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TAXONOMIC DESCRIPTION

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Sandaracinobacter neustonicus sp. nov., isolated from the sea surface microlayer in the Southwestern Pacific Ocean, and emended description of the genus *Sandaracinobacter*

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Abstract

A Gram-stain-negative, non-motile, facultatively anaerobic and rod-shaped bacterial strain, designated PAMC 28131^T, was isolated from a sea surface microlayer sample in the open water of the Pacific Ocean. Phylogenetic analysis of the 16S rRNA gene sequence of strain PAMC 28131^T revealed an affiliation to the genus *Sandaracinobacter* with the closest species *Sandaracinobacter sibiricus* RB16-17^T (sequence similarity of 98.2%). Strain PAMC 28131^T was able to grow optimally with 0.5–1.0% NaCl and at pH 6.5–7.0 and 30 °C. The polar lipids were phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid. The major cellular fatty acids (>10%) were $C_{18:1}$ *ω6c* and/or $C_{18:1}$ *ω7c*, (42.6%), $C_{17:1}$ *ω6c* (19.3%) and $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c* (15.8%), and the respiratory quinone was Q-10. The genomic DNA G+C content was 65.3mol%. The phylogenetic, phenotypic and chemotaxonomic data showed that strain PAMC 28131^T could be clearly distinguished from *S. sibiricus* RB16-17^T. Thus, strain PAMC 28131^T should be classified as representing a novel species in the genus *Sandaracinobacter*, for which the name *Sandaracinobacter neustonicus* sp. nov. is proposed. The type strain is PAMC 28131^T (=KCCM 43127^T=JCM 30734^T).

INTRODUCTION

The genus *Sandaracinobacter* was first proposed by Yurkov *et al.* [1] with *Sandaracinobacter sibiricus* as the type species. The type strain of *S. sibiricus* was originally described as *Erythrobacter sibiricus* with a freshwater bacterium isolated from microbial mats formed at moderate temperatures in hydrothermal vents of the Bol'shoi River near Lake Baikal [2]. *Erythrobacter sibiricus* was reclassified into the new genus *Erythromicrobium* as bacteriochlorophyll *a* (Bchl *a*)-containing freshwater aerobic bacteria [3], and subsequently reorganized into the new genus *Sandaracinobacter* based on results of polyphasic analysis [1]. Until now, *S. sibiricus* is the only member with a validly published name in the genus *Sandaracinobacter* [4].

The description of the genus *Sandaracinobacter* includes cells that are Gram-stain-negative, aerobic, chemoorganotrophic, yellow-orange-coloured, Bchl *a*-containing, motile by means of subpolar flagella, thin and long rods; the habitat is freshwater [1]. Here, we isolated a bacterial strain from a sea surface microlayer (SSM) sample collected offshore in the Pacific Ocean, and performed a polyphasic analysis to determine the taxonomic position of the strain.

SSM is the air–sea interface, which might serve as a reservoir of terrestrial and marine bacteria that have been transported by two plausible processes: (1) atmospheric deposition originated from terrestrial aerosols; and (2) bubbles rise adhering microbes from subsurface seawater [5, 6]. To isolate neustonic bacteria in a remote ocean area, an SSM sample was collected

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Keywords: new species; *Sandaracinobacter neustonicus*; sea surface microlayer; Pacific Ocean.
Abbreviations: Bchl *a*, bacteriochlorophyll *a*; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; Q, ubiquinone; R2A, Reasoner's 2A agar; SSM, sea surface microlayer; TSA, tryptic soy agar; VP, Voges–Proskauer; YPA, yeast extract–peptone–acetate.
The GenBank/EMBL/DBBJ accession numbers for the 16S rRNA gene sequence and the draft genome sequence of strain PAMC 28131^T (=KCCM 43127^T=JCM 30734^T) are KR052095 and VFSU00000000, respectively.
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Two supplementary tables and three supplementary figures are available with the online version of this article.

from the Southwestern Pacific Ocean (29.86° S, 171.86° E) using a rotating drum SSM sampler (SSM thickness of ~100 µm) [7] during the *Araon* expedition in April 2014. The SSM sample was supplemented with autoclaved glycerol (final concentration 20%, v/v) and preserved at −80 °C until use. For cultivation of different salt-tolerant bacteria, the glycerol-amended SSM sample was spread onto each plate containing marine agar 2216 (Difco), tryptic soy agar (TSA; Difco) and Reasoner's 2A agar (R2A; Difco). Those plates were incubated under aerobic conditions at 25 °C for 3 days. Strain PAMC 28131^T was isolated on R2A agar and subsequently purified four times on fresh R2A agar at 25 °C. The strain was able to grow well on a modified YPA agar (yeast extract, 1 g; Bacto peptone, 1 g; sodium acetate, 1 g; KCl, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; NH_4Cl, 0.3 g; K_2HPO_4, 0.3 g; vitamin B12, 20 µg; Bacto agar, 15 g; distilled water, 1 l) [1] at 30 °C. Strain PAMC 28131^T was preserved in a modified YPA broth (i.e. the sample compositions of the modified YPA agar with no addition of Bacto agar) supplemented with 30% (v/v) glycerol at −80 °C.

Despite the general recommendation to use the phylogenetically closest type strain as a reference material for comparing phenotypic, genomic (i.e. genome-wide comparison) and certain chemotaxonomic (i.e. fatty acids) properties [8], we could not include the type strain *S. sibiricus* RB16-17^T in this study. *S. sibiricus* RB16-17^T was not deposited into any public culture collections and is not currently available [2]. Alternatively, we have conducted phenotypic analyses of strain PAMC 28131^T under almost identical conditions that were employed for characterizing *S. sibiricus* RB16-17^T in the literature [1, 2]. Unless otherwise specified, characteristics of strain PAMC 28131^T were based on cultures grown aerobically on modified YPA agar at 30 °C for 3–4 days.

Direct sequencing of the 16S rRNA gene was performed as previously described [9]. The nearly complete 16S rRNA gene sequence (1417 bp) of strain PAMC 28131^T was compared using BLAST against the GenBank and EzBioCloud databases [10, 11]. The 16S rRNA gene sequences of closely related taxa obtained from the GenBank database were aligned using the RDP aligner [12] on the basis of secondary structures. Phylogenetic analyses were performed using the program MEGA (version 10.1) [13]. Neighbour-joining (NJ) [14] trees were reconstructed using the Kimura two-parameter model [15] with the options of uniform rates and pairwise deletion. Maximum-parsimony (MP) [16] trees were reconstructed using the tree-bisection-reconnection [17] heuristic search method with the options of 10 initial trees (random addition) and complete deletion. Maximum-likelihood (ML) [18] trees were reconstructed using the Kimura two-parameter model [15] with the options of the nearest-neighbour-interchange heuristic method, uniform rates and complete deletion. The robustness of the phylogenetic trees reconstructed by each tree-making algorithm was evaluated by bootstrap analyses based on 1000 replications.

Phylogenetic analyses based on 16S rRNA gene sequences showed that strain PAMC 28131^T formed a robust clade with *S. sibiricus* RB16-17^T (Fig. 1a). Strain PAMC 28131^T showed the highest 16S rRNA gene sequence similarity to *S. sibiricus* RB16-17^T (98.2%) and lower similarities to other members of the genera *Sphingomonas* (≤94.7%), *Polymorphobacter* (≤93.7%), *Novosphingobium* (≤93.5%) and *Sandarakinorhabdus* (≤93.5%). The 16S rRNA gene sequence similarity between strain PAMC 28131^T and *S. sibiricus* RB16-17^T (98.2%) is lower than the thresholds of 98.7–99% for delineating bacterial species [19], suggesting that strain PAMC 28131^T could be assigned to a novel species in the genus *Sandaracinobacter*.

To obtain the genome sequence, genomic DNA of strain PAMC 28131^T was extracted using a commercial kit (DNeasy Blood and Tissue kit, Qiagen). A sequencing library was reconstructed with Nextera DNA Preparation Kit (Illumina) and sequenced with Illumina MiSeq. *De novo* genome assembly was performed with paired-end reads (300 bp×2) using the CLC Genomics Workbench program (version 8.5). Contamination was checked for the genome contigs on the basis of 16S rRNA [20] and protein-coding genes [21]. Genome completeness was estimated by CheckM (version 1.0.8) [21]. Genome size, N50 and DNA G+C content were calculated using QUAST (version 4.5) [22]. A complete 16S rRNA gene sequence was retrieved from the genome sequence of strain PAMC 28131^T after genome annotation using the Pathosystems Resource Integration Centre (PATRIC) web service [23]. To infer a more robust phylogeny of members of the family *Sphingomonadaceae*, phylogenomic analysis of genomes of strain PAMC 28131^T and the type strains of related species was employed based on the Genome Taxonomy Database (GTDB) taxonomy using GTDB-Tk [24]. For a multiple sequence alignment of amino acids of 120 concatenated marker genes obtained by GTDB-Tk, phylogenetic analyses based on the NJ, MP and ML methods were performed with the bootstrap analysis of 1000 replications using the program MEGA (version 10.1) [13].

The 16S rRNA gene sequence of strain PAMC 28131^T retrieved from its genome sequence was identical to that determined by direct sequencing, indicative of no genome contamination. Consistently, quality estimates based on a set of marker genes gave a result of a reliable genome sequence of strain PAMC 28131^T (i.e. 100% completeness and 0.87% contamination). Other statistics for the final genome assembly of strain PAMC 28131^T are given in Table S1 (available in the online version of this article), in accordance with the minimal standards for the use of genomic data in prokaryotic taxonomy [25]. The genome size of strain PAMC 28131^T was 3.65 Mbp with the DNA G+C content of 65.3mol% (Tables 1 and S1). The phylogenomic tree based on amino acid sequences for 120 concatenated marker genes showed that strain PAMC 28131^T formed a coherent lineage with the robust clade comprising *Polymorphobacter* species and *Sandarakinorhabdus* species (Fig. 1b), indicating a distinct taxonomic position of the genus *Sandaracinobacter* in the family *Sphingomonadaceae*.

Phenotypic features of strain PAMC 28131^T were characterized with repeated experiments in duplicate on different days. Gram-staining was performed as previously described [26].

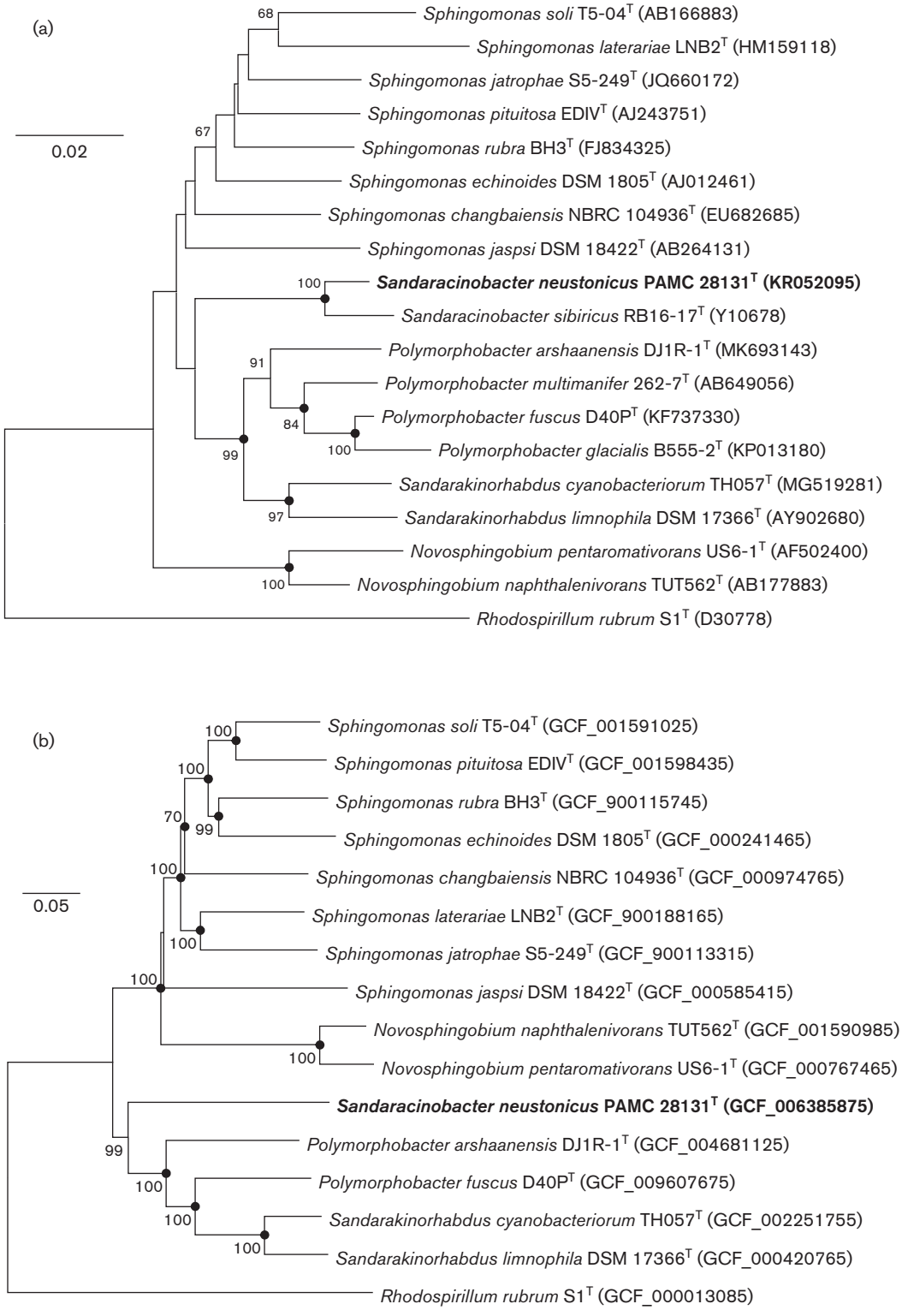

Fig. 1. Neighbour-joining trees based on (a) 16S rRNA gene sequences and (b) amino acid sequences for 120 concatenated marker genes of strain PAMC 28131^T and related members in the family *Sphingomonadaceae* with *Rhodospirillum rubrum* S1^T as an outgroup. Only bootstrap values above 70% are shown (1000 resamplings) at the branching points. Filled circles indicate that the corresponding nodes were also obtained in both the maximum-parsimony and the maximum-likelihood trees. Bars, 0.02 and 0.05 substitutions per site.

Table 1. Differential characteristics between strain PAMC 28131^T and *Sandaracinobacter sibiricus* RB16-17^T

Strains: 1, PAMC 28131^T (this study); 2, *Sandaracinobacter sibiricus* RB16-17^T [1, 2]. +, Positive; –, negative.

Characteristic	1	2
Oxygen requirement	Facultatively anaerobic	Strictly aerobic
Optimal growth:		
Temperature (°C)	30	25–30
NaCl (%, w/v)	0.5–1.0	0–1.0
pH	6.5–7.0	7.5–8.5
Motility	–	+
Bacteriochlorophyll *a*	–	+
Major carotenoid *in vivo* peaks (nm)	450, 474	424, 450, 474
Catalase activity	+	–
Hydrolysis of starch	+	–
Utilization of sole carbon source:		
Acetate	–	+
Fructose	–	+
Ribose	+	–
Sucrose	–	+
Quinone(s)	Q-10	Q-9, Q-10
DNA G+C content (mol%)	65.3	68.5*

*Thermal denaturation method [1]

Cell motility was assessed by the hanging-drop method [27]. Cells grown for 4–5 days with shaking (100 r.p.m.) at 30 °C in the modified YPA broth were observed by transmission electron microscopy (EX2, JEOL) to examine cell size and morphology. Anaerobic growth was tested on the modified YPA agar along with an obligately anaerobic bacterium *Clostridium* sp. PAMC 80033 as experimental control using the GasPak anaerobic system (BBL) at 30 °C for 3 weeks. As previously described [2], the presence of Bchl *a* and carotenoid pigment was determined for *in vivo* whole cells and acetone–methanol extracts with a spectrophotometer (S-3100, Scinco) for cells that had been grown in either the dark or the light for 7 days. Voges–Proskauer (VP) reaction was tested as previously described [26] with negative and positive control strains (*Escherichia coli* KCCM 11234^T and *Enterobacter aerogenes* KCCM 12177^T, respectively) [28, 29]. H_2S production assay was performed using triple sugar iron agar (Difco) with negative and positive control strains (*Escherichia coli* KCCM 11234^T and *Citrobacter freundii* KCCM 11931^T, respectively) [28, 30].

The cells of strain PAMC 28131^T were Gram-stain-negative, facultatively anaerobic, non-motile, rods (Fig. S1). Bchl *a* was not detected in strain PAMC 28131^T, while carotenoid pigments were present (Table 1, Fig. S2). Negative results of VP reaction and H_2S production were obtained for strain PAMC 28131^T.

The temperature range for growth was determined by assessing changes of the OD_{600} in the modified YPA broth for 3 weeks at 4, 10–30 (in increments of 5 °C), 37 and 42 °C. The pH range (pH 5.0–10.0, in increments of 0.5 pH units) for growth was determined by assessing changes of the OD_{600} in modified YPA broth using citrate 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 at 30 °C for 3 weeks. The pH for aliquots of autoclave-sterilized broth was measured before inoculation of cells to check pH change, resulting in no substantial pH changes. Salt tolerance was tested by assessing changes of the OD_{600} in the modified YPA broth supplemented with 0–5% (in increments of 0.5%, w/v) and 6–12% (in increments of 2%) NaCl at 30 °C for 3 weeks.

Strain PAMC 28131^T grew at 4–37 °C (optimum, 30 °C) and pH 6.0–8.0 (optimum, pH 6.5–7.0). Growth occurred at NaCl concentrations of 0–1.0% (w/v; optimum, 0.5–1.0%).

Catalase and oxidase tests were performed as previously described [26, 31]. Hydrolysis of starch and Tweens (20, 40, 60 and 80) were tested according to Hansen and Sørheim [32] after 7 days incubation. Hydrolysis of casein, xanthine and hypoxanthine was determined using the method of Smibert and Krieg [26]. In addition, other biochemical activities of strain PAMC 28131^T were determined by using the API ZYM, API 20NE and API 50CH (for acid production) kits (bioMérieux) according to the manufacturer's instructions. The oxidation of carbon compounds was tested using the GEN III MicroPlate system (Biolog) according to the manufacturer's instructions. Sole carbon source utilization was tested according to the method of Yurkov and Gorlenko [2] with a final concentration of 0.1% carbon source. Sole carbon source 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} at 30 °C for 3 weeks.

Strain PAMC 28131^T was positive for catalase and oxidase activities (Table 1). It hydrolysed casein, starch, Tweens 20, 40, 60 and 80, but did not hydrolyze xanthine and hypoxanthine. Other phenotypic characteristics of strain PAMC 28131^T are given in the species description and Table 1.

Polar lipids were extracted using the method described by Minnikin *et al.* [33], separated by two-dimensional TLC and identified by spraying with appropriate detection reagents [34]. The fatty acid methyl esters in whole cells grown on the modified YPA agar at 30 °C for 4 days were analysed by gas chromatography according to the instruction of the Microbial Identification System (MIDI) using the TSBA 6.21 database. Isoprenoid quinone composition was analysed by HPLC as described by Collins [35] at the Korean Culture Center of Microorganisms (KCCM).

The polar lipids of strain PAMC 28131^T were phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid (Fig. S3). The major cellular fatty acids (>10%) were summed feature 8 ($C_{18:1}$ ω6*c* and/or $C_{18:1}$ ω7*c*, 42.6%), $C_{17:1}$ ω6*c* (19.3%) and summed feature 3 ($C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*, 15.8%; Table S2). The isoprenoid quinone of strain PAMC 28131^T was Q-10 (Table 1).

Strain PAMC 28131^T can be clearly differentiated from *S. sibiricus* RB16-17^T by certain characteristics (Table 1). Strain PAMC 28131^T was a facultatively anaerobic bacterium, while *S. sibiricus* RB16-17^T was known to require oxygen to grow [2]. Catalase activity, starch hydrolysis and motility differentiated strain PAMC 28131^T from *S. sibiricus* RB16-17^T (Table 1). Bacteriochlorophyll *a*, a main feature of the genus *Sandaracinobacter* [2], was not detected in strain PAMC 28131^T, neither was a major carotenoid peak of 424 nm in the present study (Table 1). The presence of Q-10 as the sole major respiratory quinone can also differentiate strain PAMC 28131^T from *S. sibiricus* RB16-17^T (Table 1). Both strains displayed different utilizations of acetate, fructose, ribose and sucrose as sole carbon source (Table 1).

In conclusion, based on the phylogenetic, phenotypic, and chemotaxonomic data described above, we suggest that strain PAMC 28131^T represents a novel species of the genus *Sandaracinobacter*, for which the name *Sandaracinobacter neustonicus* sp. nov. is proposed.

EMENDED DESCRIPTION OF THE GENUS *SANDARACINOBACTER* YURKOV *ET AL.* 1997

The properties of this genus are as described by Yurkov *et al.* [1], with the following amendments. Strictly aerobic or facultatively anaerobic. Motility is variable. Bacteriochlorophyll *a* is variable. Cultures are intensely yellow-orange or yellow because of carotenoid pigments. Contains quinones Q-9 and Q-10, or sole quinone Q-10. The major polar lipids are phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid. The major cellular fatty acids are $C_{18:1}$ ω6*c* and/or $C_{18:1}$ ω7*c*, $C_{17:1}$ ω6*c* and $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*. DNA G+C content is 65.3–68.5 mol%.

DESCRIPTION OF *SANDARACINOBACTER NEUSTONICUS* SP. NOV.

Sandaracinobacter neustonicus (neu.sto'ni.cus. N.L. masc. adj. *neustonicus* pertaining to and living in the neuston).

Displays the following properties in addition to those given in the genus description. Gram-stain-negative. Cells are rods 0.6–0.8 µm wide and 1.4–3.1 µm long. Colonies are circular, convex, entire, opaque and yellow in colour, and 1.5–2.0 mm in diameter after 5 days incubation. Grows at 4–37 °C (optimum, 30 °C) and pH 6.0–8.0 (optimum, pH 6.5–7.0). Growth occurs at NaCl concentrations of 0–1.0% (w/v) (optimum, 0.5–1.0%). Negative for VP reaction and H_2S production. Positive for catalase and oxidase activities. Xanthine and hypoxanthine are not hydrolysed, but casein, starch and Tweens (20, 40, 60 and 80) are hydrolysed. According to the API ZYM test, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), trypsin, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase and *α*-glucosidase, but negative for *α*-galactosidase, *β*-glucuronidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase and *α*-fucosidase. According to the API 20NE test, positive for *β*-glucosidase, hydrolysis of gelatin, *β*-galactosidase, and assimilation of glucose and maltose, but negative for nitrate and nitrite reduction, indole production, fermentation, arginine dihydrolase, urease, assimilation of caprate, malate, citrate and phenylacetate. According to the API 50CH test, acid is produced from 5-ketogluconate and maltose, but not from 2-ketogluconate, *N*-acetylglucosamine, D-adonitol, aesculin, 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, D-mannitol, D-mannose, melezitose, melibiose, methyl *α*-D-glucopyranoside, methyl *α*-D-mannopyranoside, methyl *β*-D-xylopyranoside, potassium gluconate, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol or DL-xylose. According to the Biolog GEN III MicroPlate test, acetic acid, acetoacetic acid, L-arginine, L-aspartic acid, L-galactonic acid lactone, D-galactose, gelatin, D-glucose-6-PO4, glucuroamide, glycyl-L-proline, L-glutamic acid, L-histidine, *β*-hydroxy-D,L-butyric acid and Tween 40 are oxidized, but L-alanine, γ-amino-butryric acid, D-arabitol, D-aspartic acid, bromo-succinic acid, cellobiose, citric acid, dextrin, formic acid, D-fructose, D-fructose-6-PO4, DL-fucose, D-galacturonic acid, D-gluconic acid, *α*-D-glucose, D-glucuronic acid, glycerol, *α*-hydroxy-D,L-butyric acid, *p*-hydroxy-phenylacetic acid, inosine, *α*-keto-butyric acid, *α*-keto-glutaric acid, L-lactic acid, D-lactic acid methyl ester, DL-malic acid, maltose, D-mannitol, D-mannose, 3-methyl glucose, methyl pyruvate, mucic acid, myo-inositol, pectin, propionic acid, L-pyroglutamic acid, quinic acid, raffinose, L-rhamnose, D-saccharic acid, DL-serine, D-sorbitol, stachyose, sucrose, trehalose and turanose are not. In sole carbon utilization tests, butyrate, D-glucose, maltose and propionate are utilized, but citrate, ethanol, formate, fumarate, lactate, malate, methanol, pyruvate, D-sorbitol, succinate and sucrose are not.

The type strain is PAMC 28131^T (=KCCM 43127^T=JCM 30734^T), isolated from the sea surface microlayer in the Southwestern Pacific Ocean. The GenBank/EMBL/DBBJ accession numbers for the 16S rRNA gene sequence and the draft genome sequence of strain PAMC 28131^T are KR052095 and VFSU00000000, respectively. The genomic DNA G+C content of the type strain is 65.3 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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