Halorubrum alkaliphilum sp. nov., a novel haloalkaliphile isolated from a soda lake in Xinjiang, China

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A novel haloalkaliphilic archaeon, strain DZ-1^T, was isolated from a soda lake in Xinjiang, China. The taxonomy of strain DZ-1^T was studied by polyphasic methods. According to 16S rRNA gene sequence similarity, strain DZ-1^T was phylogenetically related to *Halorubrum tibetense* (97.5%), *Halorubrum vacuolatum* (95.7%) and *Halorubrum saccharovorum* (95.9%). Strain DZ-1^T was able to grow at 20–44 °C and was also physiologically different from the above-mentioned species with respect to assimilation of sugars and utilization of organic acids. The DNA G + C content of strain DZ-1^T was 62.1 mol% (T_m). The DNA–DNA relatedness of strain DZ-1^T to *H. tibetense* and *H. vacuolatum* was 22 and 13%, respectively. It was concluded that strain DZ-1^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum alkaliphilum* (type strain, DZ-1^T = AS 1.3528^T = JCM 12358^T) is proposed.

Species of the genus *Halorubrum* (McGenity & Grant, 1995) can be classified into two groups according to their growth pH ranges and origins. Group 1 includes species that cannot grow at pH 10.5, such as *Halorubrum sodomense* (Oren, 1983), *Halorubrum lacusprofundi* (Franzmann *et al.*, 1988), *Halorubrum saccharovorum* (Tomlinson & Hochstein, 1976), *Halorubrum trapanicum* (McGenity & Grant, 1995), *Halorubrum coriense* (Kamekura & Dyll-Smith, 1995), *Halorubrum distributum* (Oren *et al.*, 1997b), *Halorubrum tebenquichense* (Lizama *et al.*, 2002), *Halorubrum terrestre* (Ventosa *et al.*, 2004) and *Halorubrum xinjiangense* (Feng *et al.*, 2004). Group 2 contains the haloalkaliphiles that are able to grow at pH 10.5 and are from soda lakes, currently with only two species, *Halorubrum vacuolatum* (Kamekura *et al.*, 1997) and *Halorubrum tibetense* (Fan *et al.*, 2004). This grouping is supported by phylogenetic analysis based on 16S rRNA gene sequences (Fig. 1). 16S rRNA gene sequence similarities among the recognized members of groups 1 and 2 are less than 96.5%. During our screening for archaerhodopsin-producers, a haloalkaliphilic archaeon, strain DZ-1^T, was obtained. In this report, we present the identification and characterization of this strain.

Strain DZ-1^T was isolated from a sludge sample from a soda

lake in Xinjiang, China by enrichment and subsequently plating the enriched cultures on agar medium containing the following ingredients (l⁻¹): 7.5 g Casamino acid, 10.0 g yeast extract, 3.0 g trisodium citrate, 200 g NaCl, 2.0 g KCl, 1.0 g MgSO₄·7H₂O, 0.2 mg MnSO₄·7H₂O, 0.05 g FeSO₄·7H₂O. The medium was adjusted to pH 9.5 with sterile Na₂CO₃. This strain grew at a temperature range of 20–44 °C (optimum 38 °C), determined using a temperature-gradient incubator, and at a pH range of 8.0–10.5 (optimum 9.0–10.0) as determined with various 25 mM pH buffers (MES pH 5.5–6.5, PIPES pH 6.1–7.5, HEPES pH 6.8–8.2, Tricine pH 7.4–8.8, CHES pH 8.6–10.0 and CAPS pH 9.7–11.1). Routine cultivation was conducted at 38 °C and pH 9.5. The requirements for NaCl and magnesium for growth were determined in medium containing 1.8–5.2 M NaCl or 0–0.1 M MgSO₄. Strain DZ-1^T showed optimal growth at 3.9–4.3 M NaCl. Magnesium was not required.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997a). Cell motility and morphology were examined by phase-contrast and transmission electron microscopy of exponentially growing liquid cultures. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed on optimal growth agar medium after incubation at 38 °C for 4 days. Anaerobic growth was tested in the presence of 5 g nitrate, L-arginine or DMSO l⁻¹ in filled, stoppered tubes.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DZ-1^T is AY510708.

Fig. 1. Phylogenetic tree showing the position of strain DZ-1^T among the species representing the genus *Halorubrum* and other genera of *Halobacteriales* based on their 16S rRNA gene sequences (GenBank/EMBL/DBJ accession numbers are shown in parentheses). The 16S rRNA gene sequence of *Methanospirillum hungatei* DSM 864^T was used as outgroup. The numbers at branch points indicate the level of bootstrap support, based on 1000 resamplings; values > 60 % are shown. Bar, 0.1 expected changes per site.

Tests for catalase and oxidase activities, and hydrolysis of starch, casein, gelatin and Tween 80 were performed as described previously (Gonzalez *et al.*, 1978). Tests for nitrate reduction, H₂S formation, indole formation and the utilization of sugars, alcohols, amino acids and organic acids were carried out as described by Oren *et al.* (1997a). Antibiotic sensitivity tests were performed by spreading bacterial suspensions on culture plates and applying discs impregnated with the tested antibiotics. Phospholipids and glycolipids were separated on silica gel plates (10 cm × 10 cm) by TLC and were analysed according to the method of Xin *et al.* (2001).

The DNA base composition was determined by thermal denaturation (*T_m*) (Marmur & Doty, 1962). The 16S rRNA gene was amplified under conditions described previously (Zhang *et al.*, 2003), except that the following pair of archaeal primers was used: 5'-ATTCCGGTTGATCCTGC-CGG-3' (nt 6–25, according to *Escherichia coli* numbering) and 5'-AGGAGGTGATCCAGCCGAG-3' (nt 1540–1521). The PCR product was sequenced by GeneCore Biotechnologies (Shanghai, China) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (model ABI3700; Applied Biosystems). A BLAST search was performed to identify the nearest taxonomically identified relatives of strain DZ-1^T, the sequences of which were retrieved from GenBank (AY149498, *H. tibetense*; U17364, *H. saccharovororum*). Multiple sequence alignments of the 16S rRNA gene

sequences were performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). A phylogenetic tree (Fig. 1) was constructed using the neighbour-joining method with Kimura's two-parameter calculation model in TREECON W version 1.3b (Van de Peer & De Wachter, 1994). DNA–DNA hybridization of strain DZ-1^T to the type strains of *H. vacuolatum* and *H. tibetense* was performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huss *et al.* (1983).

16S rRNA gene sequence similarity searches indicated that strain DZ-1^T was phylogenetically related to the haloalkaliphilic species of the genus *Halorubrum*, i.e. *H. tibetense* (97.5 %) and *H. vacuolatum* (95.7 %). Strain DZ-1^T contained C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidyl glycerol and phosphatidyl glycerol phosphate methyl ester, but lacked phosphatidyl glycerol sulfate and sulfated glycolipid, similar to *H. tibetense* and *H. vacuolatum*. Phylogenetic tree analysis showed that strain DZ-1^T and *H. tibetense* and *H. vacuolatum* were in the same cluster (Fig. 1). The lipid composition, physiological properties (see the following species description) and characteristics of the DNA molecule indicated that strain DZ-1^T was a member of the genus *Halorubrum* and was phylogenetically close to the haloalkaliphilic species. However, strain DZ-1^T was distinguished from *H. tibetense* and *H. vacuolatum* in phenotypic and physiological properties: strain DZ-1^T was motile, while *H. tibetense* and *H. vacuolatum* are non-motile. Strain DZ-1^T assimilated fructose but not

Table 1. Differentiation of *Halorubrum alkaliphilum* sp. nov. from other *Halorubrum* species

Taxa: 1, *H. alkaliphilum* strain DZ-1^T; 2, *H. tibetense* AS 1.3239^T (data from Fan *et al.*, 2004); 3, *H. vacuolatum* JCM 9060^T (Grant & Larsen, 2001); 4, *H. saccharovororum* ATCC 29252^T (Grant & Larsen, 2001); 5, *H. lacusprofundi* JCM 8891^T (Grant & Larsen, 2001); 6, *H. xinjiangense* AS 1.3527^T (Feng *et al.*, 2004); 7, *H. coriense* JCM 9275^T (Grant & Larsen, 2001); 8, *H. distributum* JCM 9100^T (Grant & Larsen, 2001); 9, *H. sodomense* ATCC 33755^T (Grant & Larsen, 2001); 10, *H. trapanicum* JCM 10477^T (Grant & Larsen, 2001); 11, *H. tebenquichense* ALT6-92^T (Lizama *et al.*, 2002); 12, *H. terrestre* VKM B-1739^T (Ventosa *et al.*, 2004). ND, Not described; PGS, phosphatidyl glycerol sulfate; S-DGD, sulfated mannosyl-glucosyl-glycerol diether.

Properties	1	2	3	4	5	6	7	8	9	10	11	12
Motility	+	—	—	+	+	+	+	+	+	—	ND	+
Gas vacuole	—	—	+	—	—	—	—	—	—	—	ND	—
Growth at 2 M NaCl	+	+	—	+	+	+	—	+	+	—	—	—
Mg ²⁺ required	—	—	—	+	+	—	+	ND	+	ND	—	ND
Growth at pH 10.5	+	+	+	—	—	—	—	—	—	—	—	—
Indole production	+	—	ND	—	—	—	—	—	—	—	—	—
H ₂ S formation	+	—	ND	+	—	+	ND	+	ND	ND	+	—
Sugar assimilation:												
Glucose	+	+	+	+	+	+	+	—	+	+	+	—
Sucrose	—	+	+	+	ND	+	ND	—	+	+	ND	—
Lactose	—	+	ND	+	+	—	+	ND	ND	—	ND	ND
Fructose	+	ND	—	+	ND	+	ND	ND	+	+	+	—
Galactose	—	—	+	+	+	—	+	—	ND	+	+	ND
Maltose	+	+	ND	+	ND	+	ND	ND	+	+	ND	ND
Succinate	—	+	+	ND	ND	—	ND	ND	ND	ND	ND	ND
Acetate	—	+	+	ND	+	—	ND	ND	ND	ND	+	ND
Glycerol assimilation	+	ND	ND	+	—	—	+	+	+	+	+	—
Amino acids required	+	ND	+	+	+	—	—	ND	+	+	ND	—
Presence of PGS	—	—	—	+	+	+	+	+	+	+	—	+
Presence of S-DGD	—	—	—	+	+	+	+	+	+	+	+	+
G + C content (mol%)	62.1	63.3	62.7	71.2	65.3	68.0	ND	63.6	67.4	64.3	63.2	64.4

sucrose, lactose or galactose. Strain DZ-1^T utilized lactate but not acetate or succinate. The characteristics of strain DZ-1^T that differentiate it from other *Halorubrum* species are listed in Table 1. The DNA–DNA relatedness of strain DZ-1^T to *H. vacuolatum* and *H. tibetense* was 13 and 22 %, respectively. The G + C content of strain DZ-1^T was 62.1 mol%. Thus, strain DZ-1^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum alkaliphilum* sp. nov. is proposed.

Description of *Halorubrum alkaliphilum* sp. nov.

Halorubrum alkaliphilum [al.ka.li'phi.lum. N.L. n. *alkali* alkaline (from Arabic *al qalí* ashes of salt wort); Gr. adj. *philos* loving; N.L. neut. adj. *philum* loving, friendly to; N.L. neut. adj. *alkaliphilum* loving alkaline conditions].

Cells are short rods (0.8–1.0 × 1.8–2.0 µm) and Gram-negative. Colonies on agar plates containing 20 % (w/v) total salts are red, elevated and round. Growth is chemo-organotrophic, aerobic and occurs at concentrations of 1.8–5.2 M NaCl, at pH 8.0–10.5 and at 20–44 °C. Optimal NaCl concentration, pH and temperature for growth are 3.9–4.3 M, pH 9.0–10.0 and 38 °C, respectively. Magnesium is not required for growth. Catalase- and oxidase-positive. Anaerobic growth with nitrate, arginine or DMSO does not

occur. Nitrate reduction to nitrite is observed and H₂S is produced from cystein. Indole formation is positive. Tween 80, casein and starch are not hydrolysed. Gelatinase-negative. Amino acids are required for growth. Fructose, glucose, mannose and maltose are utilized with the production of acids. Glycerol is utilized without acid production. No growth or acid production is observed on sucrose, lactose, raffinose, rhamnose, galactose, D-ribose, mannitol or sorbitol. Lactate and glutamic acid are utilized. Resistant to penicillin G, rifampicin, chloramphenicol, neomycin and bacitracin. Cells contain C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidyl glycerol and phosphatidyl glycerol phosphate methyl ester, but lack phosphatidyl glycerol sulfate and sulfated glycolipid. The DNA G + C content is 62.1 mol% (*T*_m). Isolated from a soda lake in Xinjiang, China.

The type strain is DZ-1^T and has been deposited in the China General Microbiological Culture Collection Center (CGMCC) as AS 1.3528^T and in the Japanese Collection of Microorganisms as JCM 12358^T.

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