International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 149–155 Printed in Great Britain

Halorubrum tebenquichense sp. nov., a novel halophilic archaeon isolated from the Atacama Saltern, Chile

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A novel extremely halophilic archaeon was isolated from Lake Tebenquiche, situated in the northern part of the Atacama Saltern, Chile. The cells of these micro-organisms were mostly irregularly disc-shaped. They grew in medium containing saturated concentrations of NaCl and did not require magnesium for optimal growth. The polar lipid composition revealed the presence of mannosyl-2-sulfate-(1-4)-glycosyl-archaeol, the main glycolipid of the genus *Halorubrum*, and two new glycolipids. The G+C content of the DNA was 63·2 mol%. Phylogenetic analysis of the 16S rRNA gene placed strain ALT6-92^T within the *Halorubrum* cluster. The low DNA–DNA hybridization value justified classification in a new species for which the name *Halorubrum tebenquichense* sp. nov. is proposed. The type strain is ALT6-92^T (= CECT 5317^T = DSM 14210^T).

Keywords: extremely halophilic archaea, *Halorubrum tebenquichense*, Lake Tebenquiche, Atacama Saltern

INTRODUCTION

The extremely halophilic archaea are the dominant microbial populations of hypersaline environments. Hypersaline ecosystems such as salt lakes and marshes show great variability in total salt concentration, ionic composition and pH (Ollivier *et al*., 1994). The current classification of halophilic archaea is based on three kinds of data: phenetic data, chemical data and genetic data (16S rRNA sequence information and DNA–DNA hybridization results) (Oren *et al*., 1997).

The Atacama Saltern is located in Northern Chile and covers an area of 32000 km^2. Because of its location in the Atacama Desert at 2300 m above sea level, it is a unique environment. Previous studies have shown that the microbiota of Lake Tebenquiche, situated in the northern part of the saltern, consist of a heterogeneous group of extremophile micro-organisms (Campos *et al*., 1990; Prado *et al*., 1991, 1993).

Abbreviations: S-DGD-3, mannosyl-2-sulfate-(1-4)-glycosyl-archaeol; S-DGD-5, mannosyl-2-sulfate-(1-2)-glycosyl-archaeol.

The GenBank/EMBL/DDBJ accession number for nucleotide sequence of strain ALT6-92^T is AJ276887.

Eighty two strains of extremely halophilic archaea were collected between 1984 and 1995, in the Lake Tebenquiche and Poligonal Zone of the Atacama Saltern. Polar lipid analyses (particularly of glycolipids) suggested that 12 of them were associated with the genus *Halorubrum*. Two strains (designated ALT6-92^T and TeSe25-84) possessed similar phenotypes as well as unique features that did not correspond to any previosly described species (Grant & Larsen, 1990; Kamekura & Dyall-Smith, 1995; Kamekura *et al*., 1997). Strain ALT6-92^T was chosen for further characterization. On the basis of 16S rRNA sequences, glycolipid content and phenotypic characterization, we propose to classify this strain as a member of a new species, namely *Halorubrum tebenquichense* sp. nov.

METHODS

Strains and culture conditions. The strains were collected from Lake Tebenquiche between 1984 and 1992. The reference strains of *Halorubrum* used in this study are listed in Table 1.

HE medium (Torreblanca *et al*., 1986) was prepared with 25% (w/v) total salts, according to Subov (1931), and supplemented with 0·1% glucose. The pH was adjusted to

Table 1. DNA base composition, DNA–DNA relatedness and evolutionary distance results for *Halorubrum* strains isolated from the Atacama Saltern and species of the genus *Halorubrum*

Strain	G+C content (mol%)	Similarity (%) to:		Evolutionary distance with ALT6-92^T
		ALT6-92^T	TeSe25-84	
ALT6-92^T	63·2	100	90·57	0
TeSe25-84	62·5	95·65	100	0·0156
H. sodomense DSM 3757	68*	55·5	35·79	0·0346
H. saccharovorum DSM 1137	71·2*	40·84	23·49	0·0564
H. trapanicum CECT 397	64·3*	42	25·63	0·0331
H. distributum VKM B-1733	ND	45	30·54	0·0483
H. lacusprofundi DSM 5036	65·3–65·8*, 54·8–56·5 (minor component)	44·06	22·84	0·0682
H. coriense DSM 10284	ND	43·14	23·24	0·0394
H. vacuolatum DSM 8800	62·7*	38	40·35	0·0881

ND, Not determined.

* Data obtained from Oren (1983), Grant & Larsen (1990), Mwatha & Grant (1993) and McGenity & Grant (1995).

7·0 with 4 M NaOH. Liquid medium was solidified by adding 2% agar before sterilization.

The characterization of isolates, including morphological, physiological, biochemical, nutritional and antimicrobial-susceptibility testing, was performed for each strain, as described previously (Tomlinson & Hochstein 1976; Tomlinson *et al.*, 1986; Rodríguez-Valera & Ruiz-Berraquero, 1983; Rodríguez-Valera *et al.*, 1980, 1982; Tindall *et al.*, 1984; González *et al.*, 1978; Juez *et al.*, 1986; Torreblanca *et al.*, 1986). Gram staining was performed by using acetic acid-fixed samples as described by Dussault (1955). Cell shape was examined by optical microscopy (standard 25; Zeiss). Transmission electron microscopy was carried out with cells grown in HE broth at 25% (w/v) overlying HE agar plates at 25% (w/v) total salts. Samples were negatively stained with 2% (w/v) uranyl acetate (30 s) and washed with an acetic acid solution at 3% (w/v). Observation was made using a high-resolution transmission electron microscope at 80 kW (TEM 902; Zeiss).

Lipid analysis. Lipids were extracted by the method of Bligh & Dyer (1959) as described by Kates (1972). The lipids were separated using silica gel TLC (Kiesel gel 60 F_{254}; Merck) and simple development with a chloroform/methanol/acetic acid/water (85:22:5:10:4 by vol.) solvent system. In addition, two-dimensional chromatography was performed by using chloroform/methanol/water (65:25:4 by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4 by vol.) in the second dimension (Tindall *et al.*, 1987; Oren *et al.*, 1996). Phospholipids were visualized with an ammonium molybdate/sulfuric acid spray. Glycolipid spots were detected by spraying the plates with 0·5% α-naphthol in 50% methanol and then with 5% H_2SO_4 in ethanol and heating them at 150 °C (Kates, 1972).

DNA isolation, G+C-content determination and DNA–DNA hybridization. The DNA was isolated and purified by the method described by Lind & Ursing (1986). The G+C content (mol%) was determined by the thermal denaturation method (T_m) (Marmur & Doty, 1962) with a Perkin-Elmer Lambda 3B spectrophotometer fitted with a temperature program accessory. DNA–DNA hybridization studies were performed with the non-radioactive method described by Ziemke *et al.* (1998). Reference DNA was double-labelled using DIG-11-dUTP and biotin-16-dUTP (Boehringer Mannheim). The labelling reaction was carried out using the Boehringer Mannheim nick-translation kit.

PCR amplification of the 16S rRNA gene coding sequence, and sequencing. Purified genomic DNA was used for PCR amplification of the 16S rDNA gene. The following two primers were designed to complement the highly conserved regions of the *Halobacterium salinarum* and *Halorubrum* spp. 16S rDNA: forward primer F8 (5′-TTGATCCTG-CCGGAGGCCATTG-3′) and reverse primer R1462 (5′-ATCCAGCCGCAGATTCCCCTAC-3), corresponding to positions 8–30 and 1462–1441, respectively. The oligonucleotides were produced by Pharmacia Biotech and then were diluted to 100 pmol μl^{-1}. The PCR was performed in a thermal cycler (model 480; Perkin-Elmer) for 30 cycles, starting with 1 min of denaturation at 94 °C, followed by 1 min of annealing at 55 °C and 2 min of elongation at 72 °C. The mixture reaction (in a total volume of 100 µl) contained: 2 µl genomic DNA, 10 µl buffer (100 mM Tris/HCl, pH 8·3; 500 mM KCl), 4 µl $MgCl_2$ (25 mM), 1 µl dNTP mixture (dATP, dCTP, dGTP, dTTP at 10 mM; Ultrapure dNTP set from Pharmacia Biotech) and 1 µl *Taq* DNA polymerase (Amplitaq DNA polymerase; Perkin-Elmer). Each primer was used at a concentration of 20 pmol μl^{-1}. The PCR products were analysed by electrophoresis in 1·7% agarose gels in TAE buffer, using λPST1 as the size marker. The products were purified with Microcon-100 concentrators (Amicon).

The following four sequencing primers were used to determine the complete 16S rRNA sequence: r1 (5′-TACC-GTGAGGCGTCCTGTTAA-3′); r2 (5′-TTGTCTCGAC-CATTGTAGCC-3′); r3 (CCCGCCAATTCCTTTAAGT-TTC-3′) and r4 (5′-TGGCACCGGTCTTGCCCAG-3′). These primers were designed to complement regions 1011–1032, 1197–1178, 870–849 and 458–440, respectively. The purified PCR products were sequenced directly using a Strech Applied Biosystems ABI 373 DNA sequencer and the manufacturer's protocols for AmpliTaq^r FS with fluorescent dye-labelled dideoxynucleotides (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer) as described by Sanger *et al.* (1977). The sequences

Table 2. Phenotypic characteristics and lipid composition of the strains ALT6-92^T and TeSe25-84 (isolated from the Atacama Saltern) in comparison with other *Halorubrum* species

Symbols: +, positive result; −, negative result; −?, not detected; +/−, slight mark. All strains/species gave the same results in tests for the hydrolysis of Tween 40 (−) and Tween 80 (−), for the production of acid from arabinose (−) and for the utilization of glucose (−).

Characteristic	ALT6-92^T	TeSe25-84	*H. sacharovorum*	*H. sodomense*	*H. trapanicum*
Growth in total salt concentrations (%, w/v) of:					
10	−	+	−	−	−
15	+	+	−	+	−
Growth at 50 °C	+	+	+*	+*	−
Growth at pH 6	−	−	+	−	−
Catalase	+	+	+*	+*	+*
Oxidase	+	+	+*	+*	+*
Hydrolysis of:					
Tween 20	−	−	−	+	−
Tween 60	−	−	−	+	−
Starch	−	−	−*	+*	−*
Gelatin	−	−	−*	−*	−*
Aesculin	+	−	−	+	−
Nitrate reduction	+	+	−?*	+*	+*
Indole	−	−	−*	−*	−*
Acid from:					
Xylose	−	−	+	+	−
Fructose	−	+	+	+	+
Glucose	−	−	+	+	+
Utilization of:					
Fructose	+	+	−	−	−
Galactose	+	+	−	−	−
Mannose	+	+	+	−	−
Trehalose	+	+	−	−	−
Starch	+	−	+	+	−
Pyruvate	+	+	−	−	−
Acetate	+	+	−	+	−
Polar lipids					
Phosphatidylglycerol (diether analogue)	+	+	+*	+*	+*
Phosphatidylglycerolsulfate (diether analogue)	−?	+/−	+*	+*	+*
Phosphatidylglycerolphosphate (diether analogue)	+	+	+*	+*	+*
Mannosyl-2-sulfate-(1-4)-glycosyl-archaeol	+	+	+*	+*	−*
Mannosyl-2-sulfate-(1-2)-glycosyl-archaeol	−	−	−*	−*	+*
X_1 (unknown glycolipid)	+	+	−	−	−
X_2 (unknown glycolipid)	+	+	−	−	−

* Data were obtained from Tomlinson & Hochstein (1976), Oren (1983), Grant & Larsen (1990) and McGenity & Grant (1995).

were analysed using the ABI PRISM 373 xl Collection 2.0 and Sequencing Analysis 3.3 programs for Macintosh G3.

Phylogenetic analysis on the basis of 16S rRNA sequence. The sequence obtained was compared with previously described 16S rRNA sequences of halophilic archaea deposited in the EMBL database. The sequences were aligned by using CLUSTAL W 1.74 (Thompson *et al.*, 1994), and phylogenetic trees were constructed by using programs in version 3.5.1 of PHYLIP (Felsenstein, 1993). We determined a matrix of evolutionary distances from the sequence alignment data, using the Jukes–Cantor model (Jukes & Cantor, 1969). A phylogenetic tree was constructed from the distance matrix by using the program FITCH, which uses the least-squares algorithm (Fitch & Margoliash, 1967).

RESULTS

Characterization and culture conditions

The specific phenotypic characteristics and lipid compositions of the *Halorubrum* strains studied are shown in Table 2. In addition, all strains were pleomorphic, showing irregular shapes (discs, squares, ovals), and were Gram-negative. They grew optimally in the

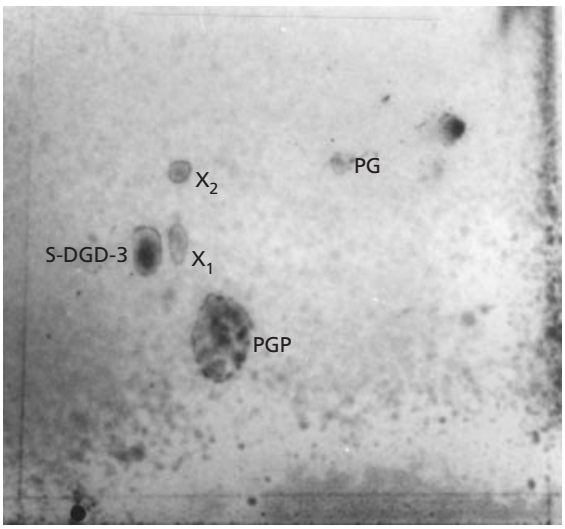

Fig. 1. Polar lipid composition of strain ALT6-92^T separated by two-dimensional TLC (for conditions, see Methods). PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; S-DGD-3, mannosyl-2-sulfate-(1-4)-glycosyl-archaeol; X_1 and X_2, unknown glycolipids. Direction of development: first dimension, vertical; second dimension, horizontal. The origin is in the bottom left-hand corner.

presence of 5·2 M NaCl (30 %, w/v, total salts) at 40 °C in the HE medium. They did not require magnesium for growth. The pH range was alkaline, and all of the strains grew between pH 7·0 and pH 10·0. The cultures were orange-red. Growth was susceptible to novobiocin (30 μg) and bacitracin (10 U) and resistant to penicillin G (10 U), kanamycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg) and streptomycin (10 μg).

The strains (ALT6-92^T and TeSe25-84) were isolated from water and sediment of Lake Tebenquiche, respectively, and proved to have very similar phenotypic properties and lipid compositions (Table 2). Strain ALT6-92^T was studied in further detail and was designated as the type strain of a new species of the genus *Halorubrum*. The cells were mostly irregularly disc-shaped and were catalase- and oxidase-positive. They did not hydrolyse gelatin, starch, Tween (20, 40, 60 or 80) or DNase. Indole was not formed. This strain produced H_2S from cysteine, reduced nitrate and hydrolysed aesculin. Acid was not produced. Growth on galactose, mannose, trehalose, glycerol, acetate and pyruvate as sole carbon and energy sources was observed.

Lipid analyses

We studied the lipid composition of strain ALT6-92^T together with some of the reference strains included in Table 1, to compare results. TLC of extracts of strain ALT6-92^T revealed that this organism contained phosphatidylglycerophosphate, phosphatidylglycerol and three glycolipids (Fig. 1). The major glycolipid was mannosyl-2-sulfate-(1-4)-glycosyl-archaeol (S-DGD-3), a glycolipid characteristic of some species of genus *Halorubrum*. In addition, two unknown diglycosyldiethers, X_1 and X_2, were observed. Phosphatidylglycerosulfate was not detected in this strain (Table 2; Fig. 1), nevertheless, it was present in other strains in the genus *Halorubrum*.

G+C content determination

The G+C contents of the 12 strains in the study and the reference strains were determined. The G+C contents of the DNAs were between 60·5 and 63·2 mol %. The G+C content for strain ALT6-92^T was 63·2 ± 0·5 mol %.

Phylogenetic analysis

A nearly complete 16S rRNA gene sequence was obtained for strain ALT6-92^T. The evolutionary distance between strain ALT6-92^T and others members of the genus *Halorubrum* was low (between 0·0346 and 0·0881), confirming its affiliation with other strains in the genus *Halorubrum*. Strain ALT6-92^T possessed the highest similarity with *Halorubrum sodomense* (96 %). The phylogenetic tree (Fig. 2) constructed for the Jukes–Cantor model and the least-squares algorithm of Fitch and Margoliash confirmed that strain ALT6-92^T was closely related to the *Halorubrum* cluster.

DNA–DNA hybridization

The DNA–DNA hybridization experiments were performed with members of the genus *Halorubrum*. The pooled standard deviation of the different experiments was 1–4 %, indicating good correspondence between duplicates. The values obtained for strain ALT6-92^T with strain TeSe25-84 showed that the isolates formed a homogeneous cluster with a high degree of internal genomic similarity (90–95 %) and should be considered as members of the same species. Closer investigation with respect to other members of the genus *Halorubrum* (Table 1) indicated that strain ALT6-92^T was a member of this genus, but that it was sufficiently different from previously described *Halorubrum* species to warrant the description of a novel species.

DISCUSSION

Previous studies of the Atacama Saltern have found a heterogeneous prokaryotic population that is represented by halotolerant bacteria, moderately halophilic bacteria and extremely halophilic archaea and cyanobacteria (Campos *et al*., 1990). Strain ALT6-92^T was isolated from the water of Lake Tebenquiche.

The polar lipids and glycolipids have proved useful, particularly for the differentiation of the genera of haloarchaea, e.g. *Haloarcula* and *Haloferax* (Torreblanca *et al*., 1986), *Halobaculum* (Oren *et al*., 1995) and *Natrialba* (Kamekura & Dyall-Smith, 1995). Although members of the genus *Halorubrum* have slight differences in their glycolipid content, with few

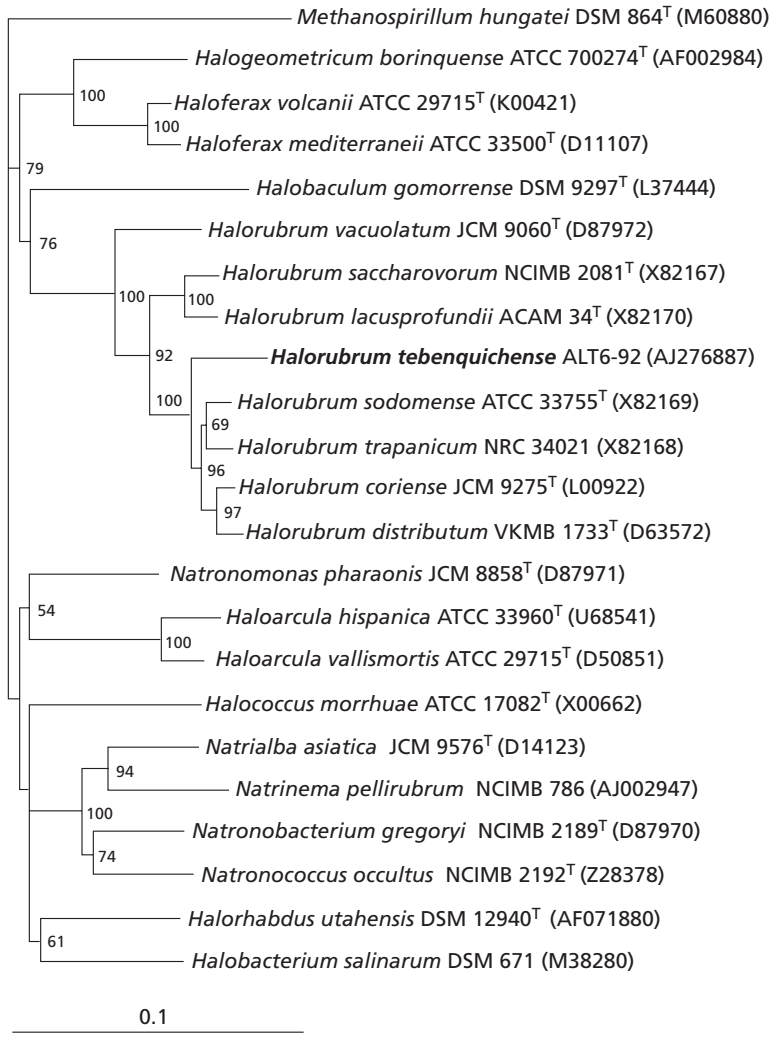

Fig. 2. Phylogenetic tree showing the position of *Halorubrum tebenquichense* ALT6-92^T among the species representatives of genus *Halorubrum* and some genera of extremely halophilic archaea. The sequence data used were obtained from the EMBL database (accession numbers are given in parentheses). The sequence of *Methanospirillum hungatei* DSM 864^T was used as the outgroup. Bootstrap values (greater than 50) are shown at the nodes. Bar, 0·1 substitution per nucleotide position.

Table 3. Differential characteristics of the strain ALT6-92^T and the described species of the genus *Halorubrum*

Data were obtained from Torreblanca *et al*. (1986), Grant & Larsen (1990), Mwatha & Grant (1993), Kamekura & Dyall-Smith (1995) and McGenity & Grant (1995). +, Positive result; −, negative result; ND, not determined.

Characteristic	ALT6–92^T	*H. sodomense*	*H. saccharovorum*	*H. trapanicum*	*H. vacuolatum*	*H. lacusprofundi*	*H. coriensis*
Cell morphology	Irregular discs	Rods	Rods	Pleomorphic rods	Pleomorphic short rods	Pleomorphic rods	Pleomorphic short rods and cup shapes
Cell dimensions (μm)	1·2–0·8 × 1·0–1·5	0·5 × 2·5–5	0·6–1·2 × 2·5	0·7–10 × 1·5–3·0	0·7–10 × 1·5–3·0	> 12	5–0·5
Range for growth in NaCl (M)	2·5–5·2	0·5–4·3	1·5–5·2	ND	2·5–5·1	> 1	2·2–2·7
Starch hydrolysis	−	+	−	−	−	−	ND
Acid from:							
Glucose	−	+	+	+	ND	−	+
Xylose	−	+	+	−	ND	ND	ND
Glycolipids*	S-DGD-3 X_1, X_2	S-DGD-3	S-DGD-3	S-DGD-5	−	S-DGD-3	S-DGD-3
G+C content (mol%)†	63·2 (T_m)	68 (Bd)	71·2 (Bd)	64·3 (Bd)	62·7 (T_m)	65·3–65·8 (Bd) 54·8–56·5	ND

* X_1, X_2, unknown glycolipids; S-DGD-3, mannosyl-2-sulfate-(1-4)-glycosyl-archaeol; S-DGD-5, mannosyl-2-sulfate-(1–2)-glycosyl-archaeol.

† T_m, melting temperature; Bd, buoyant density.

exceptions they all contain a glycosyl-mannosyl core-sulfated diglicosyl diether (McGenity & Grant, 1995). Even though this glycolipid has not been detected in *Halorubrum vacuolatum*,the presence of S-DGD-3 is characteristic of the other members of this genus (Mwatha & Grant, 1993). Strain ALT6-92^T possessed

a polar lipid pattern similar that to found in the genus *Halorubrum* (Trincone *et al.*, 1990). Both phosphatidylglycerophosphate and phosphatidylglycerol were detected, the major glycolipid being S-DGD-3; two unknown glycolipids (X_1 and X_2) were also present. Analysis of 16S rDNA gene sequences further confirms that strain ALT6-92^T is a member of the genus *Halorubrum*, there being $> 95\%$ sequence similarity and evolutionary distances of 0·0346–0·0881 with other species of the genus.

DNA hybridization is acknowledged as the superior method for the elucidation of relationships between closely related taxa, such as strains and species (Stackebrandt & Goebel, 1994). The DNA–DNA relatedness between ALT6-92^T and species of the genus *Halorubrum* was in the range 38–55%. Thus, strain ALT6-92^T appears to represent a novel genospecies within the genus *Halorubrum* (Wayne *et al.*, 1987). Morever, differential phenotypic characteristics distinguish ALT6-92^T from the known species of *Halorubrum* (Table 3). This organism is of particular importance, as it grew in total salt concentrations of 2·5–5·2 M and at pH values in the range 7·0–10·0. Magnesium was not required for growth.

The phenotypic and chemotaxonomic characteristics, together with the molecular and phylogenetic results, correspond with the minimal standards for the description of members of the *Halobacteriales* (Oren *et al.*, 1997) and justify the creation of a new species.

Description of *Halorubrum tebenquichense* sp. nov.

Halorubrum tebenquichense (te.ben.qui.chen′se. N.L. adj. *tebenquichense* of the Tebenquiche, the hypersaline lake of Atacama Saltern).

Cell are irregularly disc-shaped (1·2–0·8 × 1·0–1·5 μm). Gram-negative. The colonies are red-orange in agar plates containing 25% (w/v) total salts, and orange in medium at 15–20% (w/v) total salts. Chemoorganotrophic and aerobic. Grows in the presence of 2·5–5·2 M NaCl at 40 °C. The temperature range is 35–50 °C and the pH range is 7·0–10·0. Magnesium is not required for growth. Catalase- and oxidase-positive. H_2S is produced from cysteine, and nitrate is reduced. Aesculin is hydrolysed. No acid is produced from sugars and polyols. Glucose, fructose, galactose, mannose, trehalose, acetate and pyruvate are used as carbon and energy sources. Susceptible to novobiocin (30 μg) and bacitracin (10 U). The polar lipids are glycerol diether analogues of phosphatidylglycerol and phosphatidylglycerophosphate. Phosphatidylglycerosulfate is not detected. The major glycolipid is S-DGD-3 and two minor glycolipids (X_1 and X_2). Isolated from hypersaline Lake Tebenquiche situated in the Atacama Saltern, Chile. The G+C content of the DNA is 63·2 mol% (T_m). The type strain is ALT6-92^T and has been deposited in the Colección Española de Cultivos Tipo, Valencia, Spain as CECT 5317^T (= DSM 14210^T).

ACKNOWLEDGEMENTS

This study was supported, in part, by grants from the Volkswagen Stiftung (project 1/64.465), Germany, the Junta de Andalucia (project CVI 190), Spain, and DGI-UCV, Chile.

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