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TAXONOMIC DESCRIPTION
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Kiloniella antarctica sp. nov., isolated from a polynya of Amundsen Sea in Western Antarctic Sea

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Abstract

A taxonomic study was conducted on strain soj2014^T, which was isolated from the surface water of a polynya in the Antarctic Sea. Comparative 16S rRNA gene sequence analysis showed that strain soj2014^T belongs to the family *Kiloniellaceae* and is closely related to *Kiloniella spongiae* MEBiC09566^T, '*Kiloniella litopenaei*' P1-1^T and *Kiloniella laminariae* LD81^T (98.0 %, 97.8 % and 96.2 % 16S rRNA gene sequence similarity, respectively). The DNA–DNA hybridization values between strain soj2014^T and closely related strains were below 28.6 %. The G+C content of the genomic DNA of strain soj2014^T was 45.5 mol%. The predominant cellular fatty acids were summed feature 8 (composed of $C_{18:1}\omega6c/C_{18:1}\omega7c$, 57.0 %) and summed feature 3 (composed of $C_{16:1}\omega6c/C_{16:1}\omega7c$, 23.5 %). Strain soj2014^T was Gram-stain-negative, slightly curved, spiral-shaped, and motile with a single polar flagellum. The strain grew at 0–30 °C (optimum, 25 °C), in 1.5–5.1 % (w/v) NaCl (optimum, 2.1–2.4 %) and at pH 5.5–9.5 (optimum, 7.5–8.0). It also had differential carbohydrate utilization traits and enzyme activities compared with closely related strains. Based on these phylogenetic, phenotypic and chemotaxonomic analyses, strain soj2014^T represents a distinct species, separable from the reference strains, and is, therefore, proposed as a novel species, *Kiloniella antarctica* sp. nov. The type strain is soj2014^T (=KCTC 42186^T=JCM 30386^T).

The genus *Kiloniella,* within the family *Kiloniellaceae* of the order *Kiloniellales* in the class *Alphaproteobacteria*, was first described by Wiese *et al.* [1] to accommodate a Gram-stain-negative, rod-shaped, oxidase- and catalase-positive, motile, facultatively anaerobic, mesophilic and chemoheterotrophic marine bacteria with moderate halotolerance. The genus *Kiloniella* was proposed by isolating a strain of *Kiloniella laminariae* from the marine brown alga, *Laminaria saccharina*, from the Baltic Sea. Recently, two additional species of the genus *Kiloniella* were reported, *Kiloniella spongiae* and '*K. litopenaei*' [2, 3]. Here, we provide a polyphasic taxonomic characterization of a *Kiloniella*-like bacterial strain, soj2014^T, which was isolated from the surface water of a polynya in the Antarctic Sea.

A bacterial strain soj2014^T was isolated from Antarctic polynya seawater (112° 00′ W, 73° 30′ S) during phytoplankton blooms. Water column samples were stored in conical tubes and transported to the laboratory in a refrigerator within 1 month of collection. The seawater sample was diluted 10-fold in sterile artificial sea water medium (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) at 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) and incubated at 10 °C for 1 week. A single cream colony was isolated and purified. The isolated strain soj2014^T was cultivated at 25 °C for 4 days onto marine agar 2216 (MA; BD) for biochemical and physiological characterizations and preserved at −80 °C in marine broth 2216 (MB; BD) supplemented with glycerol (30 %, v/v). The reference strains, *K. spongiae* MEBiC09566^T, '*K. litopenaei*' P1-1^T and *K. laminariae* LD81^T were obtained from the Korean Culture Centre of Microorganisms, Deutsch

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Keywords: polynya; carbohydrate utilization; enzyme activities.
Abbreviations: AL, Aminolipid; ME, Minimum Evolution; ML, Maximum Likelihood; NJ, Neighbor joining; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol.
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The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain soj2014^T is KM101108.
Two supplementary figures are available with the online Supplementary Material.

Sammlung von Mikroorganismen und Zellkultren GmbH and the Belgian Co-ordinated Collections of Micro-organisms, respectively, for physiological and biochemical comparisons with strain soj2014^T. Unless otherwise stated, strain soj2014^T and the reference strains were grown on MA for 5 days at 25 °C.

Genomic DNA was extracted using a commercial genomic DNA extraction kit (GeneAll). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′; *E. coli* positions 8–27) and 1492R (5′-TACGGYTACCTTG TTACGACTT-3′; *E. coli* positions 1492–1513) [4]. The purified PCR product was sequenced by the fluorescent dye terminator method [5] using primers 27F, 518R (5′-GTA TTACCGCGGCTGCTGG-3′), 785F (5′-GGATTAGA TACCCTGGTA-3′) and 1492R [4, 6] by Cosmo Genetech. The 16S rRNA gene sequences were assembled using SeqMan software (DNAStar) [7]. The 16S rRNA gene sequence of strain soj2014^T determined in this study was 1445 bp in length. The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Sequence alignments were performed using SILVA (www.arb-silva.de/aligner), by considering the secondary structure of the rRNA gene [8]. Gaps were edited in the BioEdit program [9]. The evolutionary distances were calculated using the Kimura two-parameter model [10]. Phylogenetic trees were reconstructed using the neighbour-joining (NJ) [11], minimum-evolution (ME) [12] and maximum-likelihood (ML) [13] methods implemented in the MEGA7 program [14]. Strain soj2014^T was determined to belong to the genus *KiloniellaI,* as shown from the bootstrap support in Fig. 1. Trees reconstructed by the NJ, ME and ML methods supported this

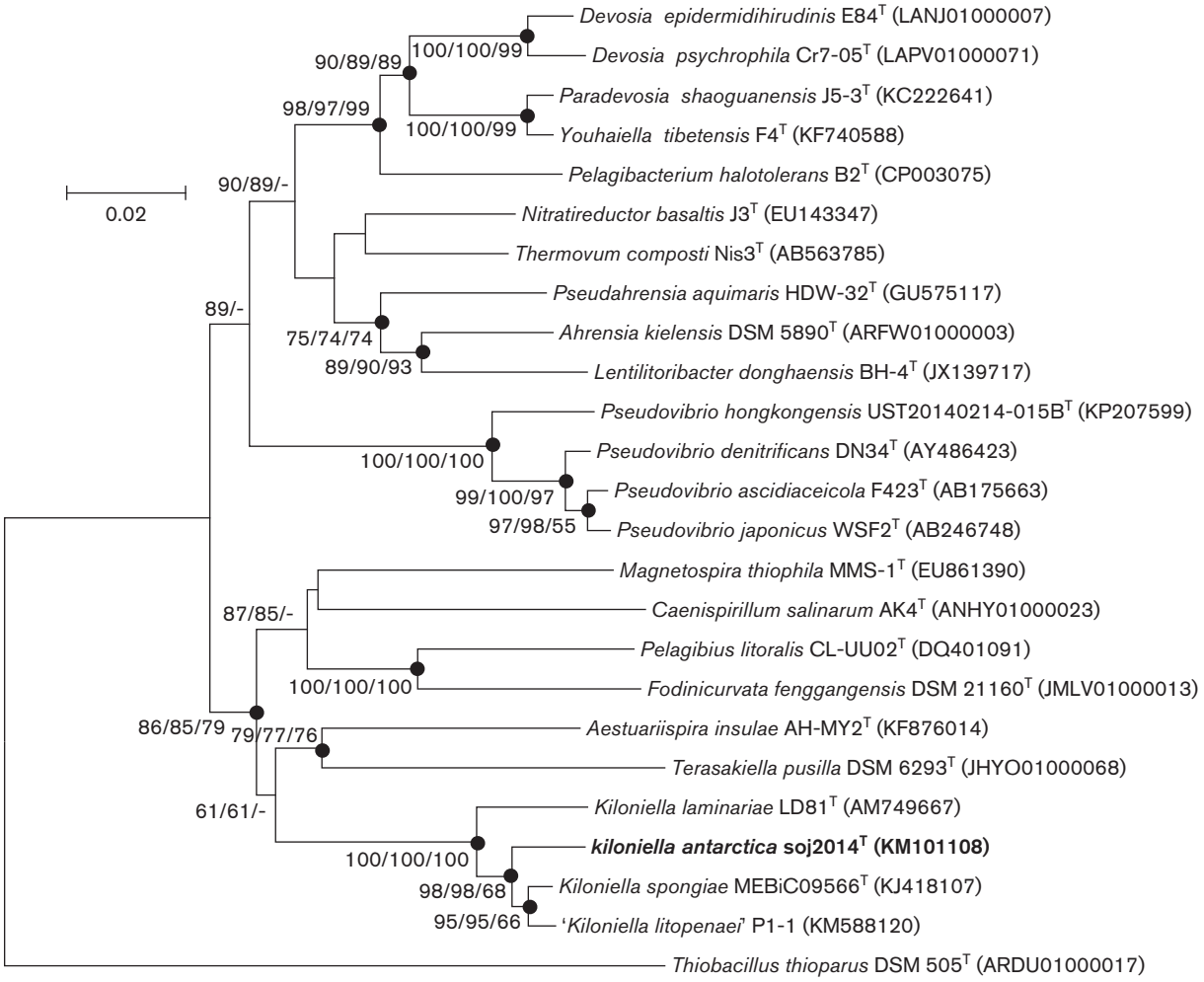

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain soj2014^T, type strains of all species of the genus *Kiloniella* with validly published names, and representatives of related genera. *Thiobacillus thioparus* DSM 505^T of *Proteobacteria* served as the outgroup. Bootstrap values ≥50 % (based on 1000 replicates) from NJ, ME and ML methods are indicated at branching points. All nodes that were recovered by all three treeing methods (NJ, ME and ML) are indicated by the filled circles. GenBank accession numbers are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.

phylogenetic position of strain soj2014^T. A pairwise comparison of the 16S rRNA gene sequences using the EzTaxon-e program (www.ezbiocloud.net/eztaxon) [15] indicated that sequence similarities with the closely related strains, *K. spongiae* MEBiC09566^T, '*K. litopenaei*' P1-1^T and *K. laminariae* LD81^T, were 98.0, 97.8 and 96.2 %, respectively.

Chromosomal DNA extracted for 16S rRNA gene amplification was used for determination of the G+C content. RNA in the DNA solution was removed by incubation with a mixture of ribonuclease A and T1 (each, 20 units ml^{-1}) at 30 °C for 1 h. The G+C content of the chromosomal DNA was determined according to Gonzalez and Saiz-Jimenez [16]. The G+C content of the chromosomal DNA of strain soj2014^T was 45.5 mol%, which was similar to those of *K. spongiae* MEBiC09566^T (44.6 mol%) and '*K. litopenae*' P1-1^T (46.1 mol%), but distinct to that of *K. laminariae* LD81^T (51.1 mol%). DNA–DNA hybridization was carried out with strain soj2014^T and the reference strains (*K. spongiae* MEBiC09566^T and '*K. litopenae*' P1-1^T), using the method described by Ezaki *et al.* [17]. All reciprocal hybridizations were carried out according to Stackebrandt and Goebel [18]; *K. laminariae* LD81^T was excluded from this experiment as it showed less than a 97 % 16S rRNA gene sequence similarity with strain soj2014^T. The genomic DNA of each strain was extracted using a genomic DNA extraction kit (GeneAll), biotinylated with photobiotin and hybridized with single-stranded unlabelled chromosomal DNA fragments of the other strains. The mean values of two independent determinations of DNA–DNA relatedness between strain soj2014^T and strain *K. spongiae* MEBiC09566^T and '*K. litopenae*' P1-1^T were 28.6 and 13.5 %, respectively. The mean values, from reciprocal hybridizations, of DNA–DNA relatedness between soj2014^T and strain *K. spongiae* MEBiC09566^T and '*K. litopenae*' P1-1^T were 33.7 % and 14.4 %, respectively. In current bacterial systematics, two strains that show a DNA–DNA relatedness value below 70 % are considered to represent distinct species [19]. The results of the phylogenetic analyses and DNA–DNA hybridization experiments indicate that strain soj2014^{T}is distinct from species of the genus *Kiloniella* with validly published names.

For determination of fatty acid compositions, strain soj2014^T, *K. spongiae* MEBiC09566^T, '*K. litopenaei*' P1-1^T and *K. laminariae* LD81^T were cultivated on MA at 25 °C and harvested at the exponential growth phase after 5 days for strain soj2014^T and after 3 days for reference strains, as described by Sasser [20]. Fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) and analysed by using a gas chromatograph (model 6890, Hewlett Packard) and identified using the Microbial Identification software package based on the Sherlock Aerobic Bacterial Database (TSBA60) [20]. The major fatty acids of strain soj2014^T were identified as summed feature 8 ($C_{18:1}\omega6c$/$C_{18:1}\omega7c$; 57.0 %) and summed feature 3 ($C_{16:1}\omega6c$/$C_{16:1}\omega7c$; 23.5 %). The overall fatty acid profile of strain soj2014^T was consistent with those of the closely related reference strains, but it could be differentiated from these strains by the percentages of the various fatty acids present (Table 1). Cells grown on MA for 5 days at 25 °C were collected and freeze-dried for extraction of respiratory quinones and polar lipids. Respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum and re-extracted in *n*-hexane/water (1 : 1, v/v). The crude *n*-hexane/quinone solution was purified using a Sep-Pak Vac silica cartridge (Waters) and subsequently analysed by HPLC, as previously described [21]. The respiratory quinone of strain soj2014^T was determined to be Q-9 (97 %), which is consistent with other members of the genus *Kiloniella*, with Q-8 present in lesser amounts (3 %). Polar lipids were extracted and analysed as reported [22]. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), an unidentified aminolipid (AL) and an unidentified lipid were detected in strain soj2014^T (Fig. S1, available in the online Supplementary Material). Strain soj2014^T and all the reference strains contained PE and PG, while the total numbers of AL and unidentified lipids differed between strains [2, 3].

A Gram staining kit (BD) was used according to the manufacturer's instructions. The morphology of the Gram-stained cells was examined by light microscopy (Olympus; CX21). Transmission electron microscopy (EM-109; Carl Zeiss) was performed after negative staining with 1 % (w/v)

Table 1. Cellular fatty acid composition of strain soj2014^T and the type strains of species of the genus *Kiloniella* with validly published names

Strains: 1, soj2014^T; 2, *K. spongiae* MEBiC09566 ^{T}; 3, '*K. litopenaei*' P1-1^T; 4, *K. laminariae* LD81^T. Data from this study. Values shown are mean percentages of total fatty acids. Values greater than 5 % are in enboldened text. Fatty acids that represented less than 1.0 % of the total in a strain have been omitted from the table. –, Not detected; TR, trace amounts (<1 %).

Fatty acid	1	2	3	4
Saturated				
$C_{12:0}$	1.1	1.0	1.2	1.1
$C_{16:0}$	2.8	2.0	3.3	4.0
$C_{18:0}$	4.3	5.2	4.9	4.6
Unsaturated				
$C_{14:1}\omega5c$	TR	TR	1.0	1.0
Hydroxy fatty acids				
$C_{18:0}$ 3OH cyclo	3.7	**5.0**	**5.9**	3.1
$C_{19:0}$ cyclo$\omega8c$	2.1	2.0	1.4	4.1
Summed feature*				
2; $C_{12:0}$ aldehyde/$C_{16:1}$iso/$C_{14:0}$ 3OH	2.9	3.3	3.2	2.9
3; $C_{16:1}\omega6c$/$C_{16:1}\omega7c$	**23.5**	**15.8**	**8.6**	**17.8**
8; $C_{18:1}\omega6c$/$C_{18:1}\omega7c$	**57.0**	**62.3**	**68.1**	**58.5**

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

phosphotungstic acid. Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. Cell motility was examined by the semisolid agar method [23]. 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 and 0.5 g sodium thioglycollate] in Hungate tubes sealed with butyl rubber stoppers. Cells of strain soj2014^T were Gram-stain-negative, oxidase- and catalase-positive, motile and microaerophilic. H_2S was not produced and glucose and lactose were not fermented in the Triple Sugar Iron Agar test. Colonies grown on MA for 4 days at 25 °C were cream, circular and with an entire convex shape. Cells of strain soj2014^T were slightly spiral with 1 polar flagellum and were 0.3–0.5×1.5–3.2 µm (Fig. S2).

The optimum growth at different temperatures (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 °C) was assessed after 1 day of incubation in MB. Ranges for growth were ascertained after prolonged incubation for 3 weeks. Strain soj2014^T was able to grow at 0–30 °C (optimum, 25 °C). Growth at different NaCl concentrations (0, 0.5, 1.0, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0, 3.3, 3.6, 3.9, 4.2, 4.5, 4.8, 5.1, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0 %, w/v) was measured using modified artificial sea water medium (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 and 5 g yeast extract). Strain soj2014^T grew in 1.5–5.1 % NaCl (w/v; optimum, 2.1–2.4 %). The response of strain soj2014^T to pH (pH 3.0–10.0 at intervals of 0.5 pH unit) was determined in MB incubated at 25 °C for 6 days. Three different buffers were used to achieve various pH ranges (final concentration, 10 mM): citrate buffer was used for pH 3.0–6.5, phosphate buffer was used for pH 6.5–8.0, Tris buffer was used for pH 8.0–10.0 [Tris-HCl (pH 8.5, Biosesang) adjusted with 1 M NaOH] [24]. Strain soj2014^T grew at pH 5.5–9.5 (optimum, pH 7.5–8.0).

Carbon source utilization and enzyme activity were determined with API 20NE, API ZYM (bioMérieux) and Biolog GN2 (Biolog) galleries according to the instructions of the manufacturers. Cells of strain soj2014^T and the reference strains were suspended in the solution supplied and adjusted to marine salinities by adding sea salt (to a final salinity of 2 %, w/v). After inoculation, the galleries were incubated at 25 °C for 4 days before reactions were read. Well-grown fresh colonies were used for poly-β-hydroxybutyrate (PHB) staining with Sudan Black after 4 days of incubations [24]. The presence of PHB was confirmed by light microscopy (Olympus; CX21). Nitrate reduction to N_2 by strains soj2014^T and *K. laminariae* LD81^T was measured using 25 ml Hungate tubes containing 10 ml MB with 5 mM nitrate and sealed with butyl rubber stoppers. Antibiotic resistance tests were performed with discs containing the following antibiotics on the MA surface by incubating cells at 25 °C for 3 days: ampicillin (10 µg), chloramphenicol (25 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 CLSI [25]. No activity was observed in the Biolog GN2 test with strain soj2014^T, while the reference strains showed the expected activities fom previously published data.

The detailed physiological characteristics and biochemical activities of strain soj2014^T are summarized in the species description, and some of its selected characteristics are compared with those of *K. spongiae* MEBiC09566^T, '*K. litopenaei*' P1-1^T and *K. laminariae* LD81^T in Table 2. The PHB accumulation, carbohydrate utilization and enzyme activities of strain soj2014^T were distinct from those of closely related species of the genus *Kiloniella*. Therefore, based on phylogenetic, chemotaxonomic and phenotypic characteristics, strain soj2014^T is considered to represent a novel species of the genus *Kiloniella*, for which the name *Kiloniella antarctica* sp. nov. is proposed.

DESCRIPTION OF *KILONIELLA ANTARCTICA* SP. NOV.

Kiloniella antarctica (L. fem. adj. ant.arc'ti.ca, of the Antarctic, where the organism was isolated).

Cells are Gram-stain-negative, microaerophilic, oxidase- and catalase-positive, slender and slightly curved spirals, 0.3–0.5 µm wide and 1.5–3.2 µm long. Cells are motile with a single polar flagellum. PHB does not accumulate. Colonies are creamy, convex, entirely circular and grew up to 0.5–1.0 mm on MA after 4 days of incubation. Growth occurs at 0–30 °C (optimum, 25 °C) and at pH 5.5–9.5 (optimum, pH 7.5–8.0) and in 1.5–5.1 % (w/v) NaCl (optimum, 2.1–2.4 %). Reduction of nitrate to N_2 is positive. Positive reactions for assimilation are obtained with *N*-acetyl-glucosamine, maltose and potassium gluconate, but negative reactions are obtained for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase (weak), naphtol-AS-BI-phosphohydrolase, acid phosphatase and β-glucosidase activities are present; but indole production, D-glucose fermentation and activity of arginine dihydrolase, urease, gelatinase, β-galactosidase, lipase(C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to erythromycin, chloramphenicol, tetracycline, kanamycin, gentamicin and streptomycin, but resistant to penicillin G and ampicillin. The predominant cellular fatty acids are summed feature 8 (composed of $C_{18:1}\omega6c/C_{18:1}\omega7c$) and summed feature 3 (composed of $C_{16:1}\omega6c/C_{16:1}\omega7c$). The polar lipids are phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid and an unidentified lipid. The major respiratory quinone is Q-9.

Table 2. Differential phenotypic characteristics of strain soj2014^T and type strains of closely related species of the genus *Kiloniella*

Strains: 1, soj2014^T (data from the present study); 2, *K. spongiae* MEBiC09566 ^{T} [2]; 3, *'K. litopenaei'* P1-1 ^{T} [3]; 4, *K. laminariae* LD81^T [1]. All strains were positive for the following: alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase. All strains were negative for the following: production of indole; D-glucose fermentation; assimilation of capric acid and adipic acid; arginine dihydrolase, urease, gelatinase, β-galactosidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. +, Positive reaction; –, negative reaction; ND, not determined. Values in parentheses represent the optimum. Characteristics in API 20NE and API ZYM tests that were different from those of the original description are given in parentheses following the data in the original publication.

Characteristics	1	2	3	4
Growth temperature (°C)	0–30 (25)	11–31 (25)	15–37 (28–32)	4–40 (25)
PHB accumulation	–	ND	+	+
DNA G+C content (mol%)	45.5	44.6	46.1	51.1
API 20NE*				
Assimilation of:				
D-Glucose	–	+(–)	+(–)	+
L-Arabinose	–	+	–	–
D-Mannose	–	–	+(–)	–
D-Mannitol	–	–	–	+
N-Acetyl-glucosamine	+	–	+(–)	+
Maltose	+	–	–	–
Gluconate	+	–	–	–
Malate	–	–	+(–)	+
Citrate	–	–	+(–)	+
Phenylacetic acid	–	–	+(–)	+
Activity of:				
β-Glucosidase	+	–	–	–
API ZYM*				
Esterase lipase (C8)	+	+(–)	+	–
Valine arylamidase	–	–(+)	–(+)	+
Trypsin	–	+	–	–

*API 20NE and API ZYM tests were performed for all the reference strains in this study.

The type strain, soj2014^T (=KCTC 42186^T=JCM 30386^T), was isolated from Antarctic seawater. The DNA G+C content of the type strain is 45.5 mol%.

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

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