Halorubrum tibetense sp. nov., a novel haloalkaliphilic archaeon from Lake Zabuye in Tibet, China

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A novel haloalkaliphilic archaeon, strain 8W8^T, was isolated from Lake Zabuye, on the Tibetan Plateau, China. On the basis of 16S rRNA gene sequence analysis, strain 8W8^T was shown to belong to the genus *Halorubrum* and was related to *Halorubrum vacuolatum* (96.7 % sequence similarity), *Halorubrum saccharovorum* (96.0 %), *Halorubrum lacusprofundi* (95.4 %) and *Halorubrum sodomense* (95.3 %). The phylogenetic distance from any species within the other genera of *Halobacteriales* was lower than 90 %. The major polar lipids of strain 8W8^T were C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol phosphate and phosphatidylglycerol phosphate methyl ester. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain 8W8^T from the eight *Halorubrum* species with validly published names. Therefore, strain 8W8^T represents a novel species, for which the name *Halorubrum tibetense* sp. nov. is proposed, with the type strain 8W8^T (=AS 1.3239^T=JCM 11889^T).

Based on the analysis of 16S rRNA gene sequences, the genus *Halorubrum* was formally proposed by McGenity & Grant (1995) to accommodate several species previously included in the genus *Halobacterium*: *Halorubrum trapanicum* NRC 34021^T (Petter, 1931), *Halorubrum saccharovorum* (Tomlinson & Hochstein, 1976), *Halorubrum sodomense* (Oren, 1983) and *Halorubrum lacusprofundi* (Franzmann *et al.*, 1988). Subsequently, Oren & Ventosa (1996) transferred *Halorubrobacterium coriense* (Kamekura & Dyll-Smith, 1995) and *Halorubrobacterium distributum* (Zvyagintseva & Tarasov, 1987) to the genus *Halorubrum*. *Natronobacterium vacuolatum* (Mwatha & Grant, 1993) was also reassigned into *Halorubrum* as *Halorubrum vacuolatum* by Kamekura *et al.* (1997). More recently, a novel species of the genus *Halorubrum*, *Halorubrum tebenquichense*, has been described (Lizama *et al.*, 2002). The aim of this study was to describe a novel haloalkaliphilic archaeon isolated from the Lake Zabuye in Tibet, China and we propose a novel species, *Halorubrum tibetense* sp. nov.

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

The GenBank accession number for the 16S rRNA gene sequence of strain 8W8^T is AY149598.

Lake Zabuye (31° 20' N 84° 05' E) is located on the Tibetan Plateau 4421 m above sea level. It is an alkaline chloride-sulfate salt lake (pH 9.4, 250 g salt l⁻¹) and is especially rich in lithium ions (0.1 g l⁻¹; Holland *et al.*, 1991). During a broad study of characterization of haloarchaea isolated from Lake Zabuye, strain 8W8^T was isolated using a complex medium containing (g l⁻¹): Casamino acids (Difco), 7.5; yeast extract (Difco), 10.0; trisodium citrate, 3.0; MgSO₄·7H₂O, 1.0; KCl, 10.0; LiCl, 0.1; Fe²⁺ and Mn²⁺, trace; NaCl, 200; Na₂CO₃, 10.0. The method used for enrichment and isolation was described previously (Tindall *et al.*, 1980). Cells of strain 8W8^T were irregular, rod-shaped and non-motile, as determined by phase-contrast and electron microscopy (Fig. 1).

The methods used for genomic DNA preparation, PCR amplification of 16S rRNA gene and the gene sequencing were described previously (McGenity & Grant, 1993). The almost complete 16S rDNA sequence (1470 bp) of strain 8W8^T was determined and compared to the sequences of members of the genus *Halorubrum* and other closely related haloarchaea. The phylogenetic analysis was performed by the neighbour-joining method with the Kimura two-parameter calculation model in TREECONW version 1.3b (Van de Peer & De Wachter, 1994) after multiple alignment

Fig. 1. Electron micrograph showing the morphology of cells of strain 8W8^T. Bar, 1 μm.

of data by CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Positions with any gaps and alignment uncertainty were omitted from the analysis. The 1412 unambiguous nucleotides were used for computing evolutionary distance. The phylogenetic tree (Fig. 2) indicated that strain 8W8^T clustered with the species of the genus *Halorubrum* and constituted a subgroup with one described haloalkaliphilic species (*Halorubrum vacuolatum* JCM 9060^T). Sequence similarity calculations after a neighbour-joining analysis indicated that strain 8W8^T was related to *Halorubrum vacuolatum* (96.7% sequence similarity), *Halorubrum saccharovororum* (96.0%), *Halorubrum lacusprofundi* (95.4%), *Halorubrum sodomense* (95.3%), and other species of *Halorubrum* (93.5–95.0%). Lower sequence similarities (<90.0%) were found with all species of the other genera of *Halobacteriales*. Moreover, the 16S rDNA sequence of strain 8W8^T possessed signature sequences of the genus

Halorubrum: 199–229, 656–672 and 1242–1259 (positions based on the sequence of *Escherichia coli*) and 11 of 14 signature bases specific for *Halorubrum* (Grant *et al.*, 2001).

Results of the chemotaxonomic analyses are given in the species description and Table 1. The polar lipid profile of strain 8W8^T (Fig. 3), analysed by thin-layer chromatography as previously described (Ross *et al.*, 1981), was similar to that of *Halorubrum vacuolatum* (Mwatha & Grant, 1993). Glycolipid and phosphatidylglycerol sulfate (PGS) were not present in these two species, whereas PGS was present in other species of *Halorubrum*. This feature would imply that the genus *Halorubrum* constitutes a heterogeneous group. As shown in Table 1, the genus *Halorubrum* contains non-alkaliphilic, facultatively alkaliphilic and obligately alkaliphilic species and they show different polar lipid compositions. Non-alkaliphilic members contained sulfated diglycosyl diether (S-DGD) and PGS besides phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (PGP-Me); facultatively alkaliphilic members had S-DGD besides PG and PGP-Me and obligately alkaliphilic members contained only PG and PGP-Me.

The results of the physiological characterization are given in the species description. The methods used were in accordance with the recommended minimal standards for description of new taxa in the haloarchaea (Oren *et al.*, 1997). DNA–DNA reassociation values were determined with strain 8W8^T and the type strains of closely related *Halorubrum* species using the spectrophotometric renaturation rate method (Huß *et al.*, 1983). Strain 8W8^T showed relatively low DNA–DNA hybridization percentages to the type strain of *Halorubrum saccharovororum* ATCC 29252^T (22%), *Halorubrum vacuolatum* JCM 9060^T (19%), *Halorubrum sodomense* NCIMB 2197^T (14%) and *Halorubrum lacusprofundi* JCM 8891^T (14%).

Overall, our data show that strain 8W8^T represents a novel organism of the genus *Halorubrum* and we propose to place it in a novel species with the name *Halorubrum tibetense* sp. nov.

Fig. 2. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank databases (accession numbers in parentheses). Bootstrap values based on 100 replications are listed as percentages at the branching points. Scale bar, 0.05 expected changes per site.

Table 1. Phenotypic characteristics of strain 8W8^T and some related *Halorubrum* species

Species: 1, *Halorubrum vacuolatum*; 2, *Halorubrum saccharovorum*; 3, *Halorubrum lacusprofundi*; 4, *Halorubrum sodomense*; 5, *Halorubrum coriense*; 6, *Halorubrum distributum*; 7, *Halorubrum trapanicum* NRC 34021^T; 8, *Halorubrum tebenquichense*. +, Positive; –, negative; ND, not determined.

Characteristic	8W8 ^T	1	2	3	4	5	6	7	8
Motility	–	–	+	+	+	+	+	–	ND
Gas vacuoles	–	+	–	–	–	–	–	–	ND
NaCl range for growth (M)	1.7–5.2	2.5–5.2	1.5–5.2	1.5–5.2	0.5–4.3	2–5.2	1.7–5.2	2.5–5.2	2.5–5.2
Mg ²⁺ requirement	–	–	+	+	+	+	ND	ND	–
Growth at pH 7.0	–	–	+	+	+	+	+	+	+
Growth at pH 10.0	+	+	–	–	–	–	–	–	+
Temp. range for growth (°C)	22–45	20–50	30–56	4–40	20–50	30–56	26–50	ND	35–50
Starch hydrolysis	–	–	–	–	+	ND	–	–	–
Utilization of:									
Glucose	+	+	+	+	+	+	–	+	+
Galactose	–	+	+	+	–	+	–	+	+
Sucrose	+	+	+	ND	+	+	–	+	ND
Lactose	+	ND	+	+	–	+	ND	–	ND
Acid from glucose	–	ND	+	–	+	ND	+	+	–
Presence of PGS	–	–	+	+	+	+	+	+	–
Presence of S-DGD	–	–	+	+	+	+	+	+	+
G + C content (mol%)*	63.3 ^a	62.7 ^a	71.2 ^b	65.8 ^b	67.4 ^b	ND	63.6	64.3 ^b	63.2 ^a

*G + C content was measured by melting temperature (a) or buoyant density (b).

Description of *Halorubrum tibetense* sp. nov.

Halorubrum tibetense (ti.be.ten'se. N.L. neut. adj. *tibetense* from Tibet).

Fig. 3. Polar lipid pattern of strain 8W8^T by two-dimensional TLC. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PL, unidentified phospholipids. The origin is in the bottom left corner.

Cells are irregular rod-shaped (0.5–1 × 1.5–2.5 µm). Gram-negative. Cells lyse in medium with less than 1.5 M NaCl. Colonies are 0.5–1 mm in diameter, red-pigmented, smooth, circular and convex. Haloalkaliphilic. Growth occurs optimally at 3.0–3.4 M NaCl. The pH range for growth is 8.0–10.5, with optimum pH 9.0–9.5. Optimal temperature for growth is 37–40 °C. Magnesium is not required for growth. Chemo-organotrophic and strictly aerobic. Catalase- and oxidase-positive. Gelatin, starch and casein not hydrolysed. Tweens 20, 60 and 80 hydrolysed. Nitrate reduced to nitrite. Indole not produced. Amino acids are utilized. Glucose, lactose, mannose, sucrose, maltose, sorbose, mannitol, starch, succinate and acetate are used as carbon sources. Sensitive to polymycin and novobiocin and insensitive to penicillin, chloramphenicol, streptomycin, tetracycline, ampicillin, rifampicin, erythromycin, bacitracin, neomycin and sulphafurazole. The polar lipids are C₂₀C₂₀ and C₂₀C₂₅ diether derivatives of PG and PGP-Me with five uncharacterized minor phospholipids. The G + C content of the DNA is 63.3 mol% (T_m). The type strain, 8W8^T (= AS 1.3239^T = JCM 11889^T) was isolated from Lake Zabuye (soda lake). The GenBank accession number of the 16S rRNA is AY149598.

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