Halalkalicoccus tibetensis gen. nov., sp. nov., representing a novel genus of haloalkaliphilic archaea

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A haloalkaliphilic archaeon (strain DS12^T) isolated from Lake Zabuye, the Tibetan Plateau, China, was characterized to elucidate its taxonomy. The strain was aerobic, chemo-organotrophic, and grew optimally at 40 °C, pH 9.5–10.0 and 3.4 M NaCl. Cells of strain DS12^T were non-motile cocci and stained Gram-variable. The major polar lipids of strain DS12^T were diphytanyl and phytanyl-sesterterpanyl diether derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. No glycolipids were detected. Phylogenetic analysis revealed that the strain formed a distinct lineage within the family *Halobacteriaceae*. The low 16S rRNA gene sequence similarity values to its closest relatives (91.5–92.5 %) and its signature bases both suggest that the strain has no close affinity with any members of the family *Halobacteriaceae* with validly published names. Therefore, it is proposed that strain DS12^T (= AS 1.3240^T = JCM 11890^T) represents the type strain of a novel species in a new genus, *Halalkalicoccus tibetensis* gen. nov., sp. nov.

The family *Halobacteriaceae* was proposed by Gibbons (1974) to incorporate both rods and cocci that required high concentrations [over 12 % (w/v)] of NaCl for growth and included two genera: *Halobacterium* and *Halococcus*. Recently, the availability of 16S rRNA gene sequence and polar lipid composition data has resulted in the recognition of taxonomic diversity at the genus level and 18 genera have now been described in this family (Grant *et al.*, 2001; Wainø *et al.*, 2000; Oren *et al.*, 2002; Hezayen *et al.*, 2002; Vreeland *et al.*, 2002). Of these, only three genera contain extremely halophilic coccoid bacteria, i.e. *Halococcus*, *Haloterrigena* and *Natronococcus*. The aim of this study was to describe a novel haloalkaliphilic coccus isolated from Lake Zabuye, Tibet, China. It differs from representatives of known genera

of the *Halobacteriaceae* and, thus, a new genus and species, *Halalkalicoccus tibetensis* gen. nov., sp. nov., is proposed to accommodate this strain.

Lake Zabuye (31° 20' N 84° 05' E) is located in the Tibetan Plateau at 4421 m above sea level. It is an alkaline chloride-sulfate salt lake (pH 9.4, 250 g salt l⁻¹). During a broad study of characterization of haloarchaea isolated from Lake Zabuye, strain DS12^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; and Na₂CO₃, 10.0. Methods for enrichment and isolation were as described previously (Tindall *et al.*, 1980). Strain DS12^T exhibited non-motile coccoid morphology in both liquid and solid cultures at various growth stages, as determined by phase-contrast microscopy without fixation and by Gram staining with acetic acid fixation (Fig. 1). Cells did not lyse in water like halococcal archaea.

Results of physiological and chemotaxonomic analyses are given in the species description and Table 1. The methods

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Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DS12^T is AF435112.

Fig. 1. Electron micrograph showing the morphology of cells of strain DS12^T. Bar, 1 µm.

used are in accordance with recommended minimal standard methods for the description of new taxa in the haloarchaea (Oren *et al.*, 1997). Polar lipids were extracted and analysed by the methods of Ross *et al.* (1985) with freeze-dried cells and Oren *et al.* (1995) with wet cells. Strain DS12^T exhibited alkaliphilic and extremely halophilic growth characteristics. One- and two-dimensional TLC of the polar lipid fraction (Fig. 2) revealed that strain DS12^T had diphytanyl moieties (C₂₀,C₂₀) and phytanyl-sesterterpanyl moieties (C₂₀,C₂₅) of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (PGP-Me) as major polar lipid components. Glycolipid and phosphatidylglycerol sulfate

Fig. 2. Polar lipid patterns of strain DS12^T by two-dimensional TLC. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester. The origin is in the bottom left-hand corner.

(PGS) were absent. This feature is in common with other haloalkaliphilic archaea.

Polar lipid compositions are one of the important indicators in haloarchaeal taxonomy. The non-alkaliphilic haloarchaea contain a variety of glycolipids, the distribution of which has been used to delineate various groups (Ross *et al.*, 1985; Torreblanca *et al.*, 1986; Kamekura & Dyall-Smith, 1995). However, the haloalkaliphilic archaea have a comparatively simple polar lipid pattern (phospholipids only, no glycolipids). Chemotaxonomy on the basis of lipid composition alone is difficult for haloalkaliphilic archaea. Even so, the polar lipid patterns could still be of value in distinguishing different taxonomic groups within the haloalkaliphilic archaea, as several minor phospholipids have been detected among the haloalkaliphiles (Morth & Tindall, 1985; Mwatha & Grant, 1993; Kamekura *et al.*, 1997). In our analysis of the polar lipid composition, strain DS12^T showed the simplest pattern of all known haloalkaliphiles, containing only PGP-Me and PG. The minor phospholipids associated with the genus *Natronococcus* (such as PL1 and PL2) were not detected in strain DS12^T (Fig. 2). This result indicates a distinction between members of the genus *Natronococcus* and strain DS12^T.

Genomic DNA was extracted using the method of Pitcher *et al.* (1989) except that lysozyme and Sarkosyl were not used. The 16S rRNA gene was amplified by PCR as described previously (McGenity & Grant, 1993). PCR products were directly sequenced on an ABI 373A DNA sequencer. The almost complete 16S rRNA gene sequence (1474 bp) of strain DS12^T was determined and compared to sequences of members of the family *Halobacteriaceae*. A phylogenetic tree was constructed by the neighbour-joining method with the Kimura two-parameter calculation model in TREECON W version 1.3b (Van de Peer & De Wachter, 1994) after

Table 1. Differential characteristics of strain DS12^T and some related archaeal cocci

Genera/species: 1, DS12^T; 2, *Natronococcus occultus*; 3, *Natronococcus amylolyticus*; 4, *Halococcus*; 5, *Haloterrigena turkmenica*. All strains are non-motile cocci and stained Gram-variable. Data were obtained from Grant *et al.* (2001), Kanai *et al.* (1995) and Morth & Tindall (1985). +, Positive; –, negative; ND, not determined; d, differs among species.

Characteristic	1	2	3	4	5
Optimal NaCl for growth (M)	3.4	3.5–3.6	2.5–3.5	3.5–4.5	3–4
Mg ²⁺ requirement (mM)	–	<10	ND	5–50	5–50
Growth at pH 7.0	–	–	–	+	+
Growth at pH 10.0	+	+	+	–	–
H ₂ S production	–	+	ND	+	–
Presence of glycolipids	–	–	–	+	+
Minor phospholipids	–	PL2, PL1	PL1	d	–
DNA G+C content (mol%)	61.5	61.2	63.5	59.5–67.0	59.8

multiple alignment of data using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Positions with any gaps and alignment uncertainty were omitted from the analysis. A total of 1405 unambiguous nucleotides were used for computing evolutionary distance and constructing a phylogenetic tree. Strain DS12^T had a low degree of similarity to other members of the family *Halobacteriaceae*. The highest similarity existed between DS12^T and *Halococcus dombrovskii* (92.5%). The similarities of strain DS12^T to closely related species were 91.5–92.5% to *Halococcus* species, 91.5% to *Haloterrigena turkmenica* and 89.9–91.7% to *Natronococcus* species. Lower sequence similarities (87.0–91.1%) were found to all species of other genera of the *Halobacteriaceae*. As shown in the phylogenetic tree (Fig. 3), strain DS12^T formed a separate branch within the family *Halobacteriaceae* and was distantly related to other members of the family. Despite being an alkaliphilic halococcal archaeon, strain DS12^T did not cluster with the *Natronococcus* group in the phylogenetic tree. However, it clustered consistently with the *Halococcus* group and branched just before the genus *Halococcus*, which is supported by a 100% bootstrap value. A variety of algorithms were utilized (maximum-parsimony, maximum-likelihood and neighbour-joining in the PHYLIP package), which gave very similar topologies (data not shown). As judged from the low similarity between strain DS12^T and *Natronococcus* species (89.9–91.7%), it is concluded that strain DS12^T is not phylogenetically close to members of the genus *Natronococcus*.

Based on sequence alignments, it has been demonstrated that each genus of *Halobacteriaceae* has specific signature bases (Grant *et al.*, 2001). The genus *Halococcus* has 30T, 553A, 50C, 116C, 218C, 229C, 233A, 557A, 1219C, 1289C and 1314T as its signature bases, whereas the genus

Natronococcus has 1355C, 1356G and 1366C. Numbering is based on the *Escherichia coli* sequence. When aligned with sequences of members of the *Halobacteriaceae*, the strain DS12^T 16S rRNA gene sequence showed none of the signature bases specific for *Halococcus* or *Natronococcus*. This also suggests that strain DS12^T is distinct from representatives of these two genera.

In terms of phenotypic characteristics, strain DS12^T had many properties in common with members of the genus *Natronococcus*. The G + C content of strain DS12^T was in the range of values reported for *Natronococcus* species. The significant phenotypic difference between strain DS12^T and members of the genus *Natronococcus* was the ability to produce H₂S from thiosulfate. Since the phenotypic characteristics of haloalkaliphilic archaea that permit their differentiation are comparatively very limited, the phylogenetic inference of 16S rRNA gene sequence data is particularly important in their classification (Kamekura *et al.*, 1997). Based on low 16S rRNA gene sequence similarity values and different signature bases, strain DS12^T could not be considered to be a member of the genus *Natronococcus*. Phylogenetic analysis revealed that strain DS12^T was most closely related to the genus *Halococcus*, but it could not be classified in the same genus due to the different signature bases, high pH requirement for growth and lack of glycolipids.

Extreme halophily, high DNA G + C content, diether core lipid composition, antibiotic susceptibility and 16S rRNA gene sequence data confirm that strain DS12^T is a member of the *Halobacteriaceae*. The intermediate position of strain DS12^T between *Halococcus* and *Natronococcus*, as suggested by the phenotypic and phylogenetic characteristics, indicates that the strain represents a novel species in a new

Fig. 3. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank databases (accession numbers are given in parentheses). Bootstrap values based on 100 replications are listed at branching points. Bar, 0.05 expected changes per site.

genus. It is therefore proposed that the new genus is named *Halalkalicoccus* (three-letter abbreviation: *Hac.*), with *Halalkalicoccus tibetensis* gen. nov., sp. nov. as the type species.

Description of *Halalkalicoccus* gen. nov.

Halalkalicoccus (Hal.al'ka.li.coc'cus. Gr. n. *hals*, *halos* salt; N.L. n. *alkali* alkali; N.L. masc. n. *coccus* from Gr. masc. n. *kokkos* berry; N.L. masc. n. *Halalkalicoccus coccus* existing in salted and alkaline environment).

Cells are cocci in liquid culture and on plates, occurring singly, in pairs or irregular clusters. Stain mainly Gram-negative with some cells Gram-positive in young cultures. Cells do not lyse in distilled water. Non-motile. Strictly aerobic. Alkaliphilic. Chemo-organotrophic. Possesses $C_{20}C_{20}$ and $C_{20}C_{25}$ diethers. No glycolipids or PGS detected. Isoprenoid quinones are MK-8 and MK-8(H_2). The type species is *Halalkalicoccus tibetensis*.

Description of *Halalkalicoccus tibetensis* sp. nov.

Halalkalicoccus tibetensis (ti.be.ten'sis. N.L. masc. adj. *tibetensis* from Tibet).

Cells are coccus-shaped (1.0–1.5 μ m) and non-motile, occurring singly, in pairs or irregular clusters. Cells do not lyse in distilled water. Stain mainly Gram-negative with some cells Gram-positive in young cultures. Colonies are 1–2 mm in diameter, orange pigmented, smooth, circular and convex. Haloalkaliphilic. Growth occurs in 1.4–5.2 M NaCl (optimum at 3.4 M NaCl), at pH 8.0–10.5 (optimum at pH 9.5–10.0) and at 23–47 °C (optimum at 40 °C). Magnesium is not required for growth. Chemo-organotrophic and strictly aerobic. Catalase- and oxidase-positive. Gelatin, starch, casein and Tweens 20, 40, 60 and 80 are not hydrolysed. Reduces nitrate to nitrite. Indole is not produced. H_2S is not produced from thiosulfate. Glucose, lactose, fructose, maltose, sorbose, mannitol, succinate and acetate are used as carbon sources. Does not use galactose, sucrose, mannose, arabinose, ribose, xylose, rhamnose or raffinose. Acid is not produced from glucose. Sensitive to rifampicin and novobiocin, slightly sensitive to chloramphenicol and insensitive to penicillin, streptomycin, tetracycline, ampicillin, polymyxin, bacitracin, neomycin and sulfafurazole. The polar lipids are $C_{20}C_{20}$ and $C_{20}C_{25}$ diether derivatives of PG and PGP-Me without minor phospholipids. Glycolipids and PGS not detected. MK-8 is the major isoprenoid quinone; MK-8(H_2) is present in smaller amounts.

The type strain is DS12^T (=AS 1.3240^T=JCM 11890^T), which was isolated from Lake Zabuye (soda lake), Tibet, China. The DNA G+C content of strain DS12^T is 61.5 mol% (T_m).

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