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

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A haloalkaliphilic archaeon (strain DS12T) 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 DS12T were non-motile cocci and stained Gram-variable. The major polar lipids of strain DS12T 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 DS12T (AS 1.3240T = JCM 11890T) 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; Waino 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 DS12T 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 DS12T 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
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\textsuperscript{T} exhibited alkaliphilic and extremely halophilic growth characteristics. One- and two-dimensional TLC of the polar lipid fraction (Fig. 2) revealed that strain DS12\textsuperscript{T} had diphytanyl moieties (C\textsubscript{20},C\textsubscript{20}) and phytanyl-sesterterpanyl moieties (C\textsubscript{20},C\textsubscript{25}) of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (PGP-Me) as major polar lipid components. Glycolipid and phosphatidylglycerol sulfate (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\textsuperscript{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\textsuperscript{T} (Fig. 2). This result indicates a distinction between members of the genus Natronococcus and strain DS12\textsuperscript{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\textsuperscript{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\textsuperscript{T} and some related archaeal cocci

Genera/species: 1, DS12\textsuperscript{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.

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>Optimal NaCl for growth (M)</td>
<td>3–4</td>
<td>3–5–3·6</td>
<td>2·5–3·5</td>
<td>3·5–4·5</td>
<td>3–4</td>
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<tr>
<td>Mg\textsuperscript{2+} requirement (mM)</td>
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<td>&lt;10</td>
<td>ND</td>
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<td>5–50</td>
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<td>Growth at pH 7·0</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Growth at pH 10·0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H\textsubscript{2}S production</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<td>Presence of glycolipids</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>Minor phospholipids</td>
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<td>PL1</td>
<td>d</td>
<td>−</td>
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<td>DNA G + C content (mol%)</td>
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<td>61·2</td>
<td>63·5</td>
<td>59·5–67·0</td>
<td>59·8</td>
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</table>
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 DS12T had a low degree of similarity to other members of the family Halobacteriaceae. The highest similarity existed between DS12T and Halococcus dom-browskii (92.5%). The similarities of strain DS12T to closely related species were 91.5% to Haloterrigena turkmenica and 89.9% 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 DS12T 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 DS12T 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 DS12T and Natronococcus species (89.9% to 91.7%), it is concluded that strain DS12T 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 DS12T 16S rRNA gene sequence showed none of the signature bases specific for Halococcus or Natronococcus. This also suggests that strain DS12T is distinct from representatives of these two genera.

In terms of phenotypic characteristics, strain DS12T had many properties in common with members of the genus Natronococcus. The G + C content of strain DS12T was in the range of values reported for Natronococcus species. The significant phenotypic difference between strain DS12T 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 DS12T could not be considered to be a member of the genus Natronococcus. Phylogenetic analysis revealed that strain DS12T 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 DS12T is a member of the Halobacteriaceae. The intermediate position of strain DS12T between Halococcus and Natronococcus, as suggested by the phenotypic and phylogenetic characteristics, indicates that the strain represents a novel species in a new family Halalkalicoccus tibetensis gen. nov., sp. nov.
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’us. 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).


**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 μm) 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 2–5 μm 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. Chemoorganotrophic and strictly aerobic. Catalase- and oxidase-positive. Gelatin, starch, casein and Tween 20, 40, 60 and 80 are not hydrolysed. Reduces nitrate to nitrite. Indole is not produced. H2S 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 chloromycetin and insensitive to penicillin, streptomycin, tetracycline, ampicillin, polymyxin, bacitracin, neomycin and sulfafurazole. The polar lipids are C20C20 and C20C25 diether derivatives of PG and PGP-Me without minor phospholipids. Glycolipids and PGS not detected. MK-8 is the major isoprenoid quinone; MK-8(H2) is present in smaller amounts. The type strain is DS12T (= AS 1.3240T = JCM 11890T), which was isolated from Lake Zhubayue (soda lake), Tibet, China. The DNA G+C content of strain DS12T is 61·5 mol% (Τm).

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**References**


