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Roseovarius nitratireducens sp. nov., a halotolerant bacterium isolated from a saline lake

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Abstract

A Gram-stain-negative, aerobic, non-motile and ovoid- to rod-shaped bacterial strain, designated TFZ^T, was isolated from a water sample of a saline lake in Xinjiang, China and subjected to polyphasic taxonomic investigation. Strain TFZ^T grew at 4–42 °C and pH 6.5–10.0 (optimum, 30 °C and pH 7.0) and with 0.5–18.0 % (w/v) NaCl (optimum, 1.5 %). According to phylogenetic analysis based on 16S rRNA gene sequences, strain TFZ^T was assigned to the genus *Roseovarius* with highest 16S rRNA gene sequence similarity of 97.5 % to *Roseovarius tolerans* EL-172^T, followed by *Roseovarius azorensis* SSW084^T (96.6 %) and *Roseovarius mucosus* DSM 17069^T (95.5 %). Digital DNA–DNA hybridization (DDH) and average nucleotide identity (ANI) were determined to evaluate the genomic relationship between strain TFZ^T and *R. tolerans* EL-172^T. Digital DDH estimation (22.80±2.35 %) as well as ANI (80.1 %) proved the dissimilarity of strain TFZ^T. Chemotaxonomic analysis showed that strain TFZ^T contained ubiquinone-10 as the predominant respiratory quinone and possessed summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $\omega6c$) and $C_{16:0}$ as the predominant form of fatty acid. The polar lipids of strain TFZ^T consisted of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and five unidentified lipids. The DNA G+C content was 65.4 mol% (by genome sequencing). Based on the polyphasic taxonomic characterization, strain TFZ^T is considered to represent a novel species of the genus *Roseovarius*, for which the name *Roseovarius nitratireducens* sp. nov. is proposed (type strain TFZ^T=MCCC 1K03339^T=KCTC 52967^T).

The genus *Roseovarius*, a member of the family *Rhodobacteraceae* of the *Alphaproteobacteria* [1], was first proposed by Labrenz *et al.* [2]. At the time of writing, the genus *Roseovarius* comprises 24 species with validly published names (www.bacterio.net/roseovarius.html), including the recently described species *Roseovarius aestuariivivens* [3], *Roseovarius antarcticus* [4], *Roseovarius aquimarinus* [5], *Roseovarius azorensis* [6] and *Roseovarius confluentis* [7]. Among them, eight species (*Roseovarius tolerans*, *R. antarcticus, R. aquimarinus, R. confluentis, Roseovarius indius, Roseovarius pacificus, Roseovarius halotolerans and Roseovarius litoreus*) can tolerate high salt concentrations (>10 %, w/v) [2, 4, 5, 7–11]. Apart from *R. confluentis*, collected from estuary sediment [7], most of them were isolated from saline environments: a hypersaline lake [2]; decayed whale bone [4]; sea water [5, 8, 10, 11]; and deep-sea sediment [9]. The genus *Roseovarius* is typically characterized as being aerobic, peroxidase- and catalase-positive, ovoid or rod-shaped [7]. In addition, *Roseovarius crassostreae, Roseovarius halocynthiae and Roseovarius sediminilitoris* of the genus *Roseovarius* have recently been reclassified into the genus *Aliiroseovarius*, and the species *Roseovarius marinus* has been reclassified as a member of the genus *Pacificibacter* [12, 13]. In this study, the polyphasic taxonomic identification of a novel *Roseovarius* strain, designated TFZ^T, which was isolated from a saline lake in China, is described in detail.

The bacterial strain, TFZ^T, was isolated from a water sample of a saline lake (37° 22′ N 89° 01′ E) located in Xinjiang, China. The salinity of the saline lake was measured to be 6 % (w/v). We expected to isolate some halotolerant bacteria from this lake in order to utilize them to treat high salinity wastewater. The water sample was diluted, using a ten-fold dilution series method, spread on marine agar 2216 (MA; BD Difco). After 48 h of incubation, a yellow-coloured colony was collected and named TFZ^T. Strain TFZ^T was routinely cultured on MA or marine broth 2216 (MB; BD Difco) after repeated purifying. For long-term preservation,

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Abbreviations: bchl *a*, bacteriochlorophyll *a*; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic; DDH, DNA–DNA hybridization; L, unidentified lipid; MA, marine agar; MB, marine broth; MES, 2-(*N*-morpholino) ethanesulfonic acid; MOF, marine oxidation-fermentation; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and draft genome sequence of strain TFZ^T are MF144427 and PDNI00000000, respectively.
One supplementary table and four supplementary figures are available with the online version of this article.

purified strains were preserved at −80 °C with 25 % (v/v) glycerol and also by lyophilization with 20 % (w/v) skimmed milk. Strain TFZ^T has been deposited at the Marine Culture Collection of China and the Korean Collection for Type Cultures.

According to the results of 16S rRNA gene sequence alignment, *R. tolerans* EL-172^T, *R. azorensis* SSW084^T and *Roseovarius mucosus* DSM 17069^T (obtained from the Japan Collection of Microorganisms, the Korean Collection for Type Cultures and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, respectively) were chosen as reference strains. They were routinely cultured and preserved in the same way as strain TFZ^T.

Amplification and sequencing of the 16S rRNA gene, as well as sequence alignment and phylogenetic analysis based on 16S rRNA gene sequences, were all performed as previously described [14]. The genome of TFZ^T was sequenced using an Illumina HiSeq 4000 system (Illumina) at the Beijing Genomics Institute (Shenzhen, China). The paired-end fragment libraries were sequenced according to the Illumina HiSeq 4000 system's protocol. Raw reads of low quality from paired-end sequencing (those with consecutive bases covered by fewer than five reads) were discarded. The sequenced reads were assembled using ABySS software [15]. Average nucleotide identity (ANI) was calculated with OrthoANI [16]. The DNA–DNA hybridization (DDH) similarity between strains was calculated in *silico* with the Genome-to-Genome Distance Calculator server version 2.1 [17, 18] and the recommended formula 2 was taken into account to interpret the results. Strain *R. tolerans* EL-172^T was used as the reference strain in the ANI value calculation and digital DDH. The DNA G+C mol% value was obtained from the genomic sequences.

Pairwise alignment based on the nearly full-length 16S rRNA gene sequence (1459 bp) of strain TFZ^T indicated that this isolate shared the highest sequence similarity with *R. tolerans* EL-172^T (97.5 %), *R. azorensis* SSW084^T (96.6 %) and *R. mucosus* DSM 17069^T (95.5 %). Topologies exhibited in the neighbour-joining tree (Fig. 1), the maximum-likelihood tree (Fig. S1, available in the online version of this article) and the minimum-evolution tree (Fig. S2) illustrated that strain TFZ^T clustered with the members of genus *Roseovarius* with the support of a high bootstrap value, which suggested that it should belong to this genus. Meanwhile, the ANI value between strain TFZ^T and *R. tolerans* EL-172^T was 80.1 %, which was lower than the threshold value of 95 % ANI relatedness for species demarcation [19, 20]. What is more, the digital DDH estimation (22.80±2.35 %) was below the proposed cut-off borderline of 70 % [17, 18], which also confirmed that strain TFZ^T was a novel species of the genus *Roseovarius*. The DNA G+C content of strain TFZ^T was 65.4 mol% (by genome sequencing), which is similar to the other type strains under the genus *Roseovarius* (Table 1).

After incubation on MA at 30 °C for 2 days, cells were harvested for morphology observation by transmission electron microscopy (JEM- 1230; Jeol) after uranyl acetate staining and by optical microscopy (BX40; Olympus) after Gram staining. For uranyl acetate staining section, cells were first suspended by distillation–distillation H_2O; then, the suspension was absorbed by a copper grid and stained by uranyl acetate; and, lastly, sterilized filter paper was used to soak up superfluous dye. The Gram-staining reaction was carried out according to Claus [21]. Furthermore, motility was determined by microscopic observation and inoculation in semi-solid agar medium. Gliding motility was determined as described by Bernardet *et al.* [22]. Bchl *a* was exacted and the absorption spectrum analysis was performed using a DU 800 spectrophotometer (Beckman; absorption spectrum from 300 to 1000 nm) based on the method described by Wu *et al.* [23]. Cells of strain TFZ^T were Gram-stain-negative, ovoid- to rod-shaped, measured 0.40–0.77 μm wide and 1.97–2.54 μm long (Fig. S3). They could move by gliding. Bchl *a* could not be produced by TFZ^T.

The temperature for optimal growth was tested at 4–45 °C (4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 42 and 45 °C) in duplicates. The pH range for growth was measured from pH 4.5 to pH 10.0, with an interval of 0.5 units, making use of appropriate biological buffers to maintain the stability of each pH system (50 mM MES for pH 5.0–6.0, MOPS for pH 6.5–7.5, Tricine buffer for pH 8.0–8.5 and CAPSO for pH 9.0–10.0). The salt tolerance was determined in NaCl-free MB at various NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 15.0, 16.0, 17.0 and 18.0 %, w/v); cultures incubated for 2 days were used to determine the optimal growth while those incubated for 14 days were utilized to determine the growth limits. Anaerobic growth was examined in an anaerobic jar (MGC) with an Anaero-Pack (MGC on modified MA supplemented with sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM), L-arginine (5.0 g l^{-1}) as potential electron acceptors for 30 days.

Susceptibility to antibiotics was determined by placing antibiotics disks (Hangzhou Microbial Reagents) on MA plates, and considered positive when the radius of the inhibition zone was over 2.0 mm. The antibiotics (μg per disc except stated) used were as follows: polymyxin B (300 IU), bacitracin (0.4 IU), lincomycin (2), rifampicin (5), chloramphenicol (30), gentamicin (10), kanamycin (30), tetracycline (30), novobiocin (30), penicillin (10), streptomycin (10), amoxicillin (20), ampicillin (10), norfloxacin (10), ceftazidime (30), ciprofloxacin (5), vancomycin (30), neomycin (30), clindamycin (2) and cephalexin (30).

Generally, strains investigated in all the following tests were cultured at their optimal temperatures. Hydrolysis activities of starch, CM-cellulose, Tween 20, Tween 40, Tween 60 and Tween 80 were determined as described previously [24]. Catalase and Oxidase activities were tested by using the method described by Wu *et al.* [25] and Kovacs [26],

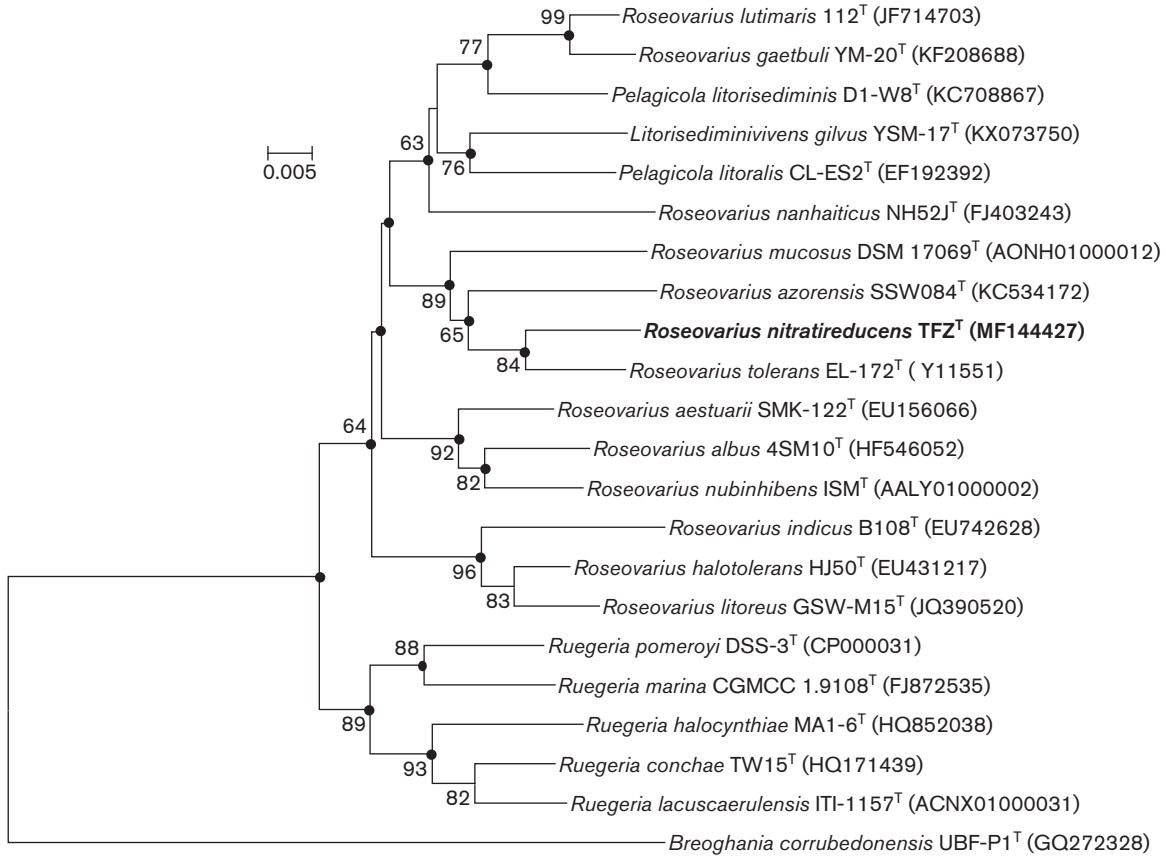

Fig. 1. Neighbour-joining phylogenetic tree of strain TFZ^T and some closely related taxa, based on 16S rRNA gene sequences (1459 bp). Bootstrap values were expressed as a percentage of 1000 replicates and only those higher than 50 % are given at the branch points. *Breoghania corrubedonensis* UBF-P1^T (GenBank accession no. GQ272328) was used as an outgroup. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and minimum-evolution trees. Bar, 0.005 substitutions per nucleotide position.

respectively. Hydrogen sulfide production, methyl red and Voges–Proskauer tests were assayed according to a previous study [27]. All experiments were performed in triplicate.

To characterize the utilization of carbon sources, bacteria were inoculated in basal medium (MB without peptone and yeast extract) containing 0.4 % varying carbon sources, including sugar, alcohol and organic acid. In the test, the basal medium with substrates but without inoculation was a blank control and growth in the basal medium with inoculation but without substrates was a negative control. Growth was measured via OD_{600nm}. Results were considered negative when OD_{600nm} measured in the test was equal to or less than the negative control; and positive when OD_{600nm} value was twice higher than the control. API 20NE and API ZYM miniaturized systems were used to test enzyme activities and additional phenotypic characteristics by following the manufacturer's instructions (bioMérieux). For the acid production test, Leifson modified marine oxidation-fermentation (MOF) medium [28] was utilized to suspend the cells for the inoculation of API 50CH (bioMérieux) strips. The API results of strain TFZ^T and the type strains of related species are given in Tables 1and S1, and in the species description.

A comparison of the physiological and biochemical characteristics of TFZ^T and the related type strains in our study showed that they shared some similarities, such as they were all negative for the methyl red test, hydrolysis of starch, CM-cellulose, and Tweens 40, 60, and 80; and positive (or weakly positive) for catalase activity, oxidase activity and production of H_2S. Moreover, the inability to grow on glucose was found for strain TFZ^T and three reference strains in our research. Despite some similarities, strain TFZ^T displayed certain distinct characteristics from other three reference strains. Notably, the NaCl tolerance of strain TFZ^T was significantly higher than that of the other three reference strains. It occurred in a wide range of NaCl concentrations and could not grow without NaCl. In addition, negative tests for producing alkaline phosphatase and producing acid from L-xylose could also be used to distinguish strain TFZ^T from the related reference strains (Table 1). Additionally,

Table 1. Differential phenotypic characteristics of strain TFZ^T and the type strains of related species

Strains: 1, TFZ^T; 2, *Roseovarius tolerans* EL-172^T; 3, *Roseovarius azorensis* SSW084^T; 4, *Roseovarius mucosus* DSM 17069^T. All data are obtained from this study unless indicated. +, Positive; −, negative. w, weakly positive. In this study, all strains were negative for hydrolysis of starch, CM-cellulose, Tweens 40, 60 and 80, and the methyl red test; and positive for catalase activity and H_2S production.

Characteristic	1	2	3	4
Motility	−	−	+	−
Cell size (width×length; μm)	0.40–0.77×1.97–2.54	0.7–1.0×1.1–2.2*	0.6–0.8×1.3–2.0‡	0.5–0.7×1.3–3.0§
Temperature range (°C)	4–42	8.5–33.5*	15–40‡	20–40§
pH range	6.5–10.0	5.9–9.0†	7.0–9.0‡	6.0–8.8§
NaCl tolerance (%, w/v)	0.5–18.0	1.0–10.0†	0.5–7.0‡	1–7§
Bchl *a*	−	+*	−‡	+§
Voges–Prokauer test	−	−	+	−
Oxidase activity	+	+	w	+
Hydrolysis of Tween 20	+	+	−	+
Reduction of nitrate to nitrite	+	+	−	−
Fermentation of glucose	−	−	−	+
Production of:				
Alkaline phosphatase	−	+	+	+
Cysteine arylamidase	−	−	+	−
Acid phosphatase	−	−	+	−
Naphthol-AS-BI-phosphohydrolase	+	w	+	w
Acid production from:				
Glycerol	+	−	+	−
L-Arabinose	+	+	−	+
D-Xylose	+	−	−	+
L-Xylose	−	+	+	+
Trehalose	−	+	−	−
Turanose	−	+	−	−
L-Fucose	−	−	−	+
Substrates:				
Maltose	+	−	+	−
Glycerol	+	+	−	+
Ethanol	+	−	+	−
Citrate	−	−	−	+
Fumaric acid	−	−	−	+
Methanol	−	−	+	−
Malate	+	+	−	−
G+C content (mol%)	65.4	62–64*	61.9‡	62.9§

*Data taken from Labrenz *et al.* [2].

†Data taken from Choi *et al.* [33].

‡Data taken from Rajasabapathy *et al.* [6].

§Data taken from Biebl *et al.* [34].

strain TFZ^T was resistant to polymyxin B, bacitracin lincomycin and tetracycline clindamycin; and sensitive to rifampicin, chloramphenicol, gentamicin, kanamycin, novobiocin, penicillin, streptomycin, amoxicillin, ampicillin, norfloxacin, ceftazidime, ciprofloxacin, vancomycin, neomycin and cephalexin.

A series of tests were performed to characterize the isoprenoid quinones and fatty acid profiles of the bacterial strain. After incubation in MB at 30 °C and at 140 r.p.m. for 2 days, cells were collected for extraction of isoprenoid quinones, which were subsequently purified by TLC and identified using a HPLC-MS system (Agilent) [29]. Isoprenoid quinones were analysed as described by Minnikin *et al.* [30]. The only respiratory quinone detected in strain TFZ^T was Q-10, which is also in accordance with the genus *Roseovarius* [2].

Cells used for the analysis of fatty acids and polar lipids were grown on MA at 30 °C for 2 days to reach the exponential stage of growth. Fatty acids were saponified, methylated

and extracted according to the standard protocol of the Sherlock Microbial Identification System (version 6.2B, MIDI) and identified based on the RTSBA6 method. The major cellular fatty acids (>10.0 %) of strain TFZ^T were summed feature 8 ($C_{18:1}\omega7c/\omega6c$) (69.2 %) and $C_{16:0}$ (10.4 %), other characteristic fatty acids were $C_{12:0}$ and $C_{12:1}$ 3-OH, which were similar to most of the three reference strains though some proportional differences existed (Table 2). Also, these results were consistent with previous research [2].

Polar lipids were extracted as described by Kates [31] and separated by two-dimensional TLC on silica gel 60 F254 plates (Merck) as described in previous research [32]. Five kinds of spray reagents were used to visualize corresponding lipids, including molybdophosphoric acid for total lipids, a-naphthol/sulphuric acid and anisaldehyde for glycolipids, molybdenum blue for phospholipids and ninhydrin for aminolipids. The polar lipid profile of TFZ^T comprised phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and five unidentified lipids (L1–5) (Fig. S4). PG, PE, and PC were also detected in the three reference strains and in the genus *Roseovarius* [2] before, which could be a significant feature to support that strain TFZ^T belonged to genus *Roseovarius.* The existence of L1 and L3 in strain TFZ^T was consistent with the three references strains in our study. In addition, the compositions of L2 and L5 in strain TFZ^T could be used to differentiate the isolate from the reference strains.

On the basis of the phenotypic, phylogenetic and chemotaxonomic characteristics described above, strain TFZ^T is considered to represent a novel species within the genus *Roseovarius* for which the name *Roseovarius nitratireducens* sp. nov. is proposed.

Table 2. Fatty acid profiles of strain TFZ^T and the type strains of related species

Strains: 1, TFZ^T; 2, *Roseovarius tolerans* EL-172^T; 3, *Roseovarius azorensis* SSW084^T; 4, *Roseovarius mucosus* DSM 17069^T. –, Not detected; TR, trace amount (<1.0 %). All data were from the present study.

Fatty acid	1	2	3	4
$C_{12:0}$	4.1	TR	2.5	4.2
$C_{12:0}$ 2-OH	–	TR	–	1.0
$C_{12:1}$ 3-OH	4.6	2.6	TR	2.8
$C_{16:0}$ 2-OH	TR	TR	TR	1.5
$C_{16:0}$	10.4	11.4	16.2	8.1
$C_{17:0}$	TR	4.9	TR	TR
$C_{18:0}$	TR	1.5	5.0	1.0
iso-$C_{18:0}$	TR	2.3	TR	5.2
11-Methyl $C_{18:1}\omega7c$	3.2	7.7	TR	TR
$C_{19:0}$ cyclo$\omega8c$	2.1	TR	TR	2.9
Summed feature 3*	2.1	TR	TR	1.1
Summed feature 8*	69.2	65.0	68.5	68.5

*Summed features represent one or more fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 comprised of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8 comprised of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

DESCRIPTION OF *ROSEOVARIUS NITRATIREDUCENS* SP. NOV.

Roseovarius nitratireducens (ni.tra.ti.re.du′cens. N.L. masc. n. *nitras -atis* nitrate; L. pres. part. *reducens* converting to a different state; N.L. part. adj. *nitratireducens* reducing nitrate).

Cells are Gram-stain-negative, aerobic, non-motile, non-spore-forming and ovoid- to rod-shaped, approximately 0.40–0.77 μm wide and 1.97–2.54 μm long. After incubation on MA at 30 °C for 2 days, colonies are elevated with a smooth surface, yellow-coloured and uniformly circular with a diameter of approximately 0.8–1.0 mm. After incubation in MB at 30 °C and at 140 r.p.m. for 2 days, cells appear pinkish. Growth occurs at 4–42 °C (optimum, 30 °C), pH 6.5–10.0 (optimum, pH 7.0) and with 0.5–18.0 % (w/v) NaCl (optimum, 1.5 %). Do not produce Bchl *a*. The strain is negative for hydrolysis of starch, CM-cellulose, Tween 40, Tween 60 and Tween 80, but positive for hydrolysis of Tween 20. In addition, catalase activity, oxidase activity and hydrogen sulfide production are positive, while results from the methyl red test and the Voges–Prokauer test are negative. With API 50CH strips, acid is produced from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, aesculin ferric citrate, D-lyxose, potassium 2-ketogluconate and potassium 5-ketogluconate. In API 20NE tests, there is a positive reaction for nitrate reduction, and negative reactions for indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis and gelatin hydrolysis. In the API ZYM kit, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase activities are present; however, alkaline phosphatase, C14 lipase, cysteine arylamidase, chymotrypsin, trypsin, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase activities are absent. In utilization of sole carbon sources tests, positive for maltose, glycerol, succinic acid, ethanol, pyruvate, malonate, acetate, glutamate and malate; negative for D-glucose, D-fructose, lactose, α-ketoglutaric acid, citrate, oxalate, fumaric acid and methanol. The isoprenoid quinone is Q-10. The most predominant cellular fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $\omega6c$). Also, $C_{16:0}$ is a dominant fatty acid next to summed feature 8. The polar lipids include PG, PE, PC and five unidentified lipids (L1–5).

The type strain is TFZ^T (=MCCC 1K03339^T=KCTC 52967^T) which was isolated from a water sample of a saline lake in Xinjiang, China. The DNA G+C content of the type strain is 65.4 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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