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TAXONOMIC DESCRIPTION

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Pseudomonas neustonica sp. nov., isolated from the sea surface microlayer of the Ross Sea (Antarctica)

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Abstract

Gram-stain-negative, aerobic and rod-shaped bacterial strains, designated SSM26^T and SSM44, were isolated from a sea surface microlayer sample from the Ross Sea, Antarctica. Analysis of the 16S rRNA gene sequences of strains SSM26^T and SSM44 revealed a clear affiliation with the genus *Pseudomonas*. Based on the results of phylogenetic analysis, strains SSM26^T and SSM44 showed the closest phylogenetic relationship with the species *Pseudomonas sabulinigri* KCTC 22137^T with the 16S rRNA gene sequence similarity level of 98.5%. Strains SSM26^T and SSM44 grew optimally at 30 °C, pH 7.0–7.5 and 0.5–10.0% NaCl (w/v). The major cellular fatty acids were $C_{18:1}\,\omega7c$ (31.3–34.9%), $C_{16:0}$ (15.5–20.2%), summed feature 3 ($C_{16:1}\,\omega7c$/$C_{16:1}\,\omega6c$; 19.5–25.4%) and $C_{12:0}$ (6.0–9.3 %). The genomic DNA G+C content of each strain was 56.2 mol%. Genomic relatedness analyses based on the average nucleotide identity and the genome-to-genome distance showed that strains SSM26^T and SSM44 constituted a single species that was clearly distinguishable from its phylogenetically close relatives. The combined phenotypic, chemotaxonomic, genomic and phylogenetic data also showed that strains SSM26^T and SSM44 could be distinguished from validly published members of the genus *Pseudomonas*. Thus, these strains should be classified as representing a novel species in the genus *Pseudomonas*, for which the name *Pseudomonas neustonica* sp. nov. is proposed with the type strain SSM26^T (=KCCM 43193^T=JCM 31284^T=PAMC 28426^T) and a sister strain SSM44 (=KCCM 43194=JCM 31285=PAMC 28427).

INTRODUCTION

The genus *Pseudomonas*, first described by Migula [1], belongs to the family *Pseudomonadaceae* in the class *Gammaproteobacteria*. Since then, many species of the genus *Pseudomonas* have been inspected in a variety of studies as they are diverse in function and some species are pathogenic for humans, animals and plants, e.g., *Pseudomonas aeruginosa* – type species of this genus – is a known human and animal pathogen, and *Pseudomonas syringae*, *Pseudomonas asplenii*, *Pseudomonas fuscovaginae* and *Pseudomonas viridiflava* are known plant pathogens [2]. At the time of writing, more than 200 species and subspecies of *Pseudomonas* have been validly published [3]. The genus *Pseudomonas* was divided into two major clusters containing seven groups based on their 16S rRNA gene sequences [4]. The six groups such as *P. syringae*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas stutzeri* and *P. aeruginosa* groups were included in one of the two major clusters, and the other cluster included only one group (*Pseudomonas pertucinogena*). Recently, phylogenetic relationships of *Pseudomonas* species based on multi-locus sequence analysis (MLSA) of housekeeping core genes revealed the presence of 18–19 groups or subgroups in the genus *Pseudomonas* [5, 6]. Here, we isolated two bacterial strains affiliated with the *P. pertucinogena* group from a sea surface

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Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; FAME, fatty acid methyl ester; GGDC, genome-to-genome distance calculator; MA, marine agar; MB, marine broth; ME, minimum-evolution; ML, maximum-likelihood; MLSA, multi-locus sequence analysis; NJ, neighbour-joining; R2A, Reasoner's 2A; SSM, sea surface microlayer; TSA, tryptic soy agar; TSB, tryptic soy broth.
The GenBank/EMBL/DBBJ accession numbers for the 16S rRNA gene sequence and the draft genome sequence of strain SSM26^T are KU716040 and RKKU00000000, respectively, and those of strain SSM44 are KU716041 and RKKV00000000, respectively.
Three supplementary tables and two supplementary figures are available with the online version of this article.

microlayer (SSM) sample, and subjected to a polyphasic taxonomic analysis. On the basis of this characterization, we propose a novel species of the genus *Pseudomonas* belonging to the *P. pertucinogena* group.

A SSM sample was collected using a customized SSM sampler employing a type of rotating drum [7] covered with polycarbonate in the Ross Sea, Antarctica during the *Araon* expedition in December 2014. The SSM sample was amended with autoclaved glycerol (final concentration 20%, v/v) and preserved at −80 °C until further analyses.

For cultivation, an aliquot (100 µl) of the glycerol-amended SSM sample was spread onto a plate containing Reasoner's 2A (R2A; Difco) agar and tryptic soy agar (TSA; Difco), and the plates were incubated aerobically at 20 °C for 2 weeks. Strains SSM26^T and SSM44 were isolated on R2A agar and TSA, respectively, and subcultured on respective fresh media. Strains SSM26^T and SSM44 were subcultured more than four times for purification. To find an appropriate culture medium, a single colony was streaked onto TSA, saline TSA (supplemented with 3% NaCl), marine agar (MA; Difco) and R2A agar, and those plates were incubated aerobically at 20 °C and 30 °C. Cells were well grown on saline TSA at 30 °C. The strains were preserved in saline tryptic soy broth (TSB; Difco; supplemented with 3% NaCl) supplemented with 30% (v/v) glycerol at −80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by the boiling method [8]. The crude extracts were used as DNA template for PCR, which included 27F and 1492R primers [9]. Direct sequencing of the purified PCR products for the 16S rRNA gene was performed using sequencing primers (27F, 518F, 800R and 1492R; [9, 10]) in an Applied Biosystems sequencer (Cosmo Genetech). Almost-complete 16S rRNA gene sequences of strains SSM26^T (1406 bp) and SSM44 (1401 bp) were obtained and compared against the GenBank and EzTaxon-e databases using BLASTN [11, 12]. The 16S rRNA gene sequences of the type strains of related species were obtained from GenBank and were aligned on the basis of secondary-structures using the RDP aligner [13]. Phylogenetic analysis was made using the programme MEGA 7.0 [14]. The model of Jukes and Cantor [15] was used to generate an evolutionary distance matrix. Phylogenetic trees based on the neighbour-joining (NJ) [16], minimum-evolution (ME) [17] and maximum-likelihood (ML) [18] methods were reconstructed using bootstrap analysis of 1000 replications.

Pseudomonas sabulinigri KCTC 22137^T (=J64^T) [19] was purchased from the Korean Collection for Type Cultures (KCTC) and used as a reference strain to compare genomic, phenotypic, biochemical and chemotaxonomical characteristics with strains SSM26^T and SSM44. Unless otherwise specified, strains SSM26^T and SSM44 were grown on saline TSA for 2–3 days at 30 °C, and *P. sabulinigri* KCTC 22137^T was grown on TSA with 5% NaCl for 2–3 days at 30 °C, which is in exponential phase.

All morphological and physiological tests of strains SSM26^T and SSM44 were carried out along with the reference strain. Gram-staining was performed as previously described [20]. Cell motility of the strain was observed by the hanging drop method [21]. Cell morphology was examined by light microscopy and transmission electron microscopy (CM200, Philips). Anaerobic growth was tested on the saline TSA using the GasPak anaerobic system (BBL) at 30 °C for 7 days. The temperature range for growth was determined on the basis of colony formation on saline TSA at 4 and 10–50 °C (in increments of 5 °C) for 3 weeks. The pH range (pH 5.0–10.0 at intervals of 0.5 pH unit) for growth was determined by measuring OD_{600} in pH-buffered saline TSB using citric acid/sodium dihydrogen 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, each at a final concentration of 50 mM, incubating at 30 °C for up to 2 weeks. Salt tolerance was determined by measuring OD_{600} at 30 °C using synthetic ZoBell broth (Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g; distilled water, 1 l) supplemented with 0–3% (at intervals of 0.5%), 4–10% (at intervals of 1%, w/v), 12 and 15% NaCl, incubating at 30 °C for up to 7 days. Catalase activity was determined by bubble formation adding 3% (v/v) H_2O_2 and oxidase activity was determined using 1% (w/v) tetramethyl-*p*-phenylenediamine [22]. Hydrolysis of starch and Tweens (20, 40, 60 and 80) tests were performed as previously described [23] after 4 day incubation of cells on the saline TSA. Decomposition of casein, hypoxanthine and xanthine were determined according to the protocols as described previously [20]. Carbon source utilization was tested according to the method of Bruns *et al.* [24] with a final concentration of 0.4% carbon source. Carbon utilization was determined 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 30 °C. In addition, other biochemical activities of strains SSM26^T and SSM44 were determined by using API 20NE and API ZYM kits (bioMérieux), and acid production was tested by using API 50CH kit (bioMérieux) according to the manufacturer's instructions except that the cell suspension was prepared as described previously [25]. The fatty acid methyl esters (FAMEs) in whole cells of strains SSM26^T and SSM44 grown on saline TSA for 2 days at 30 °C were analysed by gas chromatography (7890B, Agilent technologies) according to the instructions of the Microbial Identification System (MIDI) with the TSBA 6.21 database. FAMEs of *P. sabulinigri* KCTC 22137^T grown on TSA with 5% NaCl for 2 days at 30 °C were also analysed.

For genome comparison, genomic DNA of strains SSM26^T and SSM44 were extracted as previously described [26]. The genome sequencing of the two strains was performed using an Illumina MiSeq at ChunLab (Seoul, Republic of Korea) and assembled using the SPAdes programme version 3.7 [27]. Contamination was checked for all genomes obtained in this study on the basis of 16S [28] and protein-coding genes [26]. Genome completeness was estimated by CheckM (version 1.0.8) [29]. Genome size, N50 and DNA G+C content were

calculated using QUAST (version 4.5) [30]. 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*. [31] and digital DNA–DNA hybridization (dDDH) by the Genome-to-Genome Distance Calculator (GGDC) described by Auch *et al*. [32]. The DNA G+C content was calculated from a draft genome of each strain. Automatic genome annotation was performed using GenDB. To infer a more robust phylogeny of members of the genus *Pseudomonas*, phylogenomic analysis of genomes of strains SSM26^T and SSM44 and the type strains of related species was employed based on the Genome Taxonomy Database (GTDB) taxonomy using GTDB-Tk [33]. For a multiple sequence alignment of amino acids of 120 concatenated marker genes obtained by GTDB-Tk, phylogenetic analyses based on the NJ, ME and ML methods were performed using bootstrap analysis of 1000 replications.

The results of the morphological, physiological and biochemical analyses of strains SSM26^T and SSM44 are given in the species description and Table 1. Cells of strains SSM26^T and SSM44 were Gram-stain-negative, strictly aerobic, motile by means of a polar flagellum and rod-shaped (Fig. S2, available in the online version of this article). The 16S rRNA gene sequences of both strains were identical. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains SSM26^T and SSM44 belonged to the genus *Pseudomonas*. Strains SSM26^T and SSM44 were most closely related to the type strain *P. sabulinigri* KCTC 22137^T with a 16S rRNA gene sequence similarity of 98.5%. The 16S rRNA gene similarity between SSM26^T and other *Pseudomonas* species were ≤97.3%; specifically, *Pseudomonas litoralis* 2SM5^T (97.3%), *Pseudomonas pelagia* CL-AP6^T (97.2%), *Pseudomonas bauzanensis* BZ93^T (96.9%), *Pseudomonas pachastrellae* KMM 330^T (96.0%), *P. pertucinogena* IFO 14163^T (95.7%), *Pseudomonas formosensis* CC-CY503^T (95.6%) and *Pseudomonas oceani* KX 20^T (95.0%). In all of the phylogenetic and phylogenomic trees, strains SSM26^T and SSM44 formed a distinct branch with *P. sabulinigri* KCTC 22137^T (Figs 1 and S1). Thus, the phylogenetic position of strains SSM26^T and SSM44 showed that the strains could be assigned to a novel species in the genus *Pseudomonas*.

The genome sizes of strains SSM26^T and SSM44 were 4.33 Mbp. According to the minimal standards recommended for the use of genomic data in prokaryotic taxonomy [34], other statistics for the genomes of both strains are given in Table S2. The ANI values calculated for the estimation of the degree of pairwise genome-based relatedness between both strains was 99.9%, indicative of a single genomic species. The ANI value generated by pairwise comparison between SSM26^T and *P. sabulinigri* KCTC 22137^T was 80.2%. This level is obviously below the proposed cut-off ANI values of 95–96% for delineating bacterial species [31, 35]. The dDDH value estimated by GGDC between strains SSM26^T and SSM44 was 100%. The dDDH values between strain SSM26^T and *P. sabulinigri* KCTC 22137^T, and between strain SSM26^T and other type strains of *Pseudomonas* species in the *P. pertucinogena* group were 22.5% and 19.3–20.3%, respectively (Table S3). These results indicated that strains SSM26^T and SSM44 are members of a distinctive species of the genus *Pseudomonas* [36]. The DNA G+C content of both strains SSM26^T and SSM44 was 56.2 mol% (Table 1).

The major fatty acid profiles of strains SSM26^T and SSM44 were similar to those of *P. sabulinigri* KCTC 22137^T. Fatty acids $C_{18:1}$ ω7*c*, $C_{16:0}$, summed feature 3 (comprising $C_{16:1}$ *ω7c*/$C_{16:1}$ ω6*c*) and $C_{12:0}$ were major components for these species (Table S1).

The following phenotypes were differential characteristics of strains SSM26^T and SSM44 compared to its closest phylogenetic neighbour *P. sabulinigri* KCTC 22137^T. Strains SSM26^T and SSM44 could be distinguished from *P. sabulinigri* KCTC 22137^T by ability to hydrolyse casein, and ability to utilize D-malic acid and L-rhamnose as a sole carbon sources (Table 1). Strains SSM26^T and SSM44 differed from *P. sabulinigri* KCTC 22137^T by positive results for caprate assimilation and negative ones for fermentation (Table 1). Strains SSM26^T and SSM44 differed from *P. sabulinigri* KCTC 22137^T by inability to produce acid from D- and L-arabinose, D-ribose, D- and L-xylose, D-galactose, D-glucose, D-mannose, L-rhamnose, cellobiose, lactose, melibiose, gentiobiose, D-lyxose and D- and L-fucose.

In conclusion, based on the phylogenetic, genomic, phenotypic and chemotaxonomic characteristics described above, strains SSM26^T and SSM44 should be placed in the genus *Pseudomonas* as representing a novel species, for which the name *Pseudomonas neustonica* sp. nov. is proposed.

DESCRIPTION OF *PSEUDOMONAS NEUSTONICA* SP. NOV.

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

Cells are Gram-stain-negative, strictly aerobic, motile by means of a polar flagellum and rod-shaped (0.3–0.5 µm wide 1.1–2.0 µm long). Colonies are white, circular, smooth and convex with 0.3–0.5 mm in diameter after 3 day incubation on TSA with 3% NaCl at 30 °C. Positive for oxidase and catalase activities. The temperature and pH ranges for growth are between 10–40 °C (optimum, 30 °C) and pH 5.0–8.5 (optimum, pH 7.0–7.5), respectively. Growth occurs in the presence of NaCl with concentration 0.5–10% (w/v) (optimum 3%). Starch, casein and Tweens (20, 40, 60 and 80) are hydrolysed, but hypoxanthine and xanthine are not.

Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are positive, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, *α*- and *β*-galactosidase, *β*-glucuronidase, *α*- and *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase are negative. Nitrate reduction, indole production, hydrolysis of gelatin and fermentation are negative, and enzyme activity of arginine dihydrolase

Table 1. Selected characteristics that differentiate strains SSM26^T and SSM44 from *Pseudomonas sabulinigri* KCTC 22137^T

Strains: 1, SSM26^T; 2, SSM44; 3, *Pseudomonas sabulinigri* KCTC 22137^T. +, Positive; –, negative. All data were obtained in this study except where indicated.

Characteristic	1	2	3
DNA G+C content (mol%) by genome analysis	56.2	56.2	58.1*
Hydrolysis of casein	+	+	–
Growth conditions:			
NaCl range (optimum) (%, w/v)	0.5–10.0 (3.0)	0.5–10.0 (3.0)	0.5–10.0 (5.0–6.0)
pH range (optimum)	5.0–8.0 (7.0–7.5)	5.0–8.5 (7.0–7.5)	5.0–9.0 (7.0–7.5)
Utilization as a sole carbon source:			
D-Malic acid	+	+	–
L-Rhamnose	+	+	–
API 20NE test results:			
Fermentation	–	–	+
Urease	+	–	+
Hydrolysis of aesculin	–	+	–
Assimilation of caprate	+	+	–
Assimilation of citrate	–	–	+
Acid production from:			
D-Arabinose	–	–	+
L-Arabinose	–	–	+
D-Ribose	–	–	+
D-Xylose	–	–	+
L-Xylose	–	–	+
D-Galactose	–	–	+
D-Glucose	–	–	+
D-Mannose	–	–	+
L-Rhamnose	–	–	+
Cellobiose	–	–	+
Lactose	–	–	+
Melibiose	–	–	+
Gentiobiose	–	–	+
D-Lyxose	–	–	+
D-Fucose	–	–	+
L-Fucose	–	–	+

*Data from Kim *et al.* [19].

and β-galactosidase are absent. Urease activity and hydrolysis of aesculin are variable. Positive for assimilation of caprate and adipate, but negative for assimilation of citrate, D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, malate and phenylacetate. Acid is produced from aesculin and potassium 2-ketogluconate, but not from *N*-acetylglucosamine, D-adonitol, amygdalin, DL-arabinose, DL-arabitol, arbutin, cellobiose, dulcitol, erythritol, D-fructose, DL-fucose, D-galactose, gentiobiose, D-glucose, glycerol, glycogen, inositol, inulin, lactose, D-lyxose, maltose,

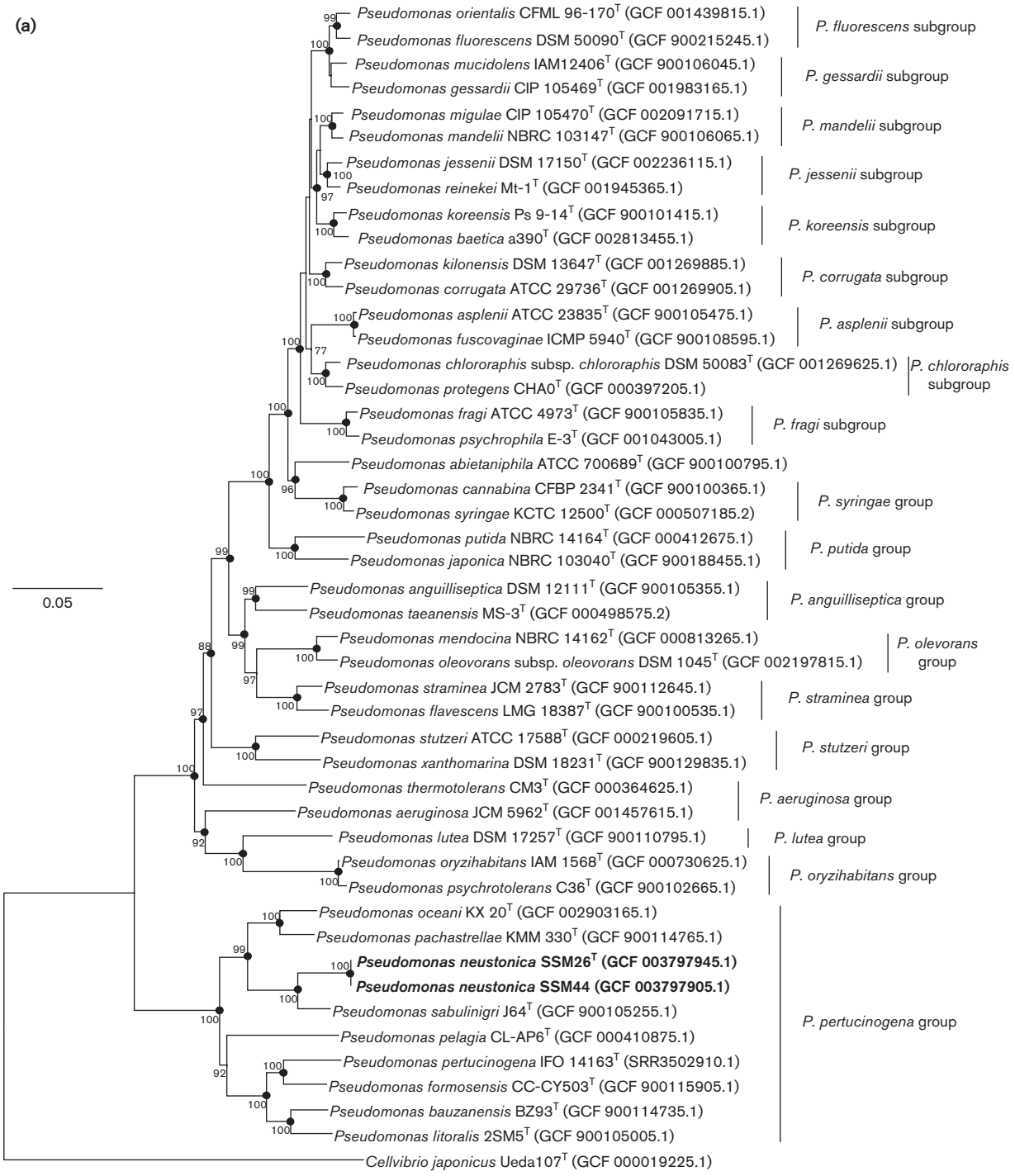
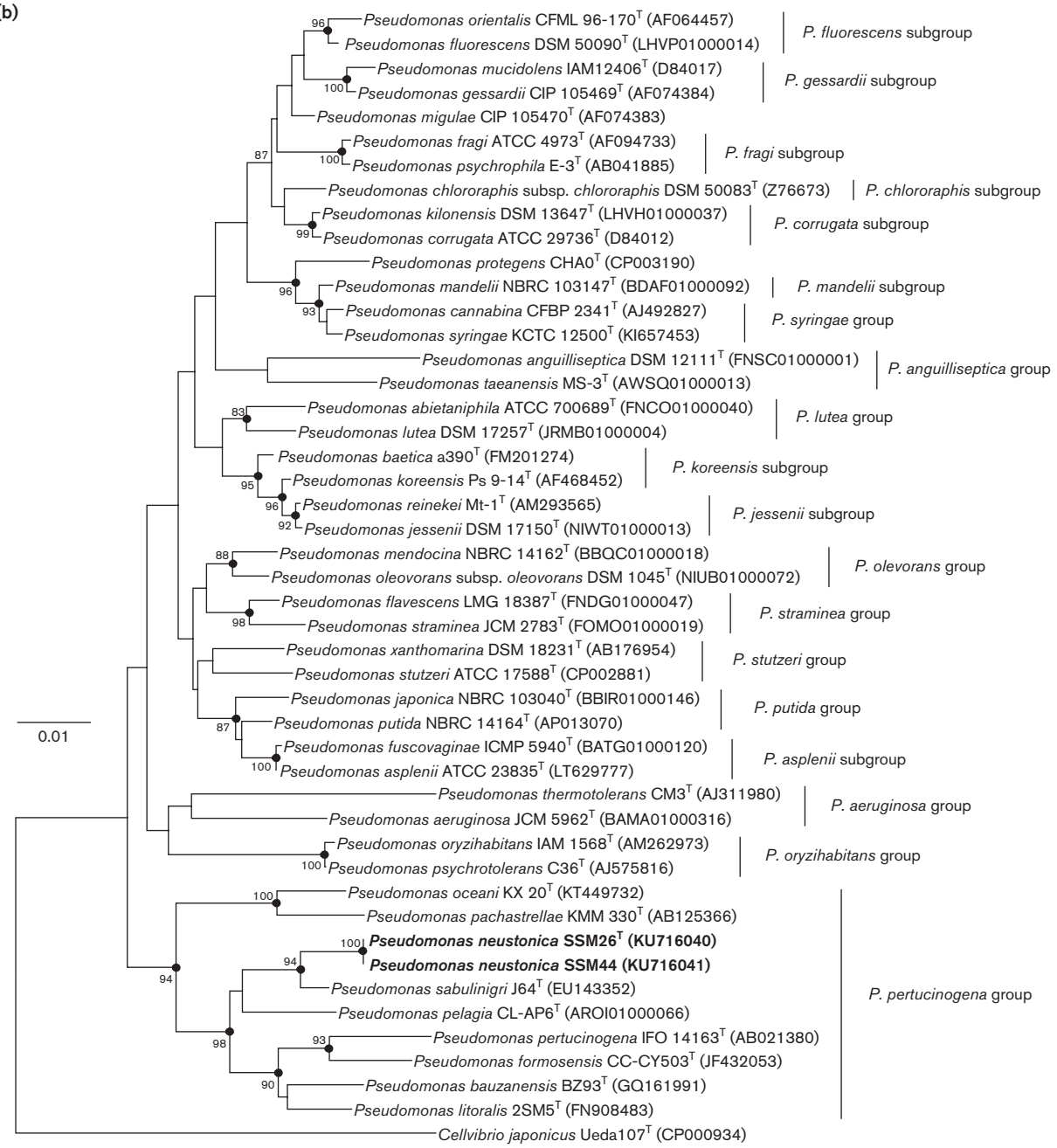


Fig. 1. Neighbour-joining trees based on (a) amino acid sequences for 120 concatenated marker genes and (b) 16S rRNA gene sequences showing the relationship between strains SSM26^T, SSM44 and the type strains of related species. *Cellvibrio japonicus* Ueda107^T was used as an outgroup. Only bootstrap values above 70% are shown (1000 resamplings) at branch points. Filled circles indicate that the corresponding nodes were also obtained in both the maximum-likelihood and the minimum-evolution trees. Bars, 0.05 and 0.01 substitutions per site.

Fig. 1. (Cont.)

(b)



D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium 5-ketogluconate, potassium gluconate, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol and DL-xylose. In sole carbon utilization tests, L-arabinose, cellobiose, D-fructose, D-glucose, lactose, D-malic acid, D-mannose, melibiose, L-proline, L-rhamnose and sucrose are utilized, but L-ascorbate, citric acid, myo-inositol, D-lyxose, raffinose, D-sorbitol and trehalose dihydrate are not utilized. The major cellular fatty acids are $C_{18:1}$ ω7*c*, $C_{16:0}$, summed feature 3 (comprising $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*) and $C_{12:0}$.

The type strain, SSM26^T (=KCCM 43193^T=JCM 31284^T), was isolated from the surface microlayer in coastal seawater of the Ross Sea (Antarctica). Strain SSM44 (=KCCM 43194=JCM 31285), isolated from the same source, is a second strain of the species. The GenBank/EMBL/DBBJ accession numbers

for the 16S rRNA gene sequence and the genome sequence of strain SSM26^T are KU716040 and RKKU00000000, respectively, and those of strain SSM44 are KU716041 and RKKV00000000, respectively. The DNA G+C contents are 56.2 mol% (by genome analysis).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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