Haloterrigena daqingensis sp. nov., an extremely haloalkaliphilic archaeon isolated from a saline–alkaline soil

Shuang Wang,¹ Qian Yang,¹ Zhi-Hua Liu,¹ Lei Sun,² Dan Wei,² Jun-Zheng Zhang,¹ Jin-Zhu Song¹ and Hai-Feng Yuan³

¹Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, PR China
²Soil Fertilizer and Environment Energy Institute of Heilongjiang Academy of Agricultural Sciences, Harbin 150086, PR China
³Nature and Ecology Institute, Heilongjiang Academy of Sciences, Harbin 150040, PR China

A haloalkaliphilic archaeon, strain JX313T, was isolated from a saline–alkaline soil from Daqing, Heilongjiang Province, China. Its morphological, physiological and biochemical features and 16S rRNA gene sequence were determined. Colonies of the strain were orange–red and cells were non-motile cocci and Gram-stain-variable. The strain required at least 1.7 M NaCl for growth, with optimal growth occurring in 2.0–2.5 M NaCl. Growth was observed at 20–50 °C and pH 8.0–10.5, with optimal growth at 35 °C and pH 10.0. The G+C content of its genomic DNA was 59.3 mol%. Phylogenetic analysis of 16S rRNA gene sequences showed that strain JX313T is associated with the genera Haloterrigena and Natrinema and is most closely related to Haloterrigena salina XH-65T (96.2% sequence similarity) and Haloterrigena hispanica FP1T (96.2%). DNA–DNA hybridization experiments revealed that the relatedness of strain JX313T to type strains of related species of the genus Haloterrigena or Natrinema was less than 50%. Furthermore, the cellular polar lipids of strain JX313T, identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and mannose-2,6-disulfate (1→2)-glucose glycerol diether (S₂-DGD), were consistent with the polar lipid characteristics of the genus Haloterrigena. Therefore, phylogenetic analysis, phenotypic assessment and chemotaxonomic data showed that JX313T represents a novel species within the genus Haloterrigena, for which the name Haloterrigena daqingensis sp. nov. is proposed. The type strain is JX313T (=CGMCC 1.8909T =NBRC 105739T).

Abbreviation: S₂-DGD, mannose-2,6-disulfate (1→2)-glucose glycerol diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JX313T is FJ545273.

A phase-contrast photomicrograph, TLC of polar lipids, minimum-evolution and maximum-parsimony phylogenetic trees and soil chemical characteristics are available as supplementary material with the online version of this paper.

Halophilic archaea belonging to the order Halobacteria are found in large numbers in saline and hypersaline environments worldwide (Kamekura & Dyall-Smith, 1995; Oren et al., 1997; Grant, 2004; Fendrihan et al., 2006). On the basis of phylogenetic analysis and DNA–DNA hybridization data, the genus Haloterrigena was established by Ventosa et al. (1999) and the first proposed species was Haloterrigena turkmenica (formerly Halococcus turkmenicus). In recent studies, some novel members of the genus Haloterrigena have been proposed on the basis of conventional physiological, biochemical and chemical characteristics and through phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization results. At the time of writing, the genus Haloterrigena comprises eight species of extremely halophilic archaeon, namely Htg. turkmenica (Ventosa et al., 1999), Htg. thermotolerans (Montalvo-Rodrı́guez et al., 2000), Htg. saccharevitans (Xu et al., 2005b), Htg. longa and Htg. limicola (Cui et al., 2006), Htg. hispanica (Romano et al., 2007), Htg. salina (Gutiérrez et al., 2008) and Htg. jeotgali (Roh et al., 2009). In this study, an aerobic haloalkaliphilic archaean designated strain JX313T was isolated. The characterization and taxonomy of strain JX313T are described, and the results indicate that strain JX313T represents a novel species of the genus Haloterrigena. Strain JX313T was isolated by enrichment culture from a soil sample collected from the surface (0–10 cm) of a soda...
meadow saline soil in Daqing, Heilongjiang Province, China (46°34′ N 125°07′ E) in July 2007. To the best of our knowledge, this is the first incidence of a haloalkaliphilic archaeon being isolated from this region.

To isolate haloalkaliphilic archaea, soil sediment was serially diluted in double-distilled water and then spread on a modified S-G agar medium (Sehgal & Gibbons, 1960) with the following composition (1−1 distilled water): 10 g yeast extract, 7.5 g Casamino acids, 3.0 g trisodium citrate, 2.0 g KCl, 0.2 g MgSO4⋅7H2O, 0.036 g FeSO4⋅7H2O, 200 g NaCl, 10.0 g Na2CO3 and 20.0 g agar. Sodium carbonate was sterilized separately and then added to the medium. The medium was adjusted to pH 9.5 with NaHCO3/Na2CO3 buffer. Plates sealed in plastic bags were incubated at 37 °C. After 7 days of incubation, representative colonies were transferred to the same medium. A pure culture was obtained by repeated streaking. Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order Halobacteriales (Oren et al., 1997). The optimal conditions for growth were determined in liquid S-G medium with 0.85–5.1 M NaCl. The pH range for growth (assayed from pH 5.0 to 11.0 at intervals of 0.5 pH units) was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CHES (pH 9.0–9.5) or CAPS (pH 10.0–11.0) to S-G medium at a concentration of 50 mM. The pH range for growth (assayed from pH 5.0 to 11.0 at intervals of 0.5 pH units) was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CHES (pH 9.0–9.5) or CAPS (pH 10.0–11.0) to S-G medium at a concentration of 50 mM. The temperature range for growth of strain JX313T was determined in S-G medium at optimal NaCl concentration and pH. Growth was monitored as the OD600. Cell morphology and motility were examined by optical and phase-contrast microscopy (BH51; Olympus). Colony morphology was analysed on solid medium by stereomicroscopy (SZ1145TR; Olympus). Gram staining was performed using acetic acid-fixed samples, as described by Dussault (1955).

Anaerobic growth was tested in the presence of nitrate, l-arginine or DMSO (each at 5 g l−1) in filled stoppered tubes. Gelatin hydrolysis was determined as described by Oren et al. (2002). The following characteristics were tested according to Xin et al. (2000) and Oren et al. (1997): hydrolysis of starch, casein and Tween 20, 40, 60 and 80, nitrate reduction, production of indole and H2S, catalase and oxidase activities and utilization of sugars, alcohols, amino acids and organic acids.

Total lipids were extracted by using a modified method of Kamekura & Kates (1988). Phospholipids and glycolipids were separated by TLC on silica gel plates (10 × 10 cm) and analysed according to description of Xin et al. (2000). Genomic DNA was prepared by the method of Marmur (1961) and the purity was checked spectrophotometrically. The DNA G+C content was determined by thermal denaturation (Tm) (Marmur & Doty, 1962) using Escherichia coli K-12 DNA as a calibration standard. The 16S rRNA gene sequence was amplified under conditions described by Feng et al. (2005) and the primer sequences referred to by Xu et al. (2005b). Sequence similarity was analysed by comparing the 16S rRNA gene sequence of strain JX313T with known sequences from the GenBank database using the BLASTN program. Sequence data were aligned with CLUSTAL W software, version 1.8 (Thompson et al., 1994). Phylogenetic trees were reconstructed by using the neighbour-joining, maximum-parsimony and minimum-evolution methods with the MEGA3 program package (Kumar et al., 2004). To evaluate the stability of the phylogenetic trees, a bootstrap analysis (1000 replications) was performed with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE in the PHYLIP software package. DNA–DNA hybridization was performed by using the thermal denaturation and renaturation method of De Ley et al. (1970) as modified by Huß et al. (1983).

A novel aerobic, extremely haloalkaliphilic archaean, strain JX313T, was isolated from soda meadow saline soil. The properties of the isolation habitat (soil) provided evidence that a high total salt concentration was required for growth of this micro-organism (see Supplementary Table S1, available in IJSEM Online). Cells of strain JX313T were coccoid and occurred singly or in pairs or irregular clusters (Supplementary Fig. S1), Gram-stain-variable and non-motile. Colonies on agar medium after incubation at 37 °C for 7 days were light red. Strain JX313T was able to grow at 1.7–5.5 M NaCl, 20–50 °C and pH 8.0–10.5. Detailed results of phenotypic and antibiotic susceptibility tests are included in the species description and Table 1.

Sequence similarity analysis of the 16S rRNA gene showed that strain JX313T was closely related to Haloterrigena salina (96.2 % similarity), Htg. hispanica FPI (96.2 %), Htg. turkenvenica VKM B-1734 (95.7 %), Htg. limicola AX-7 (95.6 %), Htg. longa ABH32 (95.5 %), Htg. thermotolerans PR5 (95.5 %), Htg. jeotgali A29 (95.1 %), Htg. sacchar- evitans AB14 (94.4 %), Natrinema versiforme XF10 (95.7 %), Nnm. gari HIS40 (95.7 %), Nnm. ejorense EJ-57 (95.4 %), Nnm. pallidum NCIMB 777 (95.4 %), Nnm. pelilurubrum NCIMB 786 (95.3 %) and Nnm. altunense AJ2 (95.0 %). The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 1, indicating that strain JX313T clustered with recognized species of the genera Haloterrigena and Natrinema. The phylogenetic position of strain JX313T was also confirmed in trees generated using the minimum-evolution and maximum-parsimony algorithms (Supplementary Fig. S2).

Strain JX313T and the reference strain Htg. thermotolerans CGMCC 1.3709T were cultured at 37 °C for 6 days and polar lipids were extracted. Polar lipid analysis indicated that strain JX313T contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and mannose-2,6-disulfate (1→2)-glucose glycerol diether (S2-DGD) as the main glycolipids (Supplementary Fig. S3). Because of the phylogenetic overlap between Haloterrigena and Natrinema, the taxonomic problems of the two genera deserve in-depth study (Montalvo-Rodríguez et al., 2000; Oren & Ventosa, 2002). Previous studies have shown that there are differences in the polar lipid composition among species of the two genera. Haloterrigena species contain the glycolipids
Table 1. Characteristics that distinguish strain JX313<sup>T</sup> from type strains of species of the genus *Haloterrigena*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Coccoid</td>
<td>Rods</td>
<td>Coccoid</td>
<td>Coccoid</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods/coccoid</td>
<td>Rods</td>
<td>Coccoid</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.8–1.3</td>
<td>0.4×1.0</td>
<td>1.2–1.6</td>
<td>1.5–2.0</td>
<td>0.5–0.6×</td>
<td>0.6–0.8×</td>
<td>3.0–10.0×</td>
<td>4.0–13.0×</td>
<td>1.5–2.0</td>
</tr>
<tr>
<td>NaCl range (M)</td>
<td>1.7–5.5</td>
<td>1.7–5.1</td>
<td>2.5–5.0</td>
<td>2.2–4.0</td>
<td>1.7–5.1</td>
<td>1.7–5.1</td>
<td>&gt;1.7</td>
<td>2.0–4.5</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>Lysis in distilled water</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
<td>35</td>
<td>37–45</td>
<td>37</td>
<td>50</td>
<td>41–45</td>
<td>40–45</td>
<td>42–45</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>Growth at pH 7.0</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at pH 10.0</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S production</td>
<td>+</td>
<td>NR</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Use of sugars as carbon source</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Indole formation</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td>+</td>
<td>NR</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NR</td>
</tr>
<tr>
<td>Tween 40</td>
<td>+</td>
<td>NR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NR</td>
</tr>
<tr>
<td>Tween 60</td>
<td>+</td>
<td>NR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>59.3</td>
<td>62.3</td>
<td>67.0</td>
<td>62.0</td>
<td>62.3</td>
<td>61.9</td>
<td>66.6</td>
<td>63.3</td>
<td>59.8</td>
</tr>
</tbody>
</table>

S<sub>2</sub>-DGD (Cui et al., 2006; Gutiérrez et al., 2008; Montalvo-Rodríguez et al., 2000; Roh et al., 2009; Ventosa et al., 1999; Xu et al., 2005b) or mannose-6-sulfate (1→2)-glucose glycerol diether (S-DGD) (Romano et al., 2007), but most *Natrinema* species have phosphatidylglycerol sulfate and unidentified glycolipids (McGenity et al., 1998; Tapingkae et al., 2007). Other data were taken from Roh et al. (2009), Gutiérrez et al. (2008), Cui et al. (2006), Xu et al. (2005b), Montalvo-Rodríguez et al. (2000) and Ventosa et al. (1999). +, Positive; –, negative; NR, not reported.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain JX313<sup>T</sup> and related taxa. The tree was constructed using MEGA3 (Kumar et al., 2004). Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 70% bootstrap support. Bar, 0.02 expected changes per site.
et al., 2008; Xin et al., 2000; Xu et al., 2005a). Therefore, it is reasonable to conclude that strain JX313<sup>T</sup> should be classified as a member of the genus *Haloterrigena* based on the higher 16S rRNA gene sequence similarities to members of the genus, clustering with members of the genus *Haloterrigena* in phylogenetic trees based on the 16S rRNA gene sequence and the presence of S<sub>2</sub>-DGD.

The DNA G+C content of strain JX313<sup>T</sup> was 59.3 mol%, which lies within the range reported for the genus *Haloterrigena* (59.2–67.0 mol%; Oren et al., 2009). DNA–DNA relatedness of strain JX313<sup>T</sup> with respect to *Htg. salina* JCM 13891<sup>T</sup>, *Htg. hispanica* DSM 18328<sup>T</sup>, *Htg. longa* JCM 13563<sup>T</sup> and *Nnm. eijinorense* CGMCC 1.6202<sup>T</sup> was 41.6, 32.8, 21.2 and 17.2%, respectively.

On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, it is suggested that strain JX313<sup>T</sup> should be classified as the type strain of a novel species of the genus *Haloterrigena* with the name *Haloterrigena daqingensis* sp. nov.

**Description of Haloterrigena daqingensis** sp. nov.

*Haloterrigena daqingensis* (da.qing'en-sis. N.L. fem. adj. *daqingensis* pertaining to Daqing, north-east China, where the type strain was isolated).

Cells are coccoid, 0.8–1.3 μm in diameter. Non-motile, occurring singly or in pairs or irregular clusters. Cells do not lyse in distilled water. Many cells are Gram-negative and a few others stain Gram-positive in young cultures. Colonies are 1–2 mm in diameter after 7 days of incubation on S-G medium at 37 °C, orange-pigmented, smooth, circular and convex. Haloalkaliphilic; growth occurs in 1.7–5.5 M NaCl (optimum at 2.0–2.5 M NaCl), at pH 8.0–10.5 (optimum at pH 10.0) and at 20–50 °C (optimum at 35 °C). Chemo-organotrophic and aerobic. Oxidase-negative and catalase-positive. Anaerobic growth does not occur with nitrate, arginine or DMSO. Nitrate reduction to nitrite is not observed. H<sub>2</sub>S is produced from Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Indole formation is negative. Negative for caseinase, amylase and gelatinase. Tweens 20, 40, 60 and 80 are hydrolysed. The following substrates are utilized as carbon sources: glycerol, acetate, pyruvate, succinate, aspartate and glutamate. Glucose, maltose, fructose, sucrose, trehalose, mannose, D-xyllose, lactose, D-ribose, rhamnose, galactose, cellobiose, arabinose, starch, mannitol, sorbitol, inositol, citrate, lactate, fumarate, arginine, lysine, alanine and glycine are not utilized for growth. Acid is produced from glycerol only. Sensitive to (μg per disc) rifampicin (5), ciprofloxacin (5), erythromycin (15) and novobiocin (30). Resistant to (μg per disc, unless otherwise indicated): streptomycin (10), tetracycline (30), vancomycin (30), kanamycin (30), lincomycin (2), neomycin (30), ampicillin (10), chloramphenicol (30), norfloxacin (10), penicillin G (10 IU) and bacitracin (0.04 IU). The major polar lipids are phosphatidyglycerol, phosphatidylglycerol methyl ester and S<sub>2</sub>-DGD. The DNA G+C content of the type strain is 59.3 mol% (T<sub>m</sub>):

The type strain, JX313<sup>T</sup> (=CGMCC 1.8909<sup>T</sup> =NBRC 105739<sup>T</sup>), was isolated from a saline and alkaline soil of Daqing, Heilongjiang, China.

**Acknowledgements**

This work was supported by grants from the 863 High-tech Project of China (2006AA10Z424) and the Key Project of Science and Technology Development of Harbin (2007A6GCN094). Gratitude is expresed to the Institute of Microbiology, Chinese Academy of Sciences, for technical assistance and to Mr Yuguang Zhou (CGMCC) for his help by providing type strains of the genus *Haloterrigena*. We are also grateful to Professor Susheng Yang (China Agricultural University) for his valuable help.

**References**


Natrinema pellirubrum nom. nov. and Natrinema pallidum nom. nov. 


