Limnobacter parvus sp. nov., a Thiosulfate-Oxidizing Bacterium Isolated from Lake Water

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Abstract

A novel bacterium, designated as strain YS8-69^T, was isolated from an inland closed lake, Xinjiang Uygur Autonomous Region, PR China. Comparative analysis of the 16S rRNA gene sequence shows the strain was affiliated to the genus *Limnobacter*, in the family *Burkholderiaceae*, with the highest similarities to *Limnobacter alexandrii* LZ-4^T (98.93%), *Limnobacter thiooxidans* DSM 13612^T (98.55%), *Limnobacter humi* NBRC 111650^T (97.66%), and *Limnobacter litoralis* KP1-19^T (97.04%). Strain YS8-69^T was a Gram stain-negative, strictly aerobic, rod shaped, catalase- and oxidase-positive bacterium, and growth was observed at 4–40 °C (optimum, 25 °C), pH 7.0–10.0 (optimum, pH 7.0), and 0–3% (w/v) NaCl (optimum, 0.5%). The principal fatty acids were $C_{16:0}$, summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$), and summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). The sole respiratory quinone was Q-8 and total polar lipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified aminolipid (AL), two unidentified glycolipids (GL1,2), an unidentified amino phosphoglycolipid (APGL), two unidentified phospholipids (PL1,2), two unidentified aminophospholipids (APL1,2), and three unidentified lipids (L1,2,3). The average nucleotide identity (ANI) values and in silico DDH between strain YS8-69^T and *L. alexandrii* LZ-4^T, *L. thiooxidans* JCM 13612^T, and *L. humi* DSM 111650^T were 73.0–80.6% and 15.8–50.2%, respectively. The genome sequence showed a length of 3,162,663 bp, with 20 contigs and 51.7% of G + C content. Based on physiological, chemotaxonomic, genotypic characteristics, and phylogenetic results, we propose that strain YS8-69^T represents a novel specie of the genus *Limnobacter*, for which the name *Limnobacter parvus* sp. nov. is proposed (type strain YS8-69^T = MCCC 1K08015^T = KCTC 92278^T).

Introduction

The biological oxidation of sulfur plays an important role in the natural sulfur cycle, and microorganisms of different trophic types dominate these oxidation reactions. Most photoautotrophic and chemoautotrophic sulfur-oxidizing bacteria can utilize thiosulfate as an electron acceptor for photosynthesis or energy metabolism. The genus *Limnobacter*, which belongs to the class *Betaproteobacteria*, is a representative group for thiosulfate-oxidizing bacteria by the "S$_4$-intermediate" (or S$_4$I) pathway [1].

The genus *Limnobacter* was first proposed by Spring et al. in 2001 [2]. Since its first description, the genus comprises 4 species: *L. thiooxidans* DSM 13612^T, *L. humi* NBRC 111650^T, *L. litoralis* KP1-19^T, and *L. alexandrii* LZ-4^T (https://lpsn.dsmz.de/search?word=Limnobacter), and the members of genus *Limnobacter* have been isolated from freshwater lake sediment [2], humus soil [3], volcanic deposit [4], and marine phycosphere microbiota [5]. The major quinone is Q-8. The predominant fatty acids are $C_{16:0}$, summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$), and summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). In the present study, a thiosulfate-oxidizing bacterium was isolated, designated strain YS8-69^T, from a brackish lake in PR China. We experimentally confirmed the thiosulfate oxidation ability of strain YS8-69^T in this study and also analyzed the SOX system associated with thiosulfate oxidation in this strain by genomic method. Based on phenotypic, chemotaxonomic, phylogenetic, genomic, and metabolic characters, we classified strain YS8-69^T in the genus *Limnobacter*, and strain YS8-69^T appears to represent a novel specie.

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Materials and Methods

Isolation and Cultivation

Strain YS8-69[T] was isolated from surface lake water (pH 8.2, 24 °C) collected from the Sayram Lake (44°29′ N, 81°10′ E), Xinjiang Uygur Autonomous Region, PR China in August 2019. The water sample was spread on R_2A [6] agar plates and incubated at 25 °C for 2 weeks. The tiny transparent colony YS8-69[T] was picked and cultured routinely on R_2A medium at 25 °C and stored at − 80 °C in R_2A medium with 30% (v/v) sterile glycerol. The reference strains *Limnobacter thiooxidans* DSM 13612[T] and *Limnobacter humi* NBRC 111650[T] were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures) and NBRC (Biological Resource Center, Japan) and cultivated under comparable conditions for comparative testing. In addition, *L. alexandrii* LZ-4[T] was selected as reference strain cited from the strain publication to compare the differences in various aspects within the genus and to make the results more convincing.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA was extracted and purified by bacterial genomic DNA fast extraction kit (Dongsheng Biotech). PCR amplification of the 16S rRNA gene was performed out using bacterial primers 27F (5′-GAGAGTTTGATC CTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTT ACGACTT-3′) [7]. The PCR products were sequenced and the almost complete 16S rRNA gene sequences of strain YS8-69[T] were obtained and then identified the phylogenetic neighbors using the EzBiocloud server (www. ezbiocloud. net/) [8] and Blastn program (www.ncbi. nlm.nih.gov/). The 16S rRNA gene sequences of the closely related and closely related-type strains were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/) database and the multiple sequence alignments were performed by Clustal W [9]. Evolutionary distances were then calculated using the Kimura two-parameter model [10]. Phylogenetic trees were reconstructed using the neighbor joining [11], maximum likelihood [12], and maximum parsimony [13] with MEGA X software [14]. Additionally, the 1000 replicates were used to estimate the bootstrap values.

Morphology, Physiology, and Biochemistry

For phenotypic tests, the strain YS8-69[T] was grown in R_2A medium for 5 days at 25 °C before use. Flagellar and cellular morphologies were examined using transmission electron microscopy (JEM1230, JEOL). Cell motility was assessed using a semisolid R_2A mediums with incubation at 25 °C for 2 weeks [15]. Growth conditions, including optimum growth temperature, tolerance to pH, and NaCl, were tested. The temperature range for growth was tested at 4, 10, 15, 20, 25, 30, 37, 40, 45, and 50 °C in R_2A (pH 7.0). The pH range for growth was determined at 0.5-pH intervals (pH 5.0–10.5) using the following buffer systems: MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), and CAPSO (pH 9.0–10.5) (at a concentration of 50 mM). Tolerance to NaCl tests was determined by NaCl-free R_2A and 0.5, 1.0, 3.0, 5.0, and 7.5% NaCl (w/v). Oxidase and catalase activity were determined by oxidase reagent (bioMérieux) and 3% (v/v) H_2O_2, respectively. Degradation of starch was tested on R_2A supplemented with 0.2% soluble starch [16]. R_2A containing 0.5% L-tyrosine was used to test the degradation of L-tyrosine. H_2S production were assayed according to the methods of Xamxidin M et al. [17]. Tweens 20, 40, 60, and 80 utilizations were tested according to a standard protocol [18]. Anaerobic growth was tested with Anaeropack System (Mitsubishi Gas Chemical) in R_2A plates supplemented with $NaNO_3$ (20 mM), $NaNO_2$ (10 mM), Na_2SO_3 (5 mM), $Na_2S_2O_3$ (20 mM), Na_2SO_4 (20 mM), and fumaric acid as growth factors over 15 days, respectively. Biochemical properties including hydrolysis of aesculin and other enzyme activities were tested using API 20NE, ZYM, 50CH (bioMérieux), and GNIII MicroPlate (Biolog) systems according to the manuals. For thiosulfate-oxidizing ability detection, at the optimum temperature, strain YS8-69[T] was inoculated into R_2A broth supplemented with $Na_2S_2O_3$·$5H_2O$ (0, 1, 3, and 5 g/L). Phenol red (0.01 g/L) was used as a pH indicator to investigate the ability of thiosulfate oxidization.

Chemotaxonomic Characterization

For cellular fatty acid analysis, cells of YS8-69[T] and the reference strains were obtained and freeze-dried after incubation in R_2A at 25 °C for 5 days. Fatty acids were then analyzed according to the standard protocol of the Microbial Identification System (midi, Microbial ID). Respiratory quinones of the isolate were extracted described by Sun et al. [19]. Polar lipids were extracted from freeze-dried cells and separated by two-dimensional TLC on silica gel plates (10×10 cm; Merck) [20]. Total polar lipids were detected by spraying with 5% phosphomolybdic acid solution followed by heating at 160 °C for 10 min. Aminolipids were detected by spraying the plate with a 0.5% (w/v) solution of ninhydrin in ethanol with heating at 55 °C for 10 min, and visualization of glycolipids were completed by spraying α-naphthol/ H_2SO_4 reagent with heating at 145 °C for 8 min and molybdenum blue reagent (Sigma) for phospholipids [21].

Genome sequence assembly, annotation, and analysis

The genomes of strain YS8-69^T and *Limnobacter humi* NBRC 111650^T were sequenced by Nova-PE150 sequencing technology with the HiSeq platform (Novogene Technology Company). The genome of *Limnobacter thiooxidans* DSM 13612^T were retrieved from the NCBI database. The assembly of the reads was performed using genome assembly and annotation pipeline on gcType online server (https://gctype.wdcm.org/index). The quality of microbial genomes was assessed using the bioinformatics tool CheckM v1.0.7 [22]. The draft genome sequence was annotated using the RAST server online (https://rast.nmpdr.org/rast.cgi) [23], and TYGS (https://tygs.dsmz.de/) server was applied to reconstruct a phylogenetic tree based on the whole proteome [24]. Metabolic pathways were predicted using the KEGG (Kyoto Encyclopedia of Genes and Genomes) online annotation server [25]. The digital DNA–DNA hybridization (*d*DDH) was conducted by the Genome-to-Genome Distance Calculator (GGDC; version2.1) according to recommended Formula 2 (http://ggdc.dsmz.de/distcalc2.php) offered by the DSMZ website [26], and the average nucleotide identity (ANI) values were calculated by OrthoANI algorithm online software [27].

Results and Discussion

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA gene sequence of strain YS8-69^T (1499 nt) was obtained. Based on the NCBI blast service and EzTaxon-e database, strain YS8-69^T was most closely related to *Limnobacter alexandrii* LZ-4^T (98.93%), *Limnobacter thiooxidans* DSM 13612^T (98.55%), *Limnobacter humi* NBRC 111650^T (97.66%), and *Limnobacter litoralis* KP1-19^T (97.04%). The topologies of the neighbor-joining, maximum-likelihood, and maximum-parsimony phylogenetic trees indicated that the genus *Limnobacter* formed a monophyletic clade. Strain YS8-69^T was clustered with *Limnobacter alexandrii* LZ-4^T and *Limnobacter thiooxidans* DSM 13612^T (Fig. 1). Phylogenetic analysis indicated that strain YS8-69^T represented a novel member of the genus *Limnobacter*.

Morphology, Physiology, and Biochemistry

Strain YS8-69^T was Gram stain-negative, strictly aerobic, rod-shaped with 0.6–0.7 μm in width and 1.2–1.5 μm in length (Fig.S1) and cells contain polyhydroxybutyrate (PHB). Motile by a single polar flagellum. Colony was transparent, circular, convex, smooth, and 1–1.5 mm in diameter after 5-day incubation at 25 °C on R$_2$A agar. The growth range of pH, temperature, and NaCl concentrations was pH 7.0–10.0 (optimum, 7.0), 4–40 °C (optimum, 25 °C), and 0–3% NaCl (w/v) (optimum, 0.5%) in R$_2$A broth. These characteristics clearly differentiated strain YS8-69^T from the related strain *Limnobacter alexandrii* LZ-4^T. The strain YS8-69^T and the other strains of the genus *Limnobacter* were positive for oxidase and catalase and all contained PHB. Except for *Limnobacter humi* NBRC 111650^T, the other strains are not produced H$_2$S. No anaerobic growth occurs on R$_2$A supplemented with NaNO$_3$ (20 mM), NaNO$_2$ (10 mM), Na$_2$SO$_3$ (5 mM), Na$_2$S$_2$O$_3$ (20 mM), Na$_2$SO$_4$ (20 mM), and fumaric acid. After 5 days cultured, thiosulfate could be oxidized by strain YS8-69^T and reference strains. Additional phenotypic characteristics are given in the species description and Table 1.

Chemotaxonomic analysis

The sole respiratory quinone detected in strain YS8-69^T was Q-8, and the polar lipids of strain YS8-69^T included diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified aminolipid (AL), two unidentified aminophospholipids (APL1,2), two unidentified glycolipids (GL1,2), an unidentified amino phosphoglycolipid (APGL), two unidentified phospholipids (PL1,2), and three unidentified lipids (L1,2,3). The major polar lipids DPG, PE, and PG were similar to those of reference strains. However, compared with the *L. alexandrii* LZ-4^T and *L. humi* NBRC 111650^T, strain YS8-69^T contained GL and APGL, but lacked one unidentified phospholipid and one unidentified aminophospholipid (Fig.S2). The fatty acid profiles of strain YS8-69^T and the reference strains are provided in Table S5. The major fatty acids ($\geq$ 10% of the total fatty acids) detected in strain YS8-69^T were C$_{16:0}$, summed feature 3 (C$_{16:1}$ ω7*c* and/or C$_{16:1}$ ω6*c*), and summed feature 8 (C$_{18:1}$ ω7*c* and/or C$_{18:1}$ ω6*c*). The result of the fatty acid analyses was similar to other species of the genus *Limnobacter*. Furthermore, strain YS8-69^T and *L. alexandrii* LZ-4^T contained C$_{17:0}$ cyclo (8.3% and 9.8%, respectively); it was not detected in other strains. The strain YS8-69^T and *L. thiooxidans* DSM 13612^T did not have unsaturated fatty acid 11-methyl C$_{18:1}$$\omega$9*c*, while other reference strains did. (Table S5).

Genome Sequence Assembly, Annotation, and Analysis

The genome completeness of strain YS8-69^T and *L. humi* NBRC 111650^T were 99.43% and 99.43%, with contamination 0.26% and 0.23%, respectively. They were regarded as excellent reference genomes for deeper analyses (genome
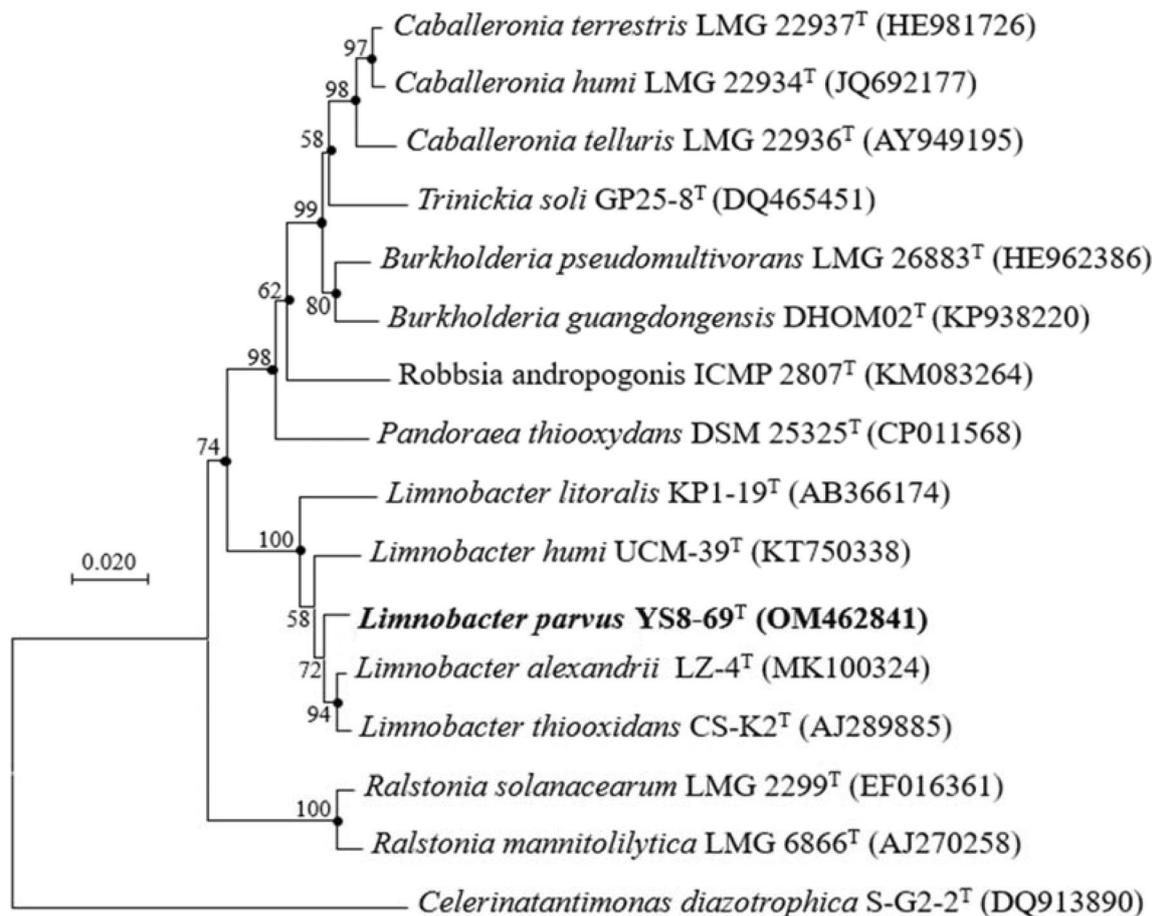
Fig. 1 Neighbor-joining phylogenetic analysis based on 16S rRNA gene sequences for strain *Limnobacter parvus* YS8-69[T] (OM462841) and related species. Percentage bootstrap values ≥ 50 (1000 replicates) shown at branch nodes. Closed circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Celerinatantimonas diazotrophica* S-G2-2[T] (DQ913890) was used as the outgroup. Bar, 0.02 nucleotide substitutions per nucleotide position

sequence estimated to be ≥ 95% completeness, with ≤ 5% contamination). The draft genome sequences of strain YS8-69[T] and *L. humi* NBRC 111650[T] were 3.16 Mb and 3.23 Mb in length with 20 and 29 contigs. The DNA G + C contents of strains YS8-69[T] and *L. humi* NBRC 111650[T] were 51.7% and 56%, respectively. The ANI values and in silico DDH between strain YS8-69[T] and the reference strains were 73–80.6 and 15.8–50.20%, which are below the species threshold of ANI 94–96% [28], and in silico DDH 70% [29]. The whole proteome-based GBDP distances using the TYGS to clarify the taxonomic positions of YS8-69[T]. The members of the genus *Limnobacter* formed a well-supported clade. Strain YS8-69[T] clustered with *Limnobacter alexandrii* LZ-4[T] and *Limnobacter thiooxidans* DSM 13612[T] together (Fig.S3). Both draft genome sequences have been deposited into the NCBI with accession numbers JANKHG000000000 and JANIGO000000000 (Table S6).

According to the KEGG and RAST analysis, genome analysis revealed that strain YS8-69[T] and all type strains of genus *Limnobacter* had thiosulfate oxidation pathway and constitute a complete sox system which is related to the oxidation of thiosulfate (Table S7 and Fig.S4). The reference strain *L. humi* NBRC 111650[T] had a completely assimilatory sulfate reduction pathway, but YS8-69[T] and other type strains of *Limnobacter* did not, and this ability was confirmed by the H_2S production experiment (Table 1). All negative traits from commercial kits are listed in Table S8.

In conclusion, strain YS8-69[T] should represent a novel species of the genus *Limnobacter* for which the name *Limnobacter parvus* sp. nov. is proposed.

Description of *Limnobacter parvus* sp. nov

Limnobacter parvus (par'vus. L. masc. adj. *parvus*, small, intended to mean the organism formed a tiny colony).

Cells are Gram stain-negative, aerobic, motile with one polar flagellum and rod shaped, approximately 0.6–0.7 μm wide and 1.2–1.5 μm long. Colonies are transparent,

Table 1 Differential phenotypic characteristics of strain YS8-69 [T] and the reference strains

Characteristic	1	2	3	4[c]
Origin	Lake water	Humus soil[a]	Lake sediment [b]	Marine phycosphere Microbiota
NaCl conc. (%) for growth (optimal)	0–3 (0.5)	0–2.5(0.5) [a]	0–4 (3) [b]	0–7 (3.5)
Temp range (°C) for growth (optimal)	10–40(25)	15–50(28–37) [a]	4–38(ND) [b]	10–42(33)
pH range for growth (optimal)	7–10(7)	5.5–9.0(6.5–7.5) [a]	5.5–10 (7.5) [b]	5.5–9 (7.5)
H_2S production	–	+	–	–
Enzyme activities (API ZYM):				
Alkaline phosphatase	+	+	–	+
Esterase(C4)	+	+	+	+
Valine arylamidase	–	–	–	+
Naphthol-AS-BI-phosphohydrolase	+	+	–	–
API 20NE tests:				
Arginine dihydrolase	-	+	-	+
Assimilation D-glucose	–	–	+	+
Assimilation L-arabinose	–	–	+	–
Assimilation potassium gluconate	–	–	+	–
Assimilation malate	+	+	+	–
Acid production from (API 50CH):				
Sucrose	+	–	–	ND
D-turanose	–	+	w	ND
D-arabinose, D-mannose, D-ribose, D-galactose, D-glucose	–	–	+	ND
DNA G+C content (%)	51.7	56	53.2	51.7

Strains: 1, YS8-69[T]; 2, *L. humi* NBRC 111650[T]; 3, *L. thiooxidans* DSM 13612[T]; 4, *L. alexandrii* LZ-4[T]

All data were obtained from this study unless stated otherwise;

+positive; w weakly positive; − negative;

[a]Data from nguyen et al. [3]

[b]Data from spring et al. [2]

[c]Data from duan et al. [5]

circular, raised, and smooth with a diameter of 1–1.5 mm after 5 days at 25 °C on R_2A plate. Growth occurs at 4–40 (optimum, 25 °C), at pH 7.0–10.0 (optimum, pH 7.0), and with 0–3% (w/v) NaCl (optimum, 0.5%). Catalase activity and oxidase activity are positive, while results for hydrolysis of starch and gelatin, Tweens (20, 40, and 60), and H_2S production test are negative. No anaerobic growth occurs on R_2A medium supplemented with $NaNO_3$ (20 mM), $NaNO_2$ (10 mM), Na_2SO_3 (5 mM), $Na_2S_2O_3$ (20 mM), Na_2SO_4 (20 mM), and fumaric acid. Acids are produced from sucrose, but not from the other substrates of the API 50CH system. In assays with the API 20NE system, assimilation of malate is positive, but others are negative. The API ZYM system are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. There are positive reactions in the Biolog GENIII MicroPlate system for aspartic acid, glutamic acid, glucuronamide, tetrazolium violet, malic acid, and acetic acid and weekly positive for D-fructose, L-fucose, D-fructose-6-PO₄, lincomycin, D-galacturonic acid, L-galactonic acid lactone, D-glucuronic acid, tetrazolium blue, L -lactic acid, D-malic acid, Tween 40, β-hydroxy-D, L butyric acid, and acetoacetic acid. The sole respiratory quinone is Q-8, and the predominant fatty acids (≥ 10% of the total fatty acids) are $C_{16:0}$, summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$), and summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminophospholipids, one unidentified aminolipid, unidentified glycolipids, unidentified amino phosphoglycolipid, and unidentified lipids. The DNA G+C content is 51.7% (by genome).

The type strain YS8-69[T] (MCCC 1K08015[T] = KCTC 92278[T]) is isolated from the lake water, collected from the Sayram Lake, Xinjiang Uygur Autonomous Region, PR China (N44°29′ E81°10′).

Declarations

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

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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