Halomicrium zhouii sp. nov., a halophilic archaeon from a marine solar saltern

Xin Yang and Heng-Lin Cui

School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, PR China

A halophilic archaeon, strain TBN51\textsuperscript{T}, was isolated from a marine solar saltern in Jiangsu, China. The colony was red-pigmented and the cells were pleomorphic, motile and Gram-staining-negative. The strain was able to grow at 20–55 °C (optimum 42 °C), in the presence of 1.4–5.1 M NaCl (optimum 2.6 M), with 0–1.0 M MgCl\textsubscript{2} (optimum 0.05 M) and at pH 5.5–9.5 (optimum pH 7.0). Cells lysed in distilled water; the minimal NaCl concentration to prevent such lysis was 8% (w/v). The major polar lipids of strain TBN51\textsuperscript{T} were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfamate and an unidentified glycolipid. The latter lipid and a minor glycolipid also detected in the novel strain were chromatographically identical to sulfated and non-sulfated mannosyl glucosyl diether, respectively. Analysis revealed that strain TBN51\textsuperscript{T} had three dissimilar 16S rRNA genes. Phylogenetic analysis based on the sequences of these genes indicated that the novel strain was most closely related to 

\textit{Halomicrium mukohataei} JCM 9738\textsuperscript{T} (89.2–94.8% sequence similarity) and 

\textit{Halomicrium katesii} DSM 19301\textsuperscript{T} (88.8–94.8%). In similar comparisons of \textit{rpoB} gene sequences, strain TBN51\textsuperscript{T} also appeared most closely related to 

\textit{Hmc. mukohataei} JCM 9738\textsuperscript{T} (88.5% sequence similarity) and 

\textit{Hmc. katesii} DSM 19301\textsuperscript{T} (88.1%). The genomic DNA G+C content of strain TBN51\textsuperscript{T} was 69.1 mol%. The results of DNA–DNA hybridizations indicated that strain TBN51\textsuperscript{T} represented a novel species since it showed relatedness values of only 23% with 

\textit{Hmc. mukohataei} JCM 9738\textsuperscript{B} and 21% with 

\textit{Hmc. katesii} DSM 19301\textsuperscript{T}. It was concluded that strain TBN51\textsuperscript{T} represents a novel species of the genus \textit{Halomicrium}, for which the name \textit{Halomicrium zhouii} sp. nov. is proposed. The type strain is TBN51\textsuperscript{T} (=CGMCC 1.10457\textsuperscript{T}=JCM 17095\textsuperscript{T}).

The genus \textit{Halomicrium} was proposed by Oren \textit{et al.} (2002) to accommodate \textit{Halomicrium mukohataei}, a species transferred from the genus \textit{Haloarcula}, which was first isolated by Ihara \textit{et al.} (1997), from soil collected on salt flats in Argentina. \textit{Halomicrium katesii} was subsequently described by Kharroub \textit{et al.} (2008). Although \textit{Hmc. mukohataei} and \textit{Hmc. katesii} are phylogenetically loosely affiliated with the genus \textit{Haloarcula}, they both have two characteristic glycolipids, mannosyl glucosyl diether (DGD-1) and sulfated mannosyl glucosyl diether (S-DGD-1), that allow them to be differentiated from established species of the genus \textit{Haloarcula} (Yang \textit{et al.}, 2007; Kharroub \textit{et al.}, 2008; Oren \textit{et al.}, 2009). The reported genomic DNA G+C contents of members of the genus \textit{Halomicrium – 52.4 and 52.9 mol%} for two strains of \textit{Hmc. katesii} (Kharroub \textit{et al.}, 2008) and 65 mol% for the type strain of \textit{Hmc. mukohataei} (Ihara \textit{et al.}, 1997) – vary more widely than the values reported for members of the genus \textit{Haloarcula}, which all fall between 60.1 and 65 mol% (Yang \textit{et al.}, 2007). \textit{Halomicr- bium mukohataei} JCM 9738\textsuperscript{B} and each of the established species in the genus \textit{Haloarcula} that have been investigated harbour at least two different 16S rRNA gene copies in their genomes (Cui \textit{et al.}, 2009; Namwong \textit{et al.}, 2011). The different 16S rRNA genes in each \textit{Haloarcula} species are divergent at about 5% of their nucleotide positions while the corresponding genes of \textit{Hmc. mukohataei} JCM 9738\textsuperscript{B} show greater intraspecific divergence (9%) than any other 16S rRNA genes that have yet been investigated (Cui \textit{et al.}, 2009). During our surveys on the halophilic archaeal diversity of marine solar salterns in eastern China, a halophilic archaeon that harboured three divergent 16S rRNA genes and appeared related to the genus \textit{Halomicrium} was obtained. The taxonomic position of this archaeon, strain TBN51\textsuperscript{T}, has now been investigated, using a polyphasic approach.

\textbf{Abbreviations:} DGD-1, mannosyl glucosyl diether; PGP, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulphate; S-DGD-1, sulfated mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the \textit{rrnA}, \textit{rrnB} and \textit{rrnC} 16S rRNA and \textit{rpoB} gene sequences of strain TBN51\textsuperscript{T} are HM063952, HQ215546, HQ215547 and JN120804, respectively.

Two supplementary figures are available with the online version of this paper.
Strain TBN51T was isolated from a sample of sediment collected from the Taibei marine solar saltern (34° 43′ 38″ N, 119° 17′ 48″ E), which is an artificial saltern near the city of Lianyungang, in Jiangsu province, China. At the time the sample was collected, the brine in the saltern had a temperature of 25 °C, a pH of 7.2 and a total salinity of 285 g l⁻¹. The neutral oligotrophic halorarchael medium (NOM) used for the isolation contained the following ingredients (l⁻¹): 0.05 g yeast extract, 0.25 g fish peptone, 1.0 g sodium pyruvate, 5.4 g KCl, 0.3 g K₂HPO₄, 0.25 g CaCl₂, 0.25 g NH₄Cl, 26.8 g MgSO₄·7H₂O, 23.0 g MgCl₂·6H₂O, 184.0 g NaCl (Cui et al., 2010). The pH of this medium was adjusted to 7.0–7.2 with 1 M NaOH and the medium was sometimes solidified with 2.0 % agar. Strain TBN51T was routinely grown aerobically at 37 °C in NOM-3, which was similar to the isolation medium but with the following modifications or additions (l⁻¹): 1.0 g yeast extract, 0.25 g fish peptone, 0.25 g sodium formate, 0.25 g sodium acetate, 0.25 g sodium lactate, 0.25 g sodium pyruvate.

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa in the order Halobacterales (Oren et al., 1997). Halomicrobium mukohataei JCM 9738T, Halomicrobium katesii DSM 19301T, Halosimplex carlsbadense JCM 11122T, Haloferax volcanii DSM 19301T were used as reference strains. Gram staining was performed by following the method outlined by Dussault (1955). Cell morphology and motility in exponentially growing liquid cultures were examined using a light microscope equipped with phase-contrast optics (E400; Nikon). The minimum salt concentration to prevent cell lysis was evaluated by suspending washed cells in serial dilutions of sterile saline containing NaCl at 0–150 g l⁻¹ and then examining the stability of the cells under a light microscope. Growth and gas formation with nitrate as electron acceptor were tested in 9 ml stoppered tubes, each completely filled with liquid NOM medium supplemented with NaNO₃ (5 g l⁻¹) and each containing an inverted Durham tube. The formation of gas from nitrate was detected by the presence of gas bubbles in the Durham tubes and the formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of L-arginine (5 g l⁻¹) or DMSO (5 g l⁻¹) was also tested in 9 ml stoppered tubes that were completely filled with medium. Starch hydrolysis was determined on plates of NOM agar supplemented with soluble starch (2 g l⁻¹) and detected by flooding the plates with Lugol’s iodine solution. Gelatin hydrolysis was investigated by growing colonies on NOM agar plates amended with gelatin (5 g l⁻¹) and flooding the plates with Frazier’s reagent (McDade & Weaver, 1959) after growth was established. Esterase activity was detected as described by Kates (1986). Starch hydrolysis was determined on plates of NOM agar amended with soluble starch (2 g l⁻¹) and each plate containing Frazier’s reagent (McDade & Weaver, 1959) after growth was established. Esterase activity was detected as described by Kates (1986). Growth on single carbon sources, fish peptone and sodium pyruvate were omitted from the NOM medium and the compound to be tested was added at a concentration of 5 g l⁻¹. Antimicrobial susceptibilities were determined on NOM agar plates with antimicrobial discs, as described by Gutiérrez et al. (2008).

Polar lipids were extracted using a chloroform/methanol system and analysed using one- and two-dimensional TLC on aluminium-backed silica-gel plates (silica gel 60 F₂₅₄ Merck), as described previously (Kates, 1986). In the two-dimensional TLC, the first solvent was chloroform/methanol/water (65 : 25 : 4, by vol.) and the second solvent, which was also used for the one-dimensional TLC, was chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). Phosphate stain reagent (Vaskovsky & Kostetsky, 1968) and α-naphthol stain were used for the detection of phospholipids and glycolipids, respectively. A general detection reagent, sulfuric acid/ethanol (1 : 2, by vol.), was also used, to detect total polar lipids. Glycerol diether moieties were detected using the procedure described by Ross et al. (1981). Isoprenoid quinones were extracted, purified and analysed by HPLC according to Collins (1985). The identities of the isoprenoid quinones were confirmed by HPLC-MS analysis.

Genomic DNA was extracted from the novel and reference strains as described by Ng et al. (1995). The 16S rRNA genes were then amplified, cloned and sequenced according to the protocol described by Cui et al. (2009). The PCR-mediated amplification and sequencing of the genes coding for RNA polymerase subunit B′ (rpoB′) were carried out according to Minegishi et al. (2010). Multiple sequence alignments were performed using the CLUSTAL W program integrated in the MEGA5 software package (http://www.megasoftware.net/; Kumar et al., 2008). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms, again using the MEGA5 software. Levels of sequence similarity between the novel strain and related halophilic archaea were calculated using the pairwise-distance computing function of MEGA5. Genomic DNA G+C content was evaluated at 260 nm, in a Beckman-Coultor DU800 spectrophotometer equipped with a high-performance temperature controller, by using the thermal denaturation method (Marmur & Doty, 1962; Owen & Pitcher, 1985) with Hmc. mukohataei JCM 9738T as the reference. The same spectrophotometer was used for the DNA–DNA hybridization analyses, which were performed according to the thermal denaturation and renaturation method of De Ley et al. (1970), as modified by Huß et al. (1983). The DNA–DNA hybridizations were carried out, in triplicate, in 2× SSC at 73 °C.

Cells of strain TBN51T were motile and pleomorphic when grown in NOM-3 liquid medium (Fig. S1, available in IJSEM Online). The cells were Gram-staining-negative and...
the colonies were red-pigmented. Strain TBN51<sup>T</sup> was able to grow at 20–55 °C (optimum 42 °C), in the presence of 1.4–5.1 M NaCl (optimum 2.6 M), with 0–1.0 M MgCl<sub>2</sub> (optimum 0.05 M) and at pH 5.5–9.5 (optimum pH 7.0). Cells lysed in distilled water. The minimal NaCl concentration needed to prevent cell lysis was 8 % (w/v). Strain TBN51<sup>T</sup> reduced nitrate to nitrite but was unable to grow under anaerobic conditions using nitrate, DMSO or L-arginine. It was positive for catalase and oxidase activities but did not form indole. Strain TBN51<sup>T</sup> produced H<sub>2</sub>S from sodium thiosulfate but did not hydrolyse starch, gelatin, casein or Tween 80. The main phenotypic characteristics differentiating strain TBN51<sup>T</sup> from *Hmc. mukohataei* JCM 9738<sup>T</sup> and *Hmc. katesii* DSM 19301<sup>T</sup> are shown in Table 1. More detailed results of the phenotypic tests for strain TBN51<sup>T</sup> are given in the species description.

The major polar lipids of strain TBN51<sup>T</sup> were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and a major glycolipid. The latter lipid and a minor glycolipid that was also detected were chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DG-1), respectively. Chromatographically identical polar lipid profiles were seen with *Hmc. mukohataei* JCM 9738<sup>T</sup> and *Hmc. katesii* DSM 19301<sup>T</sup> (Fig. S2). The polar lipid profile of strain TBN51<sup>T</sup> supported the strain’s inclusion in the genus Halomicrobium. Strain TBN51<sup>T</sup> had C20 : C20 diether core lipids. Unsaturated and dihydrogenated menaquinones with eight isoprene units, MK-8 and MK-8(H<sub>2</sub>), were found in both strain TBN51<sup>T</sup> and *Hmc. mukohataei* JCM 9738<sup>T</sup>. Strain TBN51<sup>T</sup> had three dissimilar 16S rRNA gene sequences: *rrnA*, *rrnB* and *rrnC*. The *rrnA* sequence (GenBank accession no. HM063952) showed 96.9 % and 92.7 % similarities to the *rrnB* (HQ215546) and *rrnC* (HQ215547) sequences, respectively, while the level of sequence similarity between *rrnB* and *rrnC* was 95.7 %. *Halomicrobium katesii* DSM 19301<sup>T</sup> also had three dissimilar 16S rRNA gene sequences: *rrn1*, *rrn2* and *rrn3*. The *rrn1* sequence (JN120801) showed 90.2 % similarity with *rrn2* (JN120802) and 90.5 % similarity with *rrn3* (JN120803), while the level of sequence similarity between *rrn2* and *rrn3* was 99.6 %. The 9.8 % divergence between two of the 16S rRNA gene sequences in *Hmc. katesii* DSM 19301<sup>T</sup> exceeded the highest corresponding divergence (9.0 %) seen in *Hmc. mukohataei* JCM 9738<sup>T</sup>. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain TBN51<sup>T</sup> was most closely related to *Hmc. mukohataei* JCM 9738<sup>T</sup> (89.2–94.8 % sequence similarities) and *Hmc. katesii* DSM 19301<sup>T</sup> (88.8–94.8 %). In a neighbour-joining tree, the three 16S rRNA gene sequences of strain TBN51<sup>T</sup> formed a tight clade that was loosely clustered with *Hmc. mukohataei* JCM 9738<sup>T</sup> and *Hmc. katesii* DSM 19301<sup>T</sup> (Fig. 1). The phylogenetic position of strain TBN51<sup>T</sup> in the trees generated using maximum-parsimony or maximum-likelihood algorithms was similar (data not shown). In the phylogenetic analysis based on the 16S rRNA gene sequences, the genera *Halomicrobium* and *Haloarcula* did not form two separate monophyletic groups, indicating that at least one complementary molecular marker should be included in the phylogeny of these two genera.

The *rpoB* gene sequences amplified from the DNA of strains TBN51<sup>T</sup> and *Hmc. katesii* DSM 19301<sup>T</sup> were both

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>Red Pleomorphic</td>
<td>Red-orange Pleomorphic</td>
<td>Red Rods</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NaCl range (M)</td>
<td>1.4–5.1</td>
<td>2.5–4.3</td>
<td>3.4–5.2</td>
</tr>
<tr>
<td>Anaerobic growth with nitrate</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of nitrate to nitrite</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gas formation from nitrate</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose*</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose*</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetate*</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamate†</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Presence of phosphatidylglycerol sulfate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>69.1</td>
<td>65</td>
<td>52.4</td>
</tr>
</tbody>
</table>

*Substrate utilized as single carbon and energy source for growth.
†Substrate utilized as single carbon, nitrogen or energy source for growth.
1827 bp. In terms of the rpoB gene sequences, strain TBN51T again appeared most closely related to Hmc. mukohataei JCM 9738T (88.5% sequence similarity) and Hmc. katesii DSM 19301T (88.1%), and Hmc. mukohataei JCM 9738T appeared closely related to Hmc. katesii DSM 19301T (98.3%). In all of the phylogenetic trees constructed on the basis of rpoB gene sequences, strain TBN51T was loosely clustered with Hmc. mukohataei JCM 9738T and Hmc. katesii DSM 19301T and these three taxa formed a monophyletic group that was separate from the genus Haloarcula.

Together, the results of the phylogenetic analyses based on 16S rRNA and rpoB gene sequences support the placement of strain TBN51T in the genus Halomicrobium. At 69.1 mol%, the genomic DNA G+C content of strain TBN51T was higher than the 65 mol% reported for Hmc. mukohataei (Oren et al., 2002) and the 52.4–52.9 mol% reported for Hmc. katesii (Kharroub et al., 2008). The DNA–DNA relatedness values between strain TBN51T and Hmc. mukohataei JCM 9738T (23%) or Hmc. katesii DSM 19301T (21%) indicated that the new strain represented a novel species (Stackebrandt & Goebel, 1994).

Based on the phenotypic, chemotaxonomic and phylogenetic data, strain TBN51T represents a novel species of the genus Halomicrobium, for which the name Halomicrobium zhouii sp. nov. is proposed.

Description of Halomicrobium zhouii sp. nov.

Halomicrobium zhouii (zhou’i.i. N.L. gen. masc. n. zhouii of Zhou, named in honour of Professor Pei-Jin Zhou, for his contributions to the study of halophilic archaea).

Cells are Gram-staining-negative, motile and pleomorphic (rods and irregular shapes) under optimal growth conditions. Colonies on agar plates containing 3.6 M NaCl are red, elevated and round. Growth occurs at 20–55 °C (optimum 42 °C), in the presence of 1.4–5.1 M NaCl (optimum 2.6 M), with 0–1.0 M MgCl2 (optimum 0.05 M) and at pH 5.5–9.5 (optimum pH 7.0). Cells lyse in distilled water. The minimum NaCl concentration needed to prevent cell lysis is 8% (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Reduces nitrate to nitrite and produces H2S from sodium thiosulfate. Does not form indole or hydrolyse...
starch, gelatin, Tween 80 or casein. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, lactose, glycercol, acetate, pyruvate and D-lactate. L-Glutamate is utilized as a single carbon, nitrogen or energy source for growth. No growth occurs on the following substrates when offered as single carbon and energy sources: D-fructose, L-sorbose, D-ribose, D-xylene, maltose, starch, D-mannitol, D-sorbitol, succinate, L-malate, fumarate and citrate. The following substrates are not utilized as single carbon, nitrogen or energy sources for growth: glycine, L-alanine, L-arginine, L-aspartate, L-lysine and L-ornithine. Acid is produced from D-glucose, D-mannose, D-galactose, sucrose and lactose. Sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), rifampicin (5), mycostatin (100) and nitrofurantoin (300). Resistant to the following antimicrobial compounds: trimethoprim (5), erythromycin (15), ampicillin (10), penicillin G (10 IU per disc), chloramphenicol (30), neomycin (30), ciprofloxacin (5), streptomycin (10), kanamycin (30), vancomycin (30), norfloxacin (10), tetracycline (30), gentamicin (10) and nalidixic acid (30). Major polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulphate and a glycolipid that is chromatographically identical to sulfated mannosyl glucosyl diether. Mannosyl glucosyl diether is present as a minor glycolipid.

The type strain is TBN51T (=CGMCC 1.10457 =JCM 17095T), which was isolated from Taipei marine solar saltern in Jiangsu province, China. The genomic DNA G+C content of strain TBN51T is 69.1 mol%.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant 30970006), the Chinese Academy of Sciences’ Institute of Microbiology, via a grant (SKLMR-20100604) awarded to the State Key Laboratory of Microbial Resources, and an MEL Young Scientist Visiting Fellowship (MELRS0931) in the State Key Laboratory of Marine Environmental Science, Xiamen University.

References


