Halobellus limi sp. nov. and *Halobellus salinus* sp. nov., isolated from two marine solar salterns

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Two halophilic archaea, strains TBN53^T and CSW2.24.4^T, were characterized to elucidate their taxonomic status. Strain TBN53^T was isolated from the Taibei marine solar saltern near Lianyungang city, Jiangsu province, China, whereas strain CSW2.24.4^T was isolated from a saltern crystallizer in Victoria, Australia. Cells of the two strains were pleomorphic, stained Gram-negative and produced red-pigmented colonies. Strain TBN53^T was able to grow at 25–55 °C (optimum 45 °C), with 1.4–5.1 M NaCl (optimum 2.6–3.9 M NaCl), with 0–1.0 M $MgCl_2$ (optimum 0–0.1 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0), whereas strain CSW2.24.4^T was able to grow at 25–45 °C (optimum 37 °C), with 2.6–5.1 M NaCl (optimum 3.4 M NaCl), with 0.01–0.7 M $MgCl_2$ (optimum 0.05 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0–7.5). Cells of the two isolates lysed in distilled water. The minimum NaCl concentrations that prevented cell lysis were 8 % (w/v) for strain TBN53^T and 12 % (w/v) for strain CSW2.24.4^T. The major polar lipids of the two strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and phosphatidylglycerol sulfate, with two glycolipids chromatographically identical to sulfated mannosyl glucosyl diether and mannosyl glucosyl diether, respectively. Trace amounts of other unidentified lipids were also detected. On the basis of 16S rRNA gene sequence analysis, strains TBN53^T and CSW2.24.4^T showed 94.1 % similarity to each other and were closely related to *Halobellus clavatus* TNN18^T (95.0 and 94.7 % similarity, respectively). Levels of *rpoB′* gene sequence similarity between strains TBN53^T and CSW2.24.4^T, and between these strains and *Halobellus clavatus* TNN18^T were 88.5, 88.5 and 88.1 %, respectively. The DNA G+C contents of strains TBN53^T and CSW2.24.4^T were 69.2 and 67.0 mol%, respectively. The level of DNA–DNA relatedness between strain TBN53^T and strain CSW2.24.4^T was 25 %, and these two strains showed low levels of DNA–DNA relatedness with *Halobellus clavatus* TNN18^T (30 and 29 % relatedness, respectively). Based on these phenotypic, chemotaxonomic and phylogenetic properties, two novel species of the genus *Halobellus* are proposed to accommodate these two strains, *Halobellus limi* sp. nov. (type strain TBN53^T=CGMCC 1.10331^T=JCM 16811^T) and *Halobellus salinus* sp. nov. (type strain CSW2.24.4^T=DSM 18730^T=CGMCC 1.10710^T=JCM 14359^T).

Abbreviations: DGD-1, mannosyl glucosyl diether; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TBN53^T and CSW2.24.4^T are GU208828 and HQ451075, respectively. Those for the *rpoB′* gene sequences of strains TBN53^T and CSW2.24.4^T are JN180933 and JN180934, respectively.

Two supplementary figures are available with the online version of this paper.

Marine solar salterns are artificial hypersaline environments for the production of halite from seawater and harbour diverse halophilic archaea (Burns *et al.*, 2004). Recently, increasing numbers of isolates representing novel taxa have been isolated from these habitats, such as *Natronomonas moolapensis*, *Halonotius pteroides* (Burns *et al.*, 2010a, b), *Haladaptatus litoreus*, *Halogranum rubrum*, *Haloplanus vescus*, *Halopelagius inordinatus*, *Halosarcina limi*, *Halogeometricum rufum*, '*Halorussus rarus*' (Cui *et al.*, 2010a, b, c, d, e, f, g), *Halarchaeum*

acidiphilum (Minegishi *et al.*, 2010a), *Salarchaeum japonicum* (Shimane *et al.*, 2011), *Halomarina oriensis* (Inoue *et al.*, 2011), *Halogranum gelatinilyticum*, *Halogranum amylolyticum*, *Haloplanus aerogenes*, *Halolamina pelagica*, *Halorientalis regularis* and *Halobellus clavatus* (Cui *et al.*, 2011a, b, c, d). The reasons behind this rapid expansion of described taxa within the family *Halobacteriaceae* include improved cultivation methods, sampling from a wider range of geographically distinct sites and increased interest in understanding the impacts of microbial communities in diverse environments around the world. These studies have shown that members of the same genus can be found to span quite different hypersaline environments. For example, *Natronomonas* species are found in both thalassohaline and athalassohaline waters (Burns *et al.*, 2010b).

The genus *Halobellus* was proposed to accommodate the species *Halobellus clavatus* described based on two strains, TNN18^T and TBN12, which were isolated from two artificial marine solar salterns in eastern China (Cui *et al.*, 2011d). The strains of *Halobellus clavatus* formed a clade with *Haloquadratum walsbyi*, but the main characteristics such as cell morphology, nutrition, miscellaneous biochemical activities, glycolipid profiles and 16S rRNA gene sequence similarities clearly separated *Halobellus* from *Haloquadratum*. The DNA G+C contents of *Halobellus* were between 61.5 and 62.4 mol%, which are considerably higher than that of *Haloquadratum* (46.9 mol%). Members of the genus *Halobellus* were able to utilize D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose and glycerol as single carbon and energy sources, whereas *Haloquadratum walsbyi* was unable to metabolize any of these substrates (Burns *et al.*, 2007). H_2S production and indole formation were positive for *Halobellus*, but negative for *Haloquadratum*. The conspicuous morphology of *Haloquadratum walsbyi* cells, which are regularly square-shaped, also distinguishes members of that genus from *Halobellus*.

In this study, we characterized two strains isolated from marine solar salterns, one in Australia and the other in China. Strain TBN53^T was isolated from sediment of Taibei marine solar saltern (34° 43′ 38″ N 119° 17′ 48″ E), an artificial saltern near Lianyungang city, Jiangsu province, China. Brine from the Taibei marine solar saltern had a temperature of 25 °C, a pH of 7.2 and a total salinity of 285 g l^{-1}. Strain CSW2.24.4^T was isolated from a crystallizer pond at the Cheetham marine solar saltern, Moolap, Victoria, Australia (38° 9′ 50.46″ S 144° 25′ 16.44″ E), as described by Burns *et al.* (2004). Brine from the Cheetham marine solar saltern (collected at ambient daytime temperature in March 2002) had a pH of 8.1 and a total salinity of 330 g l^{-1}. Isolation media and procedures have been described previously (Burns *et al.*, 2004; Cui *et al.*, 2010a, e). The strains were routinely grown aerobically at 37 °C in NOM-3 medium (NOM series medium) with the following modifications (per litre): 1.0 g yeast extract, 0.25 g fish peptone, 0.25 g sodium formate, 0.25 g sodium acetate, 0.25 g sodium lactate and 0.25 g sodium pyruvate.

Genomic DNA from halophilic archaeal strains was prepared as described by Ng *et al.* (1995). The 16S rRNA gene was amplified via PCR by using primers 18F and 1518R (Cui *et al.*, 2009). PCR-mediated amplification and sequencing of the *rpoB′* genes were carried out according to Minegishi *et al.* (2010b). Multiple sequence alignments were performed via the CLUSTAL W program integrated in the MEGA5 software (http://www.megasoftware.net/) (Kumar *et al.*, 2008). Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms within the MEGA5 software. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Levels of gene sequence similarity were calculated by using the pairwise-distance computing function in MEGA5.

Sixteen complete 16S rRNA gene sequences of strains TBN53^T and CSW2.24.4^T were obtained. Comparisons indicated that the two strains have one type of 16S rRNA gene sequence. The gene lengths of strains TBN53^T and CSW2.24.4^T were 1472 and 1475 bp, respectively. Strains TBN53^T and CSW2.24.4^T shared 94.1 % 16S rRNA gene sequence similarity and the two strains were closely related to *Halobellus clavatus* TNN18^T (95.0 and 94.7 % similarity, respectively). They were also similar to the type strains of *Halosarcina limi* (94.7 %), *Halosarcina pallida* (94.0 %) and *Halogeometricum borinquense* (94.3 %). Phylogenetic tree reconstructions by using the neighbour-joining algorithm revealed that strain TBN53^T and strain CSW2.24.4^T clustered tightly with *Halobellus clavatus* TNN18^T (Fig. 1a), forming a monophyletic clade that branched nearest to *Haloquadratum walsbyi*, and more distantly from other recognized genera of the *Halobacteriaceae*. Although the branch lengths of the three members within the *Halobellus*-related clade appeared to be relatively long, the bootstrap confidence values indicated strong support for monophyly. The phylogenetic position was also confirmed in the trees generated with the maximum-parsimony and maximum-likelihood algorithms (data not shown). The results of phylogenetic analysis based on 16S rRNA gene sequences supported the placement of strains TBN53^T and CSW2.24.4^T in the genus *Halobellus*.

The *rpoB′* gene lengths of strains TBN53^T and CSW2.24.4^T were both 1830 bp, and the nucleotide sequences were 90.2 % similar to each other, and also closely similar to the corresponding gene of *Halobellus clavatus* TNN18^T (92.5 and 89.3 %, respectively). In phylogenetic tree reconstructions, strains TBN53^T and CSW2.24.4^T clustered tightly with *Halobellus clavatus* TNN18^T (Fig. 1b) and the three taxa formed a monophyletic group separate from the related genera *Halopelagius*, *Haloferax*, *Halosarcina* and *Haloquadratum*. The phylogenetic position was also confirmed in trees generated with the maximum-parsimony and maximum-likelihood algorithms (data not shown). Phylogenetic analysis based on *rpoB′* gene sequences supported

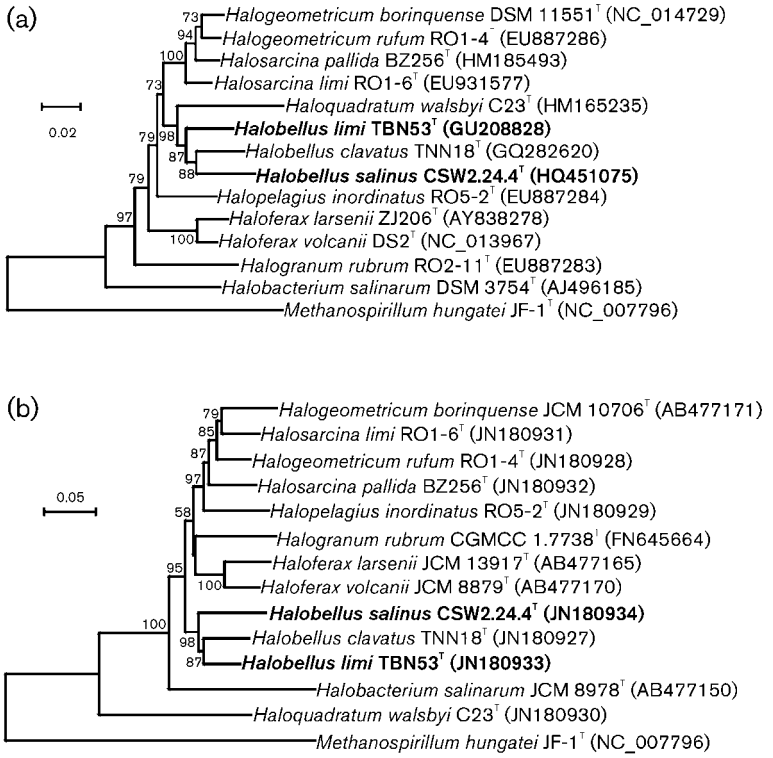

Fig. 1. Neighbour-joining phylogenetic trees based on 16S rRNA gene (a) and *rpoB′* gene (b) sequences showing the relationships between strain TBN53^T, strain CSW2.24.4^T and related members within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 70 % support. Bars, 0.02 (a) and 0.05 (b) expected changes per site. The sequence of *Methanospirillum hungatei* JF-1^T was used as an outgroup.

the placement of strains TBN53^T and CSW2.24.4^T in the genus *Halobellus*.

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren *et al.*, 1997). *Halobellus clavatus* TNN18^T, *Halopelagius inordinatus* RO5-2^T, *Haloferax volcanii* CGMCC 1.2150^T and *Halobacterium salinarum* CGMCC 1.1958^T were used as reference strains. Various tests relating to morphology and growth characteristics, nutrition, miscellaneous biochemical activities and sensitivity to antimicrobial agents were performed as described previously (Cui *et al.*, 2010b).

Cells of strains TBN53^T and CSW2.24.4^T were motile and pleomorphic when grown in NOM-3 liquid medium (Fig. S1 available in IJSEM Online). Cells stained Gram-negative and colonies were red-pigmented. Strain TBN53^T was able to grow at 25–55 °C (optimum 45 °C), with 1.4–5.1 M NaCl (optimum 2.6–3.9 M NaCl), with 0–1.0 M $MgCl_2$ (optimum 0–0.1 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0), whereas strain CSW2.24.4^T was able to grow at 25–45 °C (optimum 37 °C), with 2.6–5.1 M NaCl (optimum 3.4 M NaCl), with 0.01–0.7 M $MgCl_2$ (optimum 0.05 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0–7.5). Cells of the two strains lysed in distilled water and minimal NaCl concentrations to prevent lysis were 8 % (w/v) for strain TBN53^T and 12 % (w/v) for strain CSW2.24.4^T. Neither strain was able to grow under anaerobic conditions using nitrate, DMSO or L-arginine, but strain TBN53^T reduced nitrate to nitrite. They were positive for catalase and oxidase, but did not form indole. Strain TBN53^T produced H_2S from sodium thiosulfate, but strain CSW2.24.4^T did not. They did not hydrolyse starch, gelatin, casein or Tween 80. The main phenotypic characteristics differentiating strain TBN53^T and strain CSW2.24.4^T from the related *Halobellus clavatus* TNN18^T were optimum NaCl concentration, the requirement for Mg^{2+}, temperature optimum, reduction of nitrate to nitrite, utilization of specific carbon sources, indole formation and H_2S formation (Table 1). More detailed results of phenotypic tests and nutritional features of these strains are given in the species descriptions below.

Polar lipids were extracted by using a chloroform/methanol system and were analysed by one- and two-dimensional TLC, as described previously (Kates, 1986). Merck silica gel 60 F_{254} aluminium-backed thin-layer plates were used in TLC analysis. For two-dimensional TLC, the first dimension was developed using chloroform/methanol/water (65 : 25 : 4, by vol.) and the second dimension used chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). The latter solvent was also used in one-dimensional TLC. Two specific detection spray reagents, phosphate stain reagent for phospholipids and α-naphthol stain for glycolipids, were used. The general detection reagents, sulfuric acid/ethanol (1 : 2, v/v) and 10 % (w/v) molybdophosphoric acid in absolute ethanol, were also used to detect total polar lipids. Glycerol diether moieties were detected by using the procedure described by Ross *et al.* (1981). Isoprenoid quinones were extracted, purified and analysed by HPLC according to Collins (1985). The isoprenoid quinones were confirmed by HPLC-MS (model

Table 1. Characteristics that distinguish strain TBN53^T and strain CSW 2.24.4^T from *Halobellus clavatus* TNN18^T and *Haloquadratum walsbyi* C23^T

Strains: 1, TBN53^T; 2, CSW2.24.4^T; 3, *Halobellus clavatus* TNN18^T; 4, *Haloquadratum walsbyi* C23^T. Data are from the current study. +/−, Variable; −/w, very weak.

Characteristic	1	2	3	4
Cell morphology	Pleomorphic*	Pleomorphic*	Pleomorphic*	Squares
Motility	+	+	+	−
Optimum NaCl (M)	2.6–3.9	3.4	2.6	3.1
Mg^{2+} requirement	−	+	−	−
Temperature optimum (°C)	45	37	37	45
Reduction of nitrate to nitrite	+	−	+/−	−
Utilization of:				
D-Glucose	+	+	+	−
D-Mannose	+	−	+	−
D-Galactose	+	−	+	−
Maltose	+	−	+	−
Sucrose	+	+	+	−
Lactose	+	−	+	−
Glycerol	+	+	+	−
D-Sorbitol	+	+	−	−
DL-Lactate	+	+	+	−
Succinate	−	−	+	−
L-Malate	−	+	+	−
Fumarate	−	−	+	−
Citrate	−	+	−	−
Glycine	+	−	−	−
L-Alanine	+	−/w	−	−
L-Glutamate	+	−	−	−
Indole formation	−	−	+	−
H_2S formation	+	−	+	−
Sensitivity to antibiotics†				
Bacitracin (0.04 IU)	+	+	+	−
Erythromycin (15)	−	−	−	+
Chloramphenicol (30)	−	−	−	+
Tetracycline (30)	−	−	−	+
Glycolipid types‡	S-DGD-1, DGD-1, 3 UG	S-DGD-1, DGD-1	S-DGD-1, DGD-1, 2–3 UG	S-DGD-1
DNA G+C content (mol%)	69.2	67.0	61.5	46.9

*Various shapes seen in pure cultures including rods and cocci.

†Concentrations in μg per disc unless stated otherwise.

‡DGD-1, Mannosyl glucosyl diether; S-DGD-1, sulfated mannosyl glucosyl diether; UG, unidentified glycolipid.

LXQ; Thermo Fisher Scientific) analysis (APCI ion source, Shim-pack VP-ODS 4.6 × 250 mm column with a flow rate of 0.8 ml min^{-1}, 40 °C and 100–1200 *m/z*).

The major polar lipids of the two strains were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and two glycolipids, with one to three minor, unidentified glycolipids. The two major glycolipids were chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DGD-1) showing a pattern chromatographically similar to the polar lipid profiles of *Halobellus clavatus* TNN18^T. Other trace unidentified lipids were detected that differentiated strain TBN53^T and strain CSW2.24.4^T from *Halobellus clavatus* TNN18^T (Fig. S2). The major polar lipid compositions support the classification of strains TBN53^T and CSW2.24.4^T in the genus *Halobellus*. Strains TBN53^T and CSW2.24.4^T had C20 and C20 diether core lipids. Unsaturated and dihydrogenated menaquinones with eight isoprene units were found in the two strains. Strain TBN53^T contained MK-8 and trace amounts of MK-8(H2) and strain CSW2.24.4^T contained 95.7 % MK-8 and 4.3 % MK-8(H2).

The DNA G+C content was determined by thermal denaturation (T_m) according to the method of Marmur & Doty (1962). DNA–DNA hybridization analyses were performed according to the thermal denaturation and

renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983).

The DNA G+C contents of strains TBN53^T and CSW2.24.4^T were 69.2 and 67.0 mol%, respectively. These values are higher than those for *Halobellus clavatus* (61.5–62.4 mol%) (Cui *et al.*, 2011d). The level of DNA–DNA relatedness between strain TBN53^T and strain CSW2.24.4^T was 25 % and these two strains showed low levels of DNA–DNA relatedness with *Halobellus clavatus* TNN18^T (30 and 29 %, respectively), lower than the accepted threshold value (70 %) used to separate bacterial species (Stackebrandt & Goebel, 1994).

Based on these phenotypic, chemotaxonomic and phylogenetic properties, two novel species of the genus *Halobellus* are proposed to accommodate these two strains, *Halobellus limi* sp. nov. and *Halobellus salinus* sp. nov. Characteristics that distinguish strains TBN53^T and CSW2.24.4^T from *Halobellus clavatus* TNN18^T and *Haloquadratum walsbyi* C23^T are shown in Table 1.

Description of *Halobellus limi* sp. nov.

Halobellus limi (li′mi. L. gen. n. *limi* of/from mud).

Cells are motile and pleomorphic (rods, irregular cocci and ovals) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 2.6–3.9 M NaCl are red, elevated and round. Growth occurs at 25–55 °C (optimum 45 °C), with 1.4–5.1 M NaCl (optimum 2.6–3.9 M NaCl), with 0–1.0 M $MgCl_2$ (optimum 0–0.1 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration needed to prevent cell lysis is 8 % (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is observed. Formation of gas from nitrate is negative. H_2S is produced from sodium thiosulfate. Indole formation is negative. Does not hydrolyse starch, gelatin, Tween 80 or casein. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose, glycerol, D-mannitol, D-sorbitol, pyruvate and DL-lactate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: glycine, L-alanine and L-glutamate. The following substrates are not utilized as single carbon and energy sources for growth: D-fructose, L-sorbose, D-ribose, D-xylose, starch, acetate, succinate, L-malate, fumarate and citrate. The following substrates are not utilized as single carbon, nitrogen or energy sources for growth: L-arginine, L-aspartate and L-lysine. Acid is produced from D-glucose, D-mannose, D-galactose, sucrose and lactose. Susceptible to (μg per disc, unless otherwise indicated) novobiocin (30), bacitracin (0.04 IU), anisomycin (50 mg), rifampicin (5), mycostatin (100) and nitrofurantoin (300), but resistant to trimethoprim (5), erythromycin (15), neomycin (30), chloramphenicol (30), ampicillin (10), penicillin G (10 IU), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are PG, PGP-Me and PGS, and a major glycolipid and a minor glycolipid chromatographically identical to S-DGD-1 and DGD-1, respectively. Three unidentified glycolipids may be present.

The type strain, TBN53^T (=CGMCC 1.10331^T=JCM 16811^T), was isolated from the Taibei marine solar saltern in Jiangsu province, China. The DNA G+C content of the type strain is 69.2 mol%.

Description of *Halobellus salinus* sp. nov.

Halobellus salinus (sa′li.nus. L. masc. adj. *salinus* of or belonging to salt).

Cells are motile and pleomorphic (rods, irregular cocci and ovals) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 3.4 M NaCl are red, elevated and round. Growth occurs at 25–45 °C (optimum 37 °C), with 2.6–5.1 M NaCl (optimum 3.4 M NaCl), with 0.01–0.7 M $MgCl_2$ (optimum 0.05 M $MgCl_2$) and at pH 5.5–9.5 (optimum pH 7.0–7.5). Cells lyse in distilled water and minimal NaCl concentration to prevent cell lysis is 12 % (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is not observed. Formation of gas from nitrate is negative. H_2S is not produced from sodium thiosulfate. Indole formation is negative. Does not hydrolyse starch, gelatin, Tween 80 or casein. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, sucrose, glycerol, D-sorbitol, pyruvate, DL-lactate, L-malate and citrate. The following substrates are not utilized as single carbon and energy sources for growth: D-mannose, D-galactose, D-fructose, L-sorbose, D-ribose, D-xylose, maltose, lactose, starch, D-mannitol, acetate, succinate and fumarate. The following substrates are not utilized as single carbon, nitrogen or energy sources for growth: glycine, L-alanine, L-arginine, L-aspartate, L-glutamate, L-lysine and L-ornithine. Acid is produced from D-glucose, D-mannose, D-galactose, sucrose and lactose. Susceptible to (μg per disc, unless otherwise indicated) novobiocin (30), bacitracin (0.04 IU), anisomycin (50 mg), rifampicin (5), nitrofurantoin (300) and norfloxacin (10), but resistant to trimethoprim (5), mycostatin (100), erythromycin (15), neomycin (30), chloramphenicol (30), ampicillin (10), penicillin G (10 IU), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are PG, PGP-Me and PGS, and a major glycolipid and a minor glycolipid chromatographically identical to S-DGD-1 and DGD-1, respectively; one trace unidentified glycolipid is present.

The type strain, CSW2.24.4^T (=DSM 18730^T=CGMCC 1.10710^T=JCM 14359^T), was isolated from a crystallizer pond at the Cheetham marine solar saltern at Moolap, Victoria, Australia. The DNA G+C content of the type strain is 67.0 mol%.

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