Halovivax asiaticus gen. nov., sp. nov., a novel extremely halophilic archaeon isolated from Inner Mongolia, China

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Strain EJ-46T, a novel pleomorphic, aerobic, extremely halophilic member of the Archaea was isolated from sediment of the saline Lake Ejinor, in Inner Mongolia, China. This organism was neutrophilic and required at least 15 % (2–5 M) NaCl for growth. MgCl2 was not required. The isolate was able to grow at pH 6–0–9.0. Optimum growth occurred in media containing 20 % (3–4 M) NaCl at pH 7–0–7–5. Polar lipid analysis revealed the presence of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, derived from both C20C20 and C20C25 glycerol diethers. Four glycolipids were detected, one of which may be novel. The DNA G+C content was 60.3 mol%. 16S rRNA gene analysis revealed that strain EJ-46T was a member of the phylogenetic group defined by the family Halobacteriaceae, and the highest 16S rRNA gene similarity values of 94.9 % and 94.8 % were obtained with the haloalkaliphilic species of the genus Natronococcus, Natronococcus occultus and Natronococcus amyloiticus, respectively. Based on the phenotypic, genotypic and phylogenetic analyses, it is proposed that the novel isolate should be classified as representing a new genus and species, for which the name Halovivax asiaticus gen. nov., sp. nov. is proposed. The type strain is EJ-46T (= CGMCC 1.4248T = CECT 7098T).

The extremely halophilic, aerobic members of the Archaea are classified within the family Halobacteriaceae, order Halobacteriales, with 20 currently recognized genera: Halalkaliococcus (Xue et al., 2005), Haloarcula (Torreblanca et al., 1986), Halobacterium (Grant, 2001a), Halobaculum (Oren et al., 1995), Halobiforma (Hezayen et al., 2002), Halococcus (Grant, 2001b), Halofexus (Torreblanca et al., 1986), Halogeometricum (Montalvo-Rodriguez et al., 1998), Halomicrobium (Oren et al., 2002), Halorhabdus (Wai et al., 2000), Halorubrum (McGenity & Grant, 1995), Halosimplex (Vreeland et al., 2002), Haloterrigena (Ventosa et al., 1999), Natrivalba (Kamekura & Dyall-Smith, 1995), Natronena (McGenity et al., 1998), Natronobacterium (Tindall et al., 1984), Natronococcus (Tindall et al., 1984), Natronolimnobius (Itoh et al., 2005), Natronomonas (Kamekura et al., 1997) and Natronorubrum (Xu et al., 1999). The current taxonomic classification of this archaeal group is based largely on 16S rRNA gene sequence comparison and chemotaxonomic criteria, particularly polar lipid composition (Grant et al., 2001).

Members of the Halobacteriaceae are usually pink- to red-pigmented because of the presence of carotenoids (Kamekura & Dyall-Smith, 1995; Hezayen et al., 2001; Grant et al., 2001). They have been isolated from various hypersaline environments, such as saline lakes (e.g. Franzmann et al.,
1988; Oren et al., 1995), soda lakes (e.g. Soliman & Triper, 1982; Tindall et al., 1984), salterns (e.g. Nuttall & Dyall-Smith, 1993; Juez et al., 1986; Ihara et al., 1997), saline soils (e.g. Kobayashi et al., 1992) or salt mines (e.g. Denner et al., 1994; Norton et al., 1993). The haloarchaea are the most halophilic organisms known and are dominant in hypersaline environments in which the salt concentration exceeds 25 % (w/v) (Rodriguez-Valera et al., 1982; Norton et al., 1994). The haloarchaea are the most halophilic organisms known and are dominant in hypersaline environments in which the salt concentration exceeds 25 % (w/v) (Rodriguez-Valera et al., 1981). They often appear at such high density that they impart a typical red colour to the hypersaline brines.

Hypersaline environments are commonly found in China. In addition to many coastal salterns, a number of salt lakes, soda lakes and salt-rich deserts are located in various geographical areas of China, e.g. Xinjiang, Inner Mongolia and Tibet Autonomous Regions. A number of new members of the Halobacteriaceae have been isolated from these saline environments (Xu et al., 1999, 2001; Xin et al., 2000, 2001; Fan et al., 2004; Feng et al., 2004, 2005). In this paper, we report the isolation of strain EJ-46T from Lake Ejinor in Inner Mongolia, China. The complete 16S rRNA gene sequence of strain EJ-46T as well as the polar lipid composition were analysed and the phenotypic characteristics were compared with those of other extremely halophilic archaea to determine the taxonomic position of strain EJ-46T. On the basis of these and other taxonomic data, we found that strain EJ-46T was not identical to any of the present haloarchaeal taxa and was sufficiently different from them to justify its classification as representing a novel species within a new genus.

Strain EJ-46T was isolated from a sediment sample from the saline Lake Ejinor (45° 14’ N 116° 32’ E) in Inner Mongolia, China, by enrichment in liquid medium and subsequent plating of the enriched culture until purity was obtained on the same medium but with agar added. The medium contained (1−1): NaCl, 195 g; MgCl2·6H2O, 32·5 g; MgSO4·7H2O, 50·8 g; CaCl2, 0·8 g; KCl, 5 g; NaHCO3, 0·16 g; NaBr, 0·6 g; and yeast extract 5 g. The pH was adjusted to 7·5 with 1 M NaOH. The water of the lake had a salinity of 338·5 g l−1 and a pH of 7·4. Strain EJ-46T grew at a temperature range of 25–45 °C (optimum 37 °C) and a pH range of 6·0–9·0 (optimum 7·0–7·5). Routine cultivation was conducted at 37 °C and pH 7·5. The requirements for NaCl and MgCl2 for growth were determined in media containing 0·9–5·2 M NaCl or 0·0–0·5 M MgCl2. Strain EJ-46T was capable of growing in a wide range of NaCl concentrations, ranging from 15 (2·5 M) to 25 % (4·3 M). It grew optimally in the presence of 20 % (3·4 M) NaCl, as has been shown for most extremely halophilic archaea. MgCl2 was not required for growth.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order Halobacteriales (Oren et al., 1997). Tests for catalase and oxidase activities and hydrolysis of starch and Tween 80 were performed as described previously (Gonzalez et al., 1978). Nitrate reduction, H2S formation, indole production and the utilization of sugars, alcohols, amino acids and organic acids were assessed as described by Oren et al. (1997). The catalase reaction was positive. Oxidase activity was weakly positive. Voges–Proskauer, methyl red, nitrate reduction, indole production from tryptophan and Simmons citrate tests were negative. Casein, gelatin and Tween 80 were not hydrolysed. The susceptibility to antibiotics was determined on agar medium plates by using antibiotic discs with the following concentrations: ampicillin (10 μg), bacitracin (10 U), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), neomycin (10 μg), novobiocin (30 μg), penicillin G (10 U), rifampicin (30 μg), streptomycin (10 μg) and tetracycline (30 μg). Isolation medium in which yeast extract was added at 0·05 % (w/v), supplemented with 1 % (w/v) of the tested substrate, was used to determine the utilization of various organic substrates as carbon and energy or as carbon, nitrogen and energy sources (Torreblanca et al., 1986). The results of antibiotic susceptibility tests and utilization of various substrates are included in the species description. The formation of acid from various sugars was tested in medium containing 0·05 % (w/v) yeast extract, supplemented with 1 % (w/v) of the sugar tested (sterilized separately). Acid was produced oxidatively from D-xylose but not from D-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, sucrose, trehalose or glycerol.

Cell morphology and motility were examined using a Olympus BX41 microscope equipped with phase-contrast optics. For photography, drops of exponentially growing liquid cultures were mixed on a microscope slide with an equal volume of the agarose containing 20 % (w/v) NaCl, and then covered with a coverslip. Cells were non-motile and pleomorphic, from rods to triangles or squares or disk-shaped (Fig. 1). Colony morphology was observed on agar medium under optimal growth conditions after incubation at 37 °C for 10 days. Colonies of strain EJ-46T formed on agar plates were circular, elevated, entire, small, opaque, glossy and pale-pink pigmented.

Fig. 1. Phase-contrast micrograph of strain EJ-46T grown in liquid medium under optimum conditions. Bar, 15 μm.
Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was done using Merck HPTLC plates silica gel 60 (art. 5641) in the solvent system chloroform/methanol/acetic acid/water (85:22:5:10:4, by vol.). Glycolipids were detected as purple spots by spraying 0·5 % p-naphthol in methanol/water (1:1) and then with sulphuric acid/ethanol (1:1), followed by heating at 160 °C. Two phospholipids, phosphatidylglycerol phosphate methyl ester and phosphatidylglycerol were detected as brown spots after prolonged heating. TLC of the polar lipids suggested that strain EJ-46T contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester derived from C_{20}C_{20} and C_{20}C_{25} glycerol diethers, as shown from the two spots (Xin et al., 2000). Four glycolipids were detected, three of which, with the exception of the fast-moving one, showed the same mobility as those detected in Natrinema pellirubrum JCM 10476T (Xin et al., 2000). The slowest-moving glycolipid possessed an R_f that was smaller than that of sulphated tetracyaglycosyl archaeol, which was identified in Haloarchaeum salinarum (Kamekura, 1993). The glycolipid detected between phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester was not observed in Natrinema pellirubrum (see Supplementary Figs S1 and S2 in IJSEM Online).

Chromosomal DNA of strain EJ-46T was isolated and purified according to methods described by Wilson (1987) and Marmur (1961). The G+C content of genomic DNA was determined from the mid-point (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The DNA G+C content of strain EJ-46T was 60·3 mol%. The 16S rRNA gene of strain EJ-46T was amplified by PCR using three universal primers as described by López-Garcia et al. (2001) and Arahal et al. (1996) and almost-complete nucleotide sequences (approx. 1400 bp) were determined. The ARB software package (Ludwig et al., 2004) was used for the 16S rRNA gene sequence analysis. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated. 16S rRNA gene phylogenetic analysis performed based on the neighbour-joining method (Saitou & Nei, 1987) showed the position of strain EJ-46T (Fig. 2).

The new isolate constituted a separate phylogenetic branch within the Halobacteriaceae. The 16S rRNA gene similarity between EJ-46T and its closest phylogenetic relatives was 94·9 and 94·8 % with Natronococcus occultus and Natronococcus amylolyticus, respectively. Similar topologies were obtained when other treeing methods (maximum-parsimony and maximum-likelihood) were used. These two species of the genus Natronococcus are haloalkaliphilic cocci, with optimum growth at alkaline pH values, in contrast to EJ-46T, which is a neutrophilic rod, growing optimally at pH 7·0–7·5. In addition, alignment of the 16S rRNA gene sequence with all published sequences of haloarchaea clearly showed that strain EJ-46T does not belong to the genus Natronococcus, as it does not share any of the signature bases defined for that genus (Kamekura et al., 2004).

The phenotypic and chemotaxonomic features and the phylogenetic data based on the 16S rRNA gene comparison clearly support the placement of strain EJ-46T in a new genus and species within the haloarchaea, for which we propose the name Halovivax asiaticus gen. nov., sp. nov. The characteristics that differentiate the new genus from other related haloarchaeal genera are shown in Table 1.

**Description of Halovivax gen. nov.**

**Halovivax** (Ha.lo.vi’vax. Gr. n. hals, halos salt; L. adj. vivax long-lived, tenacious of life; N.L. masc. n. Halovivax long-living halophile).

Gram-negative. Cells are extremely pleomorphic, although most are rod-shaped. Colonies are pale-pink pigmented. Strictly aerobic; oxygen is used as the final electron acceptor. Growth occurs at pH 6·0–9·0, 25–45 °C and in 15–25 % (2·5–4·3 M) NaCl. Optimum growth occurs at pH 7·0–7·5, 37 °C and 20 % (3·4 M) NaCl. The DNA G+C content of the only species in the genus is 60·3 mol% (T_m method). Polar lipids include phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two major and one minor glycolipids similar to those of Natrinema pellirubrum and a unidentified glycolipid. Isolated from salt lakes. Phylogenetically affiliated to the Halobacteraeae. The type species of the genus is Halovivax asiaticus.

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**Fig. 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain EJ-46T and some other related haloarchaeal species. GenBank accession numbers are shown in parentheses. Bar, 1 % sequence divergence.
Table 1. Characteristics that distinguish *Halovivax asiaticus* gen. nov., sp. nov. from other related haloarchaeal genera


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>5</th>
<th>6</th>
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<tr>
<td>Morphology</td>
<td>Rod/pleomorphic rod</td>
<td>Rod/pleomorphic rod</td>
<td>Rod/pleomorphic rod</td>
<td>Rod</td>
<td>Coccus</td>
<td>Rod</td>
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<td>Cell size (µm)</td>
<td>0-4–0.5 × 4–5</td>
<td>0.12–1 × 0.5–7</td>
<td>0.2–2 × 0.5–5</td>
<td>0.5–0.7 × 10–15</td>
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<td>0.5–1 × 1–5</td>
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<td>Gas vesicles</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<td>NaCl optimum (M)</td>
<td>3.4</td>
<td>1.7–4.5/ND*</td>
<td>2.5–4.3</td>
<td>3.0</td>
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<td>3.5–4</td>
<td>3.4–4.3</td>
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<td>0.5–4.5</td>
<td>1.7–5.2</td>
<td>2.0–5.2</td>
<td>1.4–5.2</td>
<td>2.0–5.2</td>
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<td>pH optimum</td>
<td>7.0–7.5</td>
<td>Neutral to 9.5</td>
<td>6.5–7.5</td>
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<td>9.0–9.5</td>
<td>6.6–9.5</td>
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<td>Temperature optimum (°C)</td>
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<td>37</td>
<td>35–45</td>
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<td>−</td>
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<td>+/ND*</td>
<td>−</td>
<td>+</td>
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<td>−</td>
<td>+/ND*</td>
<td>−</td>
<td>−/ND*</td>
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<td>Nitrate</td>
<td>−</td>
<td>−</td>
<td>+/ND*</td>
<td>−</td>
<td>−/ND*</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>l-Arginine</td>
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<td>−/ND*</td>
<td>−/ND*</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
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<td>Acid from carbohydrates</td>
<td>V</td>
<td>+/ND*</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>V</td>
<td>ND</td>
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<td>Growth on single carbon sources</td>
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<td>V</td>
<td>+/ND*</td>
<td>−</td>
<td>−/ND*</td>
<td>−/ND*</td>
<td>ND</td>
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<td>Indole from tryptophan</td>
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<td>V</td>
<td>ND</td>
<td>ND</td>
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<td>−</td>
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<td>−</td>
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<td>V</td>
<td>−</td>
<td>V</td>
<td>V</td>
<td>−</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>−/ND*</td>
<td>V</td>
<td>+</td>
<td>V</td>
<td>+/ND*</td>
<td>+</td>
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<tr>
<td>Casein</td>
<td>+</td>
<td>−/ND*</td>
<td>−/ND*</td>
<td>ND</td>
<td>ND</td>
<td>+/ND*</td>
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<tr>
<td>Tween 80</td>
<td>+</td>
<td>−/ND*</td>
<td>V</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>Pigmentation</td>
<td>Pale-pink</td>
<td>Red/purple*</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td>White/red*</td>
<td>Light-red to pale-orange</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>60.3</td>
<td>62.7–72.1</td>
<td>62–65</td>
<td>65.0</td>
<td>63.5–64</td>
<td>60.0–63.1</td>
<td>69.9</td>
</tr>
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</table>

*Some species have different reactions or have not been tested.*
Description of Halovivax asiaticus sp. nov.

Halovivax asiaticus (a.si.a’ti.cus. L. masc. adj. asiaticus pertaining to Asia, where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.4–0.5 × 4.0–5.0 μm (Fig. 1). Colonies are circular and 1–2 mm in diameter after incubation for 10 days at 37 °C. Extremely halophilic and the cells lyse in water. MgCl₂ is not required. Growth does not occur above 50 °C. Amino acids are not required for growth. Catalase-positive and weakly positive for oxidase. Production of indole and methyl red, Voges–Proskauer and Simmons citrate tests are negative. H₂S is produced from cysteine. Acid is produced from xylose. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Produces urease. Anaerobic growth with nitrate or L-arginine does not occur. Starch, ascorbic, phosphatase and DNase are not hydrolysed. Gelatin, Tween 80 not occur above 50 °C.

The type strain, EJ-46T (= CGMCC 1.4248T = CECT 7098T), was isolated from the saline Lake Ejinor in Inner Mongolia, China.

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