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Leeuwenhoekiella polynyae sp. nov., isolated from a polynya in western Antarctica

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A Gram-stain-negative, motile by gliding, rod-shaped bacterial strain, designated SOJ2014-1^T was isolated from surface water of a polynya in the Antarctic Ocean. A comparative 16S rRNA gene sequence analysis showed that strain SOJ2014-1^T belongs to the genus *Leeuwenhoekiella* and is most closely related to *Leeuwenhoekiella marinoflava* DSM 3653^T (97.5 % 16S rRNA gene sequence similarity). The G+C content of the genomic DNA of strain SOJ2014-1^T was 38.8 mol%. Its predominant cellular fatty acids were summed feature 3 (composed of $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, iso-$C_{15:1}$ G and summed feature 9 (composed of iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$). DNA–DNA relatedness between strain SOJ2014-1^T and close relatives, *L. marinoflava* DSM 3653^T and *Leeuwenhoekiella aequorea* LMG 22550^T, was below 49 %. The respiratory quinone was MK-6. The major polar lipids were phosphatidylethanolamine, an unidentified aminolipid and two unidentified lipids. The strain grew at 0–35 °C (optimum, 25 °C) with 0–14.0 % (w/v) NaCl (optimum, 1.0–5.0 %). It was strictly aerobic and had different carbohydrate utilization traits compared with *L. marinoflava* DSM 3653^T. Based on the phenotypic, chemotaxonomic and phylogenetic analyses, strain SOJ2014-1^T is proposed as a representative of a novel species, *Leeuwenhoekiella polynyae*. The type strain is SOJ2014-1^T (=KCTC 42185^T=JCM 30387^T).

The genus *Leeuwenhoekiella*, a member of the family *Flavobacteriaceae*, was originally proposed by Nedashkovskaya *et al.* (2005) to accommodate yellow-pigmented, chemoorganotrophic, strictly aerobic, motile by gliding and Gram-stain-negative bacteria. Currently, the genus *Leeuwenhoekiella* comprises four recognized species, including *Leeuwenhoekiella marinoflava* (formerly *Cytophaga marinoflava)*, *Leeuwenhoekiella aequorea*, *Leeuwenhoekiella palythoae* (Nedashkovskaya *et al.*, 2009, 2014) and *Leeuwenhoekiella blandensis* (Pinhassi *et al.*, 2006).

A novel bacterial strain, designated SOJ2014-1^T, was isolated from Antarctic polynya seawater (112° 00′ W 73° 30′ S) obtained during the *Araon* scientific cruise (January 2014). The seawater sample was diluted 10-fold in sterile artificial seawater (ASW; l^{-1} distilled water, 1.3 g KCl, 0.02 g K_2HPO_4, 23 g NaCl, 0.5 g NH_4Cl, 0.1 g $CaCl_2.2H_2O$, 3 g $MgCl_2.6H_2O$, 0.14 g KBr and 3 mM bicarbonate) with pH 7.5. An aliquot of each dilution was spread onto modified seawater cytophaga agar (250 ml distilled water, 750 ml seawater, 0.2 g sodium acetate, 0.5 g tryptone, 0.5 g yeast extract, 0.2 g beef extract and 15 g agar) (Irgens *et al.*, 1989) and was incubated at 10 °C for 1 week. A single golden-yellow-coloured colony was isolated and purified. Strain SOJ2014-1^T was incubated at 25 °C under aerobic conditions on marine agar 2216 (MA; Difco) and preserved at −80 °C in marine broth 2216 (MB; Difco) supplemented with glycerol (25 %, w/v). *L. marinoflava* DSM 3653^T, *L. aequorea* LMG 22550^T, *L. palythoae* KCTC 22020^T and *L. blandensis* KCTC 22103^T were used as the reference strains. Unless otherwise stated, *L. marinoflava* DSM 3653^T, *L. aequorea* LMG 22550^T, *L. palythoae* KCTC 22020^T, *L. blandensis* KCTC 22103^T and strain SOJ2014-1^T were grown on MA at 25 °C.

Genomic DNA was extracted using a commercial genomic DNA extraction kit (Cosmo Genetech). The 16S rRNA

Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain SOJ2014-1^T is KM101107.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

gene was amplified from the DNA using the universal bacterial primers 27F and 1492R (Lane, 1991) and the purified PCR product was sequenced by Cosmo Genetech. The 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR). The 16S rRNA gene sequence of strain SOJ2014-1^T was determined to be 1375 bp in length. It was compared with available 16S rRNA gene sequences in the EzTaxon server (http://eztaxon-e.ezbiocloud.net) (Kim *et al.*, 2012) and the result indicated that the sequence similarities between strain SOJ2014-1^T and species of the genus *Leeuwenhoekiella* ranged from 96.0 to 97.5 %.

The 16S rRNA gene sequences of representative members of the family *Flavobacteriaceae* were retrieved from the GenBank database and aligned with the sequence of strain SOJ2014-1^T using SILVA (http://www.arb-silva.de/aligner) considering the secondary structure of the rRNA gene (Pruesse *et al.*, 2007). Gaps were edited in the BioEdit program (Hall, 1999). Phylogenetic trees were generated with the neighbour-joining (NJ; Saitou & Nei, 1987), and minimum-evolution (ME; Kidd & Sgaramella-Zonta, 1971) methods implemented in the MEGA5 program (Tamura *et al.*, 2011). Kimura's two-parameter model (Kimura, 1983) was used for the calculation of phylogenetic distances. Based on the NJ and ME methods, strain SOJ2014-1^T was determined to belong to the genus *Leeuwenhoekiella* as shown by the bootstrap support in Fig. 1. According to maximum-likelihood (Felsenstein, 1981) method, the phylogenetic affiliation of strain SOJ2014-1^T to the genus *Leeuwenhoekiella* was also supported (data not shown).

Genomic DNA extracted for 16S rRNA gene amplification was used for determination of DNA G+C content. The G+C content of the genomic DNA was determined by following the method of Gonzalez & Saiz-Jimenez (2002). The G+C content of the genomic DNA of strain SOJ2014-1^T was 38.8 mol%. DNA−DNA hybridization was conducted by the fluorometric method using photobiotin-labelled DNA in microwells as described by Ezaki *et al.* (1989). DNA−DNA hybridization between strain SOJ2014-1^T and two reference strains, *L. aequorea* LMG 22550^T and *L. marinoflava* DSM 3653^T, was conducted at 37 °C with five replicates. The DNA−DNA hybridization value between strain SOJ2014-1^T and the two reference strains varied from 37.3 to 48.8 % (Table S1, available in the online Supplementary Material). The standard criterion for defining species by DNA−DNA hybridization value (i.e. <70 %; Stackebrandt & Goebel, 1994) supports that strain SOJ2014-1^T represents a novel species within the genus *Leeuwenhoekiella*.

The cellular fatty acids of strain SOJ2014-1^T and four reference strains, *L. marinoflava* DSM 3653^T, *L. aequorea* LMG 22550^T, *L. palythoae* KCTC 22020^T and *L. blandensis*, were analysed after cultivation on MA at 25 °C at pH 7.5 for 3 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the MIDI system (Sherlock Microbial Identification System, version 6.1), analysed by GC (6890N; Agilent Technologies) and identified using the Microbial Identification software package (TSBA6 database) (Sasser, 1990). The major cellular fatty acids of strain SOJ2014-1^T were summed feature 3 (composed of $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, iso-$C_{15:1}$ G and summed feature 9 (composed of is- $C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$). Although the profiles were similar for strain SOJ2014-1^T and the reference strains, the compositions in the strains differed quantitatively (Table 1).

The quinone composition of strain SOJ2014-1^T was determined as follows. The freeze-dried cells (100 mg) were treated with chloroform/methanol (2 : 1, v/v) overnight to extract isoprenoid quinones. Preparative TLC was performed using Kieselgel 60 F254 plates (20 × 20 cm, 0.5 mm thick; Merck) with petroleum ether/diethyl ether (9 : 1, v/v) as the solvent. The resulting bands were scraped from the plate under short-wavelength UV light and were redissolved in the solvent. Finally the quinone profile was analysed using reversed-phase HPLC (LC20AD system; Shimadzu) with an ODS-2 C18 column (150 × 4.6 mm; Phenomenex) and a UV detector at 270 nm. The major respiratory quinone was MK-6. Polar lipids of strain SOJ2014-1^T were extracted, separated using two-dimensional TLC and identified according to the published procedure by Komagata & Suzuki (1987). The major phospholipids were phosphatidylethanolamine, an unidentified aminolipid and two lipids that were not stainable with any of the specific spray reagents applied to detect a phosphate group or an amino group (Fig. S1). 1

Cell morphology and size were observed using light microscopy (CX21; Olympus) and transmission electron microscopy (TEM) (Tecnai G2 Spirit; FEI). Cells cultivated at 25 °C for 4 days on MA were used for negative staining for TEM with 1 % (w/v) phosphotungstic acid. Colony morphology was observed after culture on MA at 25 °C for 3 days. Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution (Taylor & Achanzar, 1972) and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Tarrand & Gröschel, 1982). A Gram Stain kit (Difco) was used according to the manufacturer's instruction. The gliding motility was evaluated using the hanging drop technique (Bernardet *et al.*, 2002). For the oxygen requirement test, cells were incubated in the presence of thioglycollate (fluid thioglycollate medium; l^{-1} distilled water, 5 g glucose (anhydrous), 37.4 g MB, 0.75 g agar, 0.001 g resazurin, 0.5 g sodium thioglycollate) in Hungate tubes sealed with butyl rubber stoppers. Colonies of strain SOJ2014-1^T were golden-yellow-coloured with entire margins. 2 Cells of strain SOJ2014-1^T were 0.4–0.6 × 1.5–2.7 μm (Fig. S2) and rod-shaped, Gram-stain-negative, oxidase- and catalase-positive and motile by gliding. 3 The cells were gathered at the top of the thioglycollate medium in the Hungate tubes where the oxygen concentration is highest, which indicates that the strain is strictly aerobic. H_2S was not produced and glucose, lactose and sucrose were fermented in triple-sugar iron agar (Padron & Dockstader, 1972). Starch hydrolysis was determined with Lugol's iodine solution after

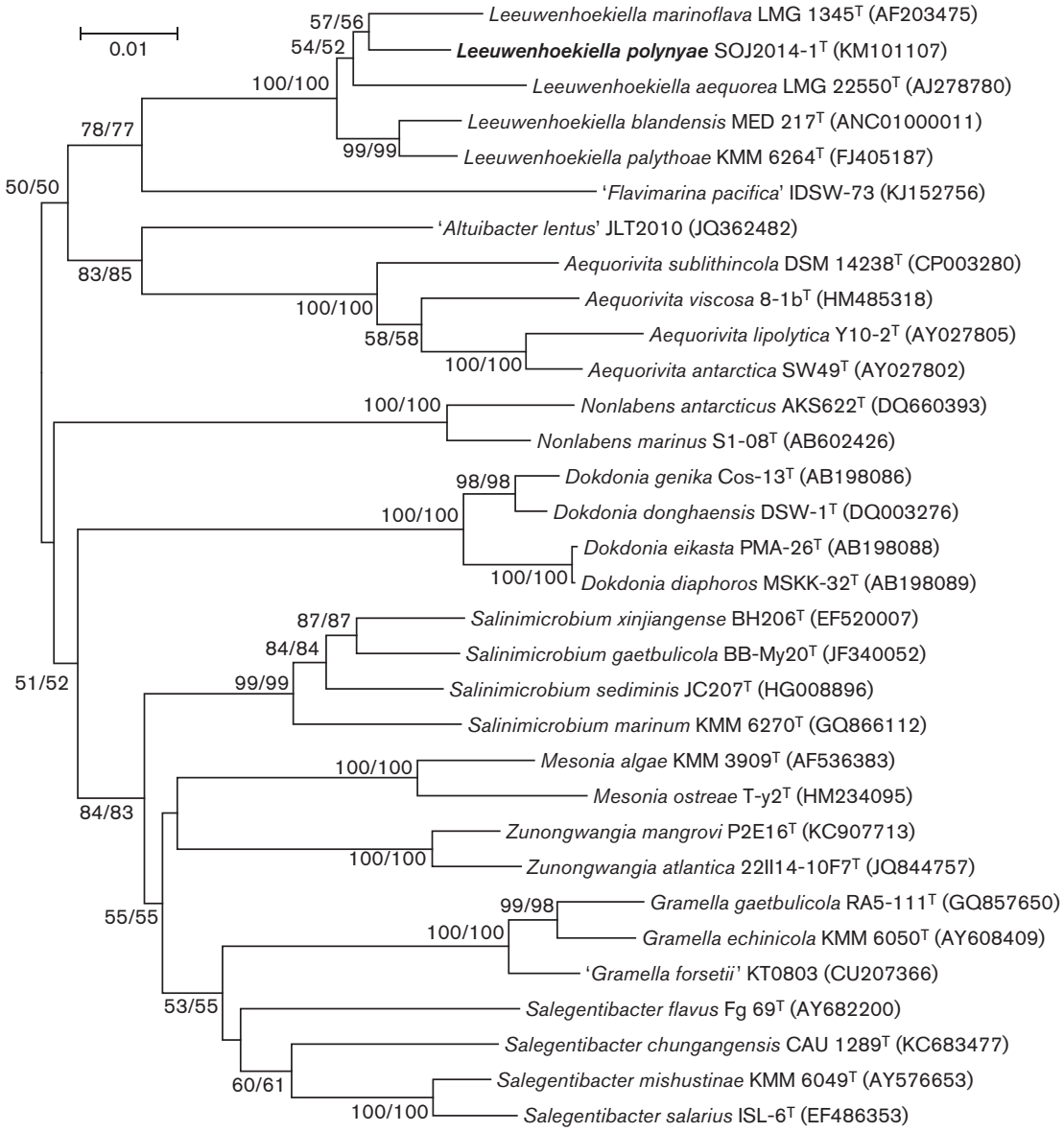

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain SOJ2014-1^T and species of the genus *Leeuwenhoekiella*. Bootstrap values $\geqslant$50 % (based on 1000 replicates) from neighbour-joining and minimum evolution methods are indicated at branch points. GenBank accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

culture on MA containing 0.2 % (w/v) starch. Hydrolysis of CM-cellulose was determined after culture on MA containing 0.5 % (w/v) CM-cellulose. The plates were stained with 0.2 % aqueous Congo red to observe the zone of clearing. Hydrolysis of casein was determined after culture on MA containing 3 % (w/v) casein. Hydrolysis of DNA was determined after culture on DNase Test Agar containing methyl green (Difco). A zone of clearing around the bacterial colonies indicated positive activity. Hydrolysis of Tween 20 was determined on the basis of a pH-based detection method using phenol red (the pH indicator) as proposed by Singh *et al.* (2006). The presence of flexirubin-type pigments was detected by the bathochromatic shift test with 20 % (w/v) KOH (Reichenbach, 1989).

Carbon source utilization and enzyme activity were determined with API 20NE, API ZYM, API 50CH (bioMérieux) and Biolog GN2 (Biolog) galleries according to the instructions of the manufacturers. After inoculation, the galleries were incubated at 25 °C for 3 days before reactions were read. Well-grown fresh colonies after 3 days of incubation were used for poly-β-hydroxybutyrate (PHB) staining with Sudan Black following the protocol of Smibert & Krieg (1994). The presence of PHB was confirmed by light

Table 1. Cellular fatty acid compositions of strain SOJ2014-1^T and the type strains of related species of the genus *Leeuwenhoekiella*

Species: 1, *Leeuwenhoekiella polynyensis* sp. nov. SOJ2014-1^T; 2, *L. marinoflava* DSM 3653^T; 3, *L. aequorea* LMG 22550^T; 4, *L. palythoae* KCTC 22020^T; 5, *L. blandensis* KCTC 22103^T. Data from this study. Values shown are percentages of total fatty acids. Those greater than 5 % are indicated in bold type. –, Not detected; TR, trace amount (<1 %).

Fatty acid	1	2	3	4	5
$C_{9:0}$	2.1	TR	1.3	–	–
$C_{16:0}$	2.4	3.5	1.4	1.7	1.9
$C_{18:0}$	1.0	1.3	1.3	–	–
anteiso-$C_{15:1}$ A	TR	TR	1.1	–	TR
anteiso-$C_{15:0}$	3.5	2.9	**5.8**	1.9	2.4
iso-$C_{15:0}$	**12.4**	**15.4**	**12.5**	**24.7**	**13.4**
iso-$C_{15:0}$ 3-OH	3.7	4.7	1.9	3.6	4.4
$C_{15:0}$ 2-OH	1.4	2.4	1.7	–	1.2
iso-$C_{15:1}$ G	**11.2**	**10.5**	**11.2**	**10.5**	**12.0**
iso-$C_{16:0}$	1.4	1.3	2.6	1.7	3.7
iso-$C_{16:0}$ 3-OH	1.4	1.1	2.4	1.9	4.1
anteiso-$C_{17:1}\omega 9c$	1.0	–	3.2	–	–
iso-$C_{17:0}$	2.2	3.7	TR	2.2	TR
iso-$C_{17:0}$ 3-OH	**17.3**	**21.3**	**12.9**	**26.7**	**23.5**
$C_{17:0}$ 2-OH	3.4	**5.9**	**8.4**	2.0	4.0
$C_{17:1}\omega 6c$	TR	–	–	–	1.6
$C_{18:1}\omega 9c$	1.1	1.6	2.1	–	–
iso-$C_{19:0}$	1.2	TR	TR	TR	1.2
Summed features*					
3 ($C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$)	**17.6**	**13.1**	**15.7**	**11.7**	**11.7**
4 (anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I)	TR	–	–	–	1.6
8 ($C_{18:1}\omega 7c/C_{18:1}\omega 6c$)	1.3	2.4	2.1	–	1.1
9 (iso-$C_{17:1}\omega 9c$ and/or 10-methyl $C_{16:0}$)	**8.5**	4.9	**9.8**	**8.6**	**8.5**

*Summed features represent groups of two or three fatty acids that could not be separated by GC with the MIDI system.

microscopy (CX21; Olympus). Susceptibility to antibiotics was determined by the disc diffusion assay using antimicrobial discs (Chang *et al.*, 2009). A bacterial suspension was spread on MA on which discs impregnated with the following antibiotics (μg per disc) were then laid: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), streptomycin (10 μg) and tetracycline (30 μg), and assessed as described by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Optimum growth at different temperatures (0–45 °C at intervals of 5 °C) was assessed after 1 day of incubation in MB. Ranges were ascertained after prolonged incubation for 3 weeks. Strain SOJ2014-1^T was able to grow at 0–35 °C (optimum, 25 °C). Growth at different NaCl concentrations was measured using 0–17.0 % (w/v) NaCl at intervals of 0.5 % in modified artificial seawater (MASW; l^{-1} distilled water, 1.3 g KCl, 0.02 g K_2HPO_4, 0.5 g NH_4Cl, 0.1 g $CaCl_2.2H_2O$, 3 g $MgCl_2.6H_2O$, 0.14 g KBr, 1.0 g peptone, 5 g yeast extract). Strain SOJ2014-1^T grew with 0–14.0 % NaCl (optimum, 1.0–5.0 %). Selective characteristics of strain SOJ2014-1^T are compared with those of other species of the genus *Leeuwenhoekiella* in Table 2. As shown in Table 2, strain SOJ2014-1^T has properties distinct from closely related species of the genus *Leeuwenhoekiella*. Therefore, based on the phylogenetic, chemotaxonomic and phenotypic characteristics, strain SOJ2014-1^T is considered to represent a novel species of the genus *Leeuwenhoekiella* for which the name *Leeuwenhoekiella polynyae* sp. nov. is proposed.

Description of *Leeuwenhoekiella polynyae* sp. nov.

Leeuwenhoekiella polynyae (po.ly′ny.ae. N.L. gen. n. *polynyae* of a polynya, an area of open water surrounded by sea ice where the organism was isolated).

Cells are Gram-stain-negative, strictly aerobic, oxidase- and catalase-positive and rod-shaped (0.4–0.6 × 1.5–2.7 μm). Cells have no flagellum. PHB is accumulated. Colonies are golden-yellow in colour, entire circular and up to 1.0–1.5 mm diameter on MA after 3 days of incubation. Growth occurs at 0–35 °C (optimum, 25 °C) and with 0–14.0 % (w/v) NaCl (optimum, 1.0–5.0 %). NaCl is not required for growth. Growth occurs chemoheterotrophically under oxic conditions. Reduction of nitrate to N_2 is negative. The hydrolysis of starch and Tween 20 is positive; but the hydrolysis of cellulose, casein, urea and DNA is negative. Flexirubin-type pigment was not detected. In the API ZYM gallery, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase activities are present; but lipase (C14), α-chymotrypsin, β-glucuronidase and α-fucosidase activities are absent. In the API 20NE gallery, positive results are demonstrated for β-glucosidase, β-galactosidase, and assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetylglucosamine, maltose, potassium gluconate, adipic acid, malic acid and phenylacetic acid. In API 50CH, positive reactions for acid production are obtained from glycerol, D-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, methyl-α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, ferric citrate, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch and glycogen. In Biolog GN2, positive reactions for α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, raffinose, L-rhamnose, sucrose, trehalose, turanose, monomethyl-succinate, D-galacturonic-acid, D-glucuronic-acid, succinic acid, bromosuccinic

Table 2. Differential phenotypic characteristics of strain SOJ2014-1^T and related species of the genus *Leeuwenhoekiella*

Strains: 1, *L. polynyae* sp. nov. SOJ2014-1^T; 2, *L. marinoflava* DSM 3653^T; 3, *L. aequorea* LMG 22550^T; 4, *L. palythoae* KCTC 22020^T; 5, *L. blandensis* KCTC 22103^T. All data were taken from this study. All strains were positive for the following: gliding motility; catalase, oxidase, α- and β-galactosidase, leucine and valine arylamidase, acid and alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase, α-glucosidase, esterase (C4), esterase lipase (C8), cystine arylamidase and trypsin activities; hydrolysis of starch; acid production of glycerol, D-galactose, D-glucose, D-fructose, D-mannose, aesculin, ferric citrate, cellobiose, maltose, lactose, sucrose, trehalose and raffinose; utilization of arabinose, glucose, lactose, mannose and sucrose; susceptibility to erythromycin, chloramphenicol and tetracycline. All strains were negative for the following: nitrate reduction; production of flexirubin-type pigments, indole and H_2S; hydrolysis of DNA, urea, cellulose and agar; α-fucosidase activity. +, Positive; −, negative; W, weakly positive. When the data obtained in the present study differ from those reported in the original descriptions of the species (Pinhassi *et al.*, 2006; Nedashkovskaya *et al.*, 2009, 2014), the original results are provided in parentheses.

Characteristic	1	2	3	4	5
Temperature range for growth (°C)	0–35	5–35 (4–37)	5–35 (4–37)	5–35 (4–38)	10–40 (10–41)
Salinity range for growth (% NaCl)	0–14	0–13 (0–15)	0–13 (0–15)	0–12	0–15 (0–17)
Hydrolysis of Tween 20	+	+	+	+	−
Acid formation from (API 50CH):					
D-Arabinose	+	−	−	W (+)	−
L-Arabinose	−	W	−	+	+
D-Xylose	W	W (−)	−	W (−)	+
L-Rhamnose	W	W (−)	−	−(+)	−
D-Mannitol	−	−	W (+)	−	W (−)
Methyl α-D-mannopyranoside	W	W	W	−	W
Methyl α-D-glucopyranoside	+	W	+	W	+
N-Acetylglucosamine	−	−	W	−	W
Amygdalin	+	−	+	+	+
Turanose	−	W	W	W	+
Enzyme activity (API ZYM)					
α-Chymotrypsin	−	+ (−)	+ (−)	+ (−)	+
β-Glucosidase	+	+	− (+)	+	+
α-Mannosidase	+	+	− (+)	+	+
Metabolism of carbon source (Biolog GN2)					
α-Cyclodextrin, L-rhamnose, D-galacturonic acid	+	+	−	+	+
N-Acetyl-D-galactosamine, melibiose	+	+	+	+	−
L-Arabinose	+	+	−	+	−
D-Mannitol	−	−	+	−	−
D-Psicose	−	−	+	−	+
Monomethyl succinate	+	−	−	−	+
Acetic acid, D-gluconic acid	−	−	+	−	−
D-Glucuronic acid	+	+	−	−	−
Succinic acid	+	−	+	−	+
Bromosuccinic acid	+	−	−	−	−
Glucose 6-phosphate, L-aspartic acid, DL-α-glycerol phosphate	−	−	+	+	+
Glycyl-L-aspartic acid	+	−	−	+	+
L-Ornithine	−	−	−	+	−
Hydroxyl-L-proline	−	+	−	−	−
Susceptibility to ampicillin	−	−	−	− (+)	+
DNA G+C content (mol%)	38.8	38.0	35.5	41.3	42.0

acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, glycerol and glucose 1-phosphate. Susceptible to erythromycin, chloramphenicol and tetracycline, but resistant to kanamycin, gentamicin, streptomycin, penicillin G and ampicillin. The predominant cellular fatty acids are summed feature 3 (composed of $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, iso-$C_{15:1}$ G and summed feature 9 (composed of iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$). The major polar lipids are phosphatidylethanolamine, an unidentified aminolipid and two unidentified lipids. The respiratory quinone is MK-6.

The type strain, SOJ2014-1^T (=KCTC 42185^T=JCM 30387^T), was isolated from Antarctic seawater. The DNA G+C content of the type strain is 38.8 mol%.

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