For cultivation, the glycerol-amended SSM sample was spread onto a plate containing marine agar 2216 (MA; Difco), and the plate was incubated under aerobic conditions at 4 °C for 2 weeks. Strain PAMC 28425$^T$ was isolated on the plate and subsequently purified four times on fresh MA at 4 °C. The strain was preserved in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at −80 °C.

The type strains *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$ were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and the Korean Collection for Type Cultures (KCTC), respectively, to compare genomic, phenotypic and chemotaxonomical characteristics with strain PAMC 28425$^T$. Unless otherwise specified, the three strains were characterized using cultures grown aerobically on MA for 2–5 days at 25 °C.

Direct sequencing of purified PCR products for the 16S rRNA gene was performed as described by Hwang *et al.* (2015). The nearly complete 16S rRNA gene sequence (1418 bp) of strain PAMC 28425$^T$ was obtained and analysed using BLAST search against the GenBank and EzTaxone databases (Altschul *et al.*, 1990; Kim *et al.*, 2012). The 16S rRNA gene sequences of closely related taxa retrieved from the GenBank database were aligned on the basis of secondary structures using the RDP aligner (Cole *et al.*, 2014). Phylogenetic analysis was made using the program MEGA 6.0 (Tamura *et al.*, 2013). Distance matrices were calculated by the Jukes and Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-likelihood methods (Felsenstein 1981) using bootstrap analysis of 1000 replications.

The fatty acid methyl esters in whole cells of strains PAMC 28425$^T$, *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$ grown on MA for 4 days at 25 °C were analysed by gas chromatography (7890B; Agilent technologies) according to the instructions of the Microbial Identification System (version 6.2; MIDI) with the TSBA6 database. For genome comparison, genomic DNA of the three strains were extracted using a commercial kit (DNeasy Blood & Tissue kit; Qiagen). The genome sequences of the three strains were determined using an Illumina MiSeq at ChunLab (Seoul, Korea) and assembled using the SPAdes program version 3.7 (Bankevich *et al.*, 2012). The degree of pairwise genome-based relatedness was estimated by both an average nucleotide identity (ANI) value following the BLAST-based ANI calculation method described by Goris *et al.* (2007) and the genome-to-genome distance calculation (GGDC) method described by Auch *et al.* (2010). The DNA G+C content was calculated from a draft genome of each strain.

Phenotypic characteristics of strain PAMC 28425$^T$ were determined in duplicate along with the those of the type strains of *P. prydzensis* and *P. mariniglutinosa*, with repetition of experiments on different days. Gram staining was performed as described by Smibert & Krieg (1994). Motility of the cells was assessed by the hanging drop method (Skerman 1967) with cells grown in MB for 2 days. Cell morphology, size and the presence of flagella were determined using transmission electron microscopy (CM200; Philips). Anaerobic growth of strain PAMC 28425$^T$ was tested along with that of a strictly aerobic bacterium (*Rhodococcus aerolatus* PAMC 27367$^T$; Hwang *et al.*, 2015) and a strictly anaerobic bacterium *Clostridium* sp. PAMC 80033 to serve as experimental controls using a GasPak Anaerobic system (BBL) at 25 °C for 3 weeks. The temperature range for growth was examined by the ability to form colonies on MA incubated at 4 and 10–45 °C (in increments of 5 °C). The pH range (pH 5.0–11.0 at intervals of 0.5 pH unit) for growth was determined by assessing turbidity measured as $OD_{600}$ in pH-buffered MB (Hwang & Cho, 2008) using citric acid/phosphate buffer for pH 5.0, MES for pH 5.5–6.5, MOPS for pH 7.0–7.5, AMPD for pH 8.0–9.5 and CAPS for pH 10.0–11.0, each at a final concentration of 50 mM, at 25 °C for up to 2 weeks. Salt tolerance was determined by assessing turbidity measured as $OD_{600}$ at 25 °C using synthetic ZoBell broth (Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g; distilled water, 1 litre) supplemented with 0–2 % (at intervals of 0.5 %) and 3–15 % (at intervals of 1 %, w/v) sea salts (Sigma).

Catalase and oxidase tests were performed according to the methods described by Smibert & Krieg (1994) and Cappuccino & Sherman (2002), respectively. Decomposition of casein and xanthine was determined as described by Smibert & Krieg (1994). Hydrolysis of starch and Tweens 20, 40, 60 and 80 was investigated as described by Hansen & Sørheim (1991). In addition, other enzyme activities and acid production from substrates were assayed using the API ZYM, API 20NE and API 50CH kits (bioMérieux) according to the manufacturer's instructions except that the cell suspension was prepared as described by Hwang *et al.* (2009). Carbon utilization was tested according to the method of Bruns *et al.* (2001) with a final concentration of 0.4 % carbon source. Carbon utilization was scored as negative when growth was equal to, or less than, that in the negative control with no carbon source. Growth was measured by monitoring changes in the $OD_{600}$ for 3 weeks at 25 °C.

Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain PAMC 28425$^T$ was affiliated with the genus *Pseudoalteromonas* (Fig. 1). Results of the 16S rRNA gene sequence similarity analysis showed that strain PAMC 28425$^T$ was closely related to *Pseudoalteromonas prydzensis* MB8-11$^T$, *Pseudoalteromonas mariniglutinosa* KMM 3635$^T$, *Pseudoalteromonas espejiana* NCIMB 2127$^T$ and *Pseudoalteromonas atlantica* IAM 12927$^T$ with

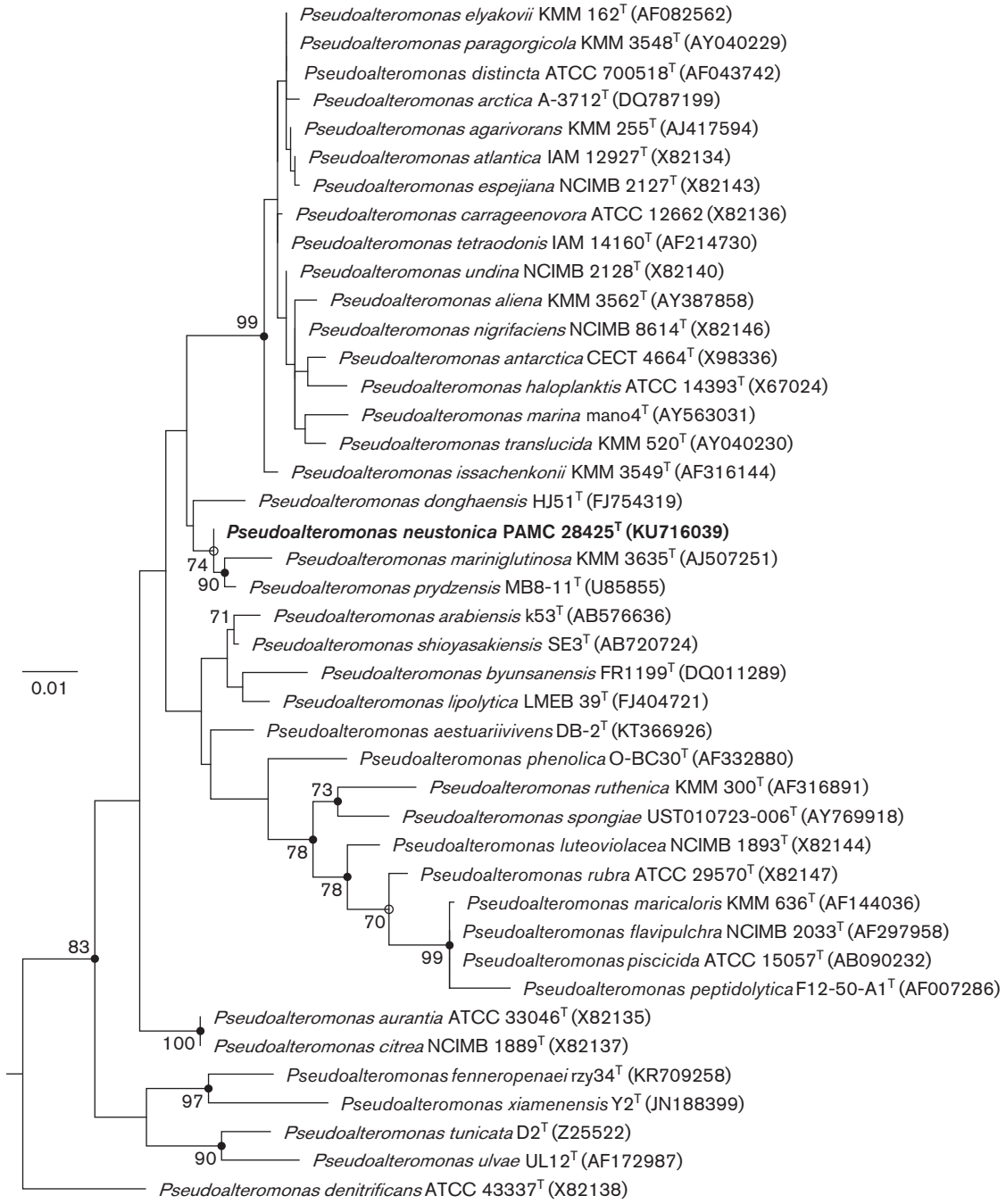


**Fig. 1.** Neighbour-joining tree derived from 16S rRNA gene sequences for strain PAMC 28425$^T$ and the members of the genus *Pseudoalteromonas* with *Psychromonas arctica* DSM 14288$^T$ (AF374385) as an outgroup (not shown). Only bootstrap values above 70 % are shown (1000 resamplings) at the branching points. Filled circles indicate that the corresponding nodes were also obtained in both the maximum-likelihood and the minimum-evolution trees, while open circles indicate that they were obtained in the latter tree only. Bar, 0.01 nucleotide substitutions per site.

similarity values of 98.6, 98.3, 98.2 and 98.0 %, respectively. However, the tree topologies inferred from three tree-making algorithms consistently showed that strain PAMC 28425$^T$ formed a clade with *Pseudoalteromonas prydzensis* MB8-11$^T$ and *Pseudoalteromonas mariniglutinosa* KMM 3635$^T$, which was obviously separated from a robust clade encompassing *Pseudoalteromonas espejiana* NCIMB 2127$^T$ and *Pseudoalteromonas atlantica* IAM 12927$^T$ (Fig. 1).

Genome relatedness for strains PAMC 28425$^T$, *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$is summarized in Table S1 (available in the online Supplementary Material). The ANI values calculated for the estimation of the degree of pairwise genome-based relatedness between strain PAMC 28425$^T$ and the two strains representing *Pseudoalteromonas prydzensis* and *Pseudoalteromonas mariniglutinosa* were 80.2–80.3 % (Table S1). This level is obviously below the proposed cut-off ANI values of 95–96 % for delineating bacterial species (Goris *et al.*, 2007; Richter & Rosselló-Móra, 2009). Consistently, DNA–DNA hybridization values estimated by GGDC were 23.2–31.3 % between strain PAMC 28425$^T$ and the two strains representing *Pseudoalteromonas prydzensis* and *Pseudoalteromonas mariniglutinosa* (Table S1), indicating that strain PAMC 28425$^T$ is a member of a distinctive species of the genus *Pseudoalteromonas* (Rosselló-Mora & Amann, 2001).

The results of morphological, physiological and biochemical analyses of strain PAMC 28425$^T$ are given in the species description, Table 1 and Fig. S1. The fatty acid profiles were generally similar among strain PAMC 28425$^T$, *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$ (Table S2). The major fatty acids

**Table 1.** Differential characteristics of strain PAMC 28425$^T$ and related species of the genus *Pseudoalteromonas*

Taxa: 1, PAMC 28425$^T$; 2, *Pseudoalteromonas prydzensis* DSM 14232$^T$; 3, *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$; 4, *Pseudoalteromonas donghaensis* HJ51$^T$; 5, *Pseudoalteromonas espejiana*; 6, *Pseudoalteromonas atlantica*; 7, *Pseudoalteromonas haloplanktis*. Data for strains 1–3 were obtained from this study unless otherwise indicated; data for taxa 4–7 were from the previous studies (Chan *et al.*, 1978; Baumann *et al.*, 1984; Akagawa-Matsushita *et al.*, 1992; Romanenko *et al.*, 2003; Bowman & McMeekin, 2005; Oh *et al.*, 2011; Park *et al.*, 2016). All strains were positive for motility; activity of oxidase, catalase and gelatinase; and utilization of propionate as a sole carbon source. All strains were negative for Gram-stain; activity of alginine dihydrolase; utilization of inositol, L-rhamnose and D-sorbitol as a sole carbon source. +, Positive; −, negative; V, variable; ND, no data available.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Isolation source | Antarctic sea surface microlayer | Antarctic sea ice | Marine diatom | Seawater | Seawater | Marine macroalgae | Seawater |
| Oxygen requirement | Facultative anaerobe | Strict aerobe | Strict aerobe | Strict aerobe | Strict aerobe | Strict aerobe | Strict aerobe |
| Temperature range (optimum) (°C) | 4–30 (25) | 4–35 (25–30) | 4–35 (25) | 4–40 (25–30) | 10–35 (30) | 5–35 (30) | 10–35 (30) |
| pH range (optimum) | 6.0–9.0 (7.5–8.5) | 6.0–8.5 (7.0–7.5) | 6.0–8.0 (6.5–7.5) | 5.5–9.5 (6.5–7.0) | ND | 5.5–8.5 | ND |
| Salt tolerance range (optimum) (%, w/v) | 1.0–7.0 (1.0–3.0) | 0.5–9.0 (0.5–3.0) | 0.5–10.0 (0.5–3.0) | 1–13 (2) | ND | ND | ND |
| Hydrolysis of aesculin | − | + | + | + | ND | + | ND |
| $\beta$-Galactosidase | − | − | − | − | + | + | − |
| Urease | − | − | − | + | + | + | + |
| Acid production from: | | | | | | | |
| D-Mannitol | − | − | + | ND | + | + | − |
| Starch | + | + | − | ND | ND | ND | ND |
| D-Arabinose, L- rhamnose | − | − | + | ND | ND | ND | − |
| L-Arabinose, cellobiose, gentiobiose, methyl $\alpha$-D-mannopyranoside | − | + | + | ND | ND | ND | ND |
| Utilization of sole carbon sources | | | | | | | |
| *N*-Acetyl-D-galactosamine | + | + | − | + | − | − | + |
| D-Mannose | − | − | − | + | V | + | + |
| L-Threonine | − | + | + | ND | + | − | V |
| Trehalose | − | − | − | ND | + | + | V |
| DNA G+C content (mol%) by genome analysis | 39.7 | 41.2 (38.0–39.0)* | 40.8 (40.3)* | (41.8)† | (43–44)* | (40.6–41.7)† | 40.8‡ (42–44)* |

*Data for the type strains of *P. prydzensis* (Bowman, 1998 ), *P. mariniglutinosa* (Romanenko *et al.*, 2003), *P. espejiana* (Chan *et al.*, 1978), and *P. haloplanktis* (Baumann *et al.*, 1984) were obtained by a thermal denaturation method.
†Data for *P. donghaensis* HJ51$^T$ (Oh *et al.*, 2011) and *P. atlantica* (Akagawa-Matsushita *et al.*, 1992) were obtained by a HPLC method.
‡Draft genome of *P. haloplankti* ATCC 14393$^T$ under the accession number of NZ_AHCA00000000 in GenBank.

of strain PAMC 28425$^T$ were summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; 31.3 %), $C_{16:0}$ (26.4 %) and summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$; 15.2 %), which are typically found as major components in members of the genus *Pseudoalteromonas* (Bowman & McMeekin, 2005). The genomic DNA G+C content of strain PAMC 28425$^T$ was 39.7 mol%, which is similar to those of the closest phylogenetic relatives *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$ (40.8–41.2 mol%; Table 1). The genomic DNA G+C content of strain PAMC 28425$^T$ is within the range of those of other species of the genus *Pseudoalteromonas* (38–48 mol%; Bowman & McMeekin, 2005).

Strain PAMC 28425$^T$ can be distinguished physiologically from other related species of the genus *Pseudoalteromonas* by the ability to grow under anaerobic conditions (Table 1). It has been regarded that strictly aerobic growth is a common property in the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995; Ivanova *et al.*, 2002); however, strain PAMC 28425$^T$ and *Pseudoalteromonas tunicata* D2$^T$ (Holmström *et al.*, 1998) indicate that facultative anaerobes are also members of this genus.

Other phenotypic characteristics can differentiate strain PAMC 28425$^T$ from the phylogenetically close relatives *Pseudoalteromonas prydzensis* DSM 14232$^T$ and *Pseudoalteromonas mariniglutinosa* KCTC 22327$^T$, for example the inability to grow in the presence of 9.0–10.0 % (w/v) sea salts, inability to hydrolyse aesculin, ability to produce acid from starch, inability to produce acid from L-arabinose, cellobiose, gentiobiose and methyl $\alpha$-D-mannopyranoside, and inability to utilize L-threonine as a sole carbon source (Table 1).

Overall, the phylogenetic, genomic, chemotaxonomic, physiological and phenotypic data obtained in the present study indicate that strain PAMC 28425$^T$ should be assigned to a novel species in the genus *Pseudoalteromonas*, for which the name *Pseudoalteromonas neustonica* is proposed.

## Emended description of the genus *Pseudoalteromona*s Gauthier *et al.* 1995 emend. Ivanova *et al*. 2002

This description is as given by Gauthier *et al.* (1995) and Ivanova *et al.* (2002) with the following modification based on an earlier study (Holmström *et al.*, 1998) and the present study. Strictly aerobic or facultatively anaerobic.

## Description of *Pseudoalteromonas neustonica* sp. nov.

*Pseudoalteromonas neustonica* (neus.to′ni.ca. N.L. fem. adj. *neustonica* pertaining to and living in the neuston).

Gram-staining-negative, facultatively anaerobic rods, approximately 0.4–1.3 μm wide and 1.3–4.0 μm long, motile by a polar flagellum. After 7 days on marine agar plates at 25 °C, colonies are orange, circular and convex, and approximately 1–2 mm in diameter. Grows at 4–30 °C (optimum of 25 °C) and pH 6.0–9.0 (optimum of pH 7.5–8.5). Growth occurs with a sea salts concentration of 1.0–7.0 % (w/v) (optimum 1.0–3.0 %). Positive for oxidase and catalase. Casein, starch, and Tweens 20, 40, 60 and 80 are hydrolysed, but xanthine is not. According to the API ZYM test, positive for acid and alkaline phosphatases, *N*-acetyl-$\beta$-glucosaminidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but negative for $\alpha$-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), $\alpha$-fucosidase, $\alpha$- and $\beta$-galactosidases, $\alpha$- and $\beta$-glucosidases, $\beta$-glucuronidase, lipase (C14), $\alpha$-mannosidase, trypsin and valine arylamidase. According to the API 20NE test, positive for gelatinase and glucose fermentation (weakly positive), but negative for arginine dihydrolase, aesculin hydrolysis, $\beta$-galactosidase (PNPG), indole production, nitrate reductase and urease. According to the API 50CH test, acid is produced from *N*-acetylglucosamine, aesculin, D-fructose, D-glucose, glycogen, maltose, D-mannose, potassium 2-ketogluconate, potassium 5-ketogluconate, starch and sucrose, but not from D-adonitol, amygdalin, DL-arabinose, DL-arabitol, arbutin, cellobiose, dulcitol, erythritol, DL-fucose, D-galactose, gentiobiose, glycerol, inositol, inulin, lactose, D-lyxose, D-mannitol, melezitose, melibiose, methyl $\alpha$-D-mannopyranoside, methyl $\alpha$-D-glucopyranoside, methyl $\beta$-D-xylopyranoside, potassium gluconate, raffinose, L-rhamnose, D-ribose, D-salicin, D-sorbitol, L-sorbose, D-tagatose, trehalose, turanose, xylitol and DL-xylose. *N*-Acetyl-D-galactosamine, L-proline and propionate are utilized as a sole carbon source, but acetate, L-arabinose, cellobiose, citrate, formic acid, D-fructose, D-galactose, D-glucose, glycerol, inositol, lactose, maltose, mannitol, D-mannose, melibiose, L-rhamnose, D-sorbitol, sucrose, L-threonine and trehalose are not utilized. The major cellular fatty acids are $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

The type strain, PAMC 28425$^T$ (=KCCM 43187$^T$=JCM 31286$^T$), was isolated from Antarctic SSM. The DNA G+C content of the type strain is 39.7 mol% (genome analysis).

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