**Natronoarchaeum rubrum** sp. nov., isolated from a marine solar saltern, and emended description of the genus *Natronoarchaeum*

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A halophilic archaeal strain, GX48\(^T\), was isolated from the Gangxi marine solar saltern near Weihai city in Shandong Province, China. Cells of the strain were rod-shaped, stained Gram-negative and formed red-pigmented colonies. Strain GX48\(^T\) was able to grow at 25–50 °C (optimum 37 °C), in the presence of 1.4–4.8 M NaCl (optimum 2.6 M NaCl), with 0–1.0 M MgCl\(_2\) (optimum 0.05 M MgCl\(_2\)) and at pH 5.5–9.5 (optimum pH 7.0). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). The major polar lipids of the strain were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two major glycolipids chromatographically identical to those of *Natronoarchaeum mannanilyticum* YSM-123\(^T\) and *Natronoarchaeum philippinense* 294-194-5\(^T\). 16S rRNA gene analysis revealed that strain GX48\(^T\) had two dissimilar 16S rRNA genes and both of them were phylogenetically related to those of the two current members of the genus *Natronoarchaeum* (96.2–98.3 % similarities). The *rpoB* gene sequence similarities between strain GX48\(^T\) and *Natronoarchaeum mannanilyticum* YSM-123\(^T\) and *Natronoarchaeum philippinense* 294-194-5\(^T\) were 96.0 % and 94.7 %, respectively. The DNA G+C content of strain GX48\(^T\) was 66.2 mol%. Strain GX48\(^T\) showed low DNA–DNA relatedness with the two members of the genus *Natronoarchaeum*. It was concluded that strain GX48\(^T\) (=CGMCC 1.10388\(^T\)=JCM 17119\(^T\)) represents a novel species of the genus *Natronoarchaeum*, for which the name *Natronoarchaeum rubrum* sp. nov. is proposed. An emended description of the genus *Natronoarchaeum* is also presented.

Members of the halophilic archaea belonging to the family *Halobacteriaceae*, have been mainly isolated from salt lakes, salt mines, marine solar salters, saline–alkaline soil, salty fermented food and salted hides (Han & Cui, 2014; Liu et al., 2013; Qiu et al., 2013; Oren, 2012; Wang et al., 2010; Zhang et al., 2013). Some of them can also be found in commercial salt (Echigo et al., 2013; Minegishi et al., 2010a, 2011; Shimane et al., 2010, 2011, 2013; Yamauchi et al., 2013a, b). The genus *Natronoarchaeum* was first proposed by Shimane et al. (2010) to accommodate the species *Natronoarchaeum mannanilyticum*, which is a mesophilic and slightly alkaliophilic haloarchaeon that was isolated from commercial salt made by the heating process in Niigata, Japan. Recently, the species *Natronoarchaeum philippinense* was described based on a strain isolated from commercial solar salt imported from the Philippines (Shimane et al., 2013). Members of the genus *Natronoarchaeum* contain phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), disulfated mannosyl glucosyl diether (S2-DGD) and an unknown glycolipid. During our surveys on the halophilic archaeal diversity of marine solar salters of Eastern China, a halophilic archaeal isolate related to the two current members of the genus *Natronoarchaeum* was obtained. In this study, we characterize strain GX48\(^T\) as a novel species of the genus *Natronoarchaeum*.

Strain GX48\(^T\) was isolated from the brine sampling from Gangxi marine solar saltern near Weihai city in Shandong Province, China (37° 22′ 57″ N 122° 27′ 49″ E) and stored at 4 °C during transport to the laboratory in 2009. The pH of the brine was 7.1 and the salinity 283 g l\(^{-1}\). The neutral oligotrophic haloarchaeal medium (NOM) was used for the isolation procedure, and contained the following ingredients (g l\(^{-1}\)): yeast extract (Oxoid) 0.05, fish peptone (Sinopharm Chemical Reagent) 0.25, sodium pyruvate 1.0, KCl 5.4, K2HPO4 0.3, CaCl2 0.25, NH4Cl 0.25, MgSO4 . 7H2O 26.8, MgCl2 .6H2O 23.0, NaCl 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The medium was solidified with 2.0 % agar. Strains were routinely grown aerobically at 37 °C in NOM medium.
Determination of morphology and growth characteristics, nutrition, miscellaneous biochemical tests and sensitivity to antimicrobial agents were performed for all strains in the same basic medium, NOM, according to the proposed minimal standards for description of new taxa in the order Halobacteriales (Oren et al., 1997). The NaCl range for growth was determined by incubating the strain in the presence of 0.9, 1.4, 1.7, 2.1, 2.6, 3.1, 3.4, 3.9, 4.3, 4.8 and 5.1 M NaCl. The pH range for growth was determined at pH 5.0–10.0 (in intervals of 0.5 pH units) using the following buffers: MES (for pH 5.5–6.7), PIPES (pH 6.1–7.5), MOPS (pH 6.5–7.9), HEPES (pH 6.8–8.2), Tricine (pH 7.4–8.8) and CHES (pH 8.6–10.0) at a concentration of 25 mM. The temperature range for growth was determined by incubating the strain at 10, 15, 20, 25, 30, 37, 40, 42, 45, 50, 55 and 60 °C. The type strains Natronoarchaeum mannanilyticum YSM-123T and Natronoarchaeum philippinense 294-194-5T were selected as reference strains in phenotypic tests. These reference strains were routinely grown aerobically at 37 °C in NOM medium.

Polar lipids were extracted using a chloroform/methanol system and analysed using one- and two-dimensional TLC, as described previously (Cui et al., 2010). Merck silica gel 60 F254 aluminium-backed thin-layer plates were used for TLC analyses. In two-dimensional TLC, the first solvent was chloroform/methanol/water (65 : 25 : 4, by vol.) and the second solvent was chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). The latter solvent mixture was also used in one-dimensional TLC. Two specific detection spray reagents, phosphate stain reagent for phospholipids and α-naphthol stain for glycolipids, were used. The general detection reagent, sulfuric acid/ethanol (1 : 2, v/v), was also used to detect total polar lipids. The presence of phospholipids and glycolipids on the two-dimensional TLC was confirmed by comparing with the one-dimensional TLC on which the polar lipid profiles of reference strains were developed.

Genomic DNA from halophilic archaeal strains was prepared as described previously (Cui et al., 2011). The 16S rRNA genes were amplified, cloned and sequenced according to the previous protocol (Cui et al., 2009). PCR-mediated amplification and sequencing of the rpoB genes were performed as described previously (Minegishi et al., 2010b). Multiple sequence alignments were performed using the CLUSTAL W program integrated in the MEGA 5 software package (http://www.megasoftware.net/). Phylogenetic trees were reconstructed using neighbour-joining, maximum-parsimony and maximum-likelihood algorithms in the MEGA 5 software (Tamura et al., 2011). Gene sequence similarity among halophilic archaea was calculated using the Pairwise-Distance computing function of MEGA 5. The DNA G+C content was determined from the mid-point value (Tm) of the thermal denaturation method (Marmur & Doty, 1962) at 260 nm with a Beckman-Coulter DU800 spectrophotometer equipped with a high-performance temperature controller. DNA–DNA hybridizations were carried out according to the thermal denaturation and renaturation method (De Ley et al., 1970; Huß et al., 1983).

Cells of strain GX48T were motile and rod-shaped when grown in NOM liquid medium (Fig. S1, available in the online Supplementary Material). Cells were Gram-stain-negative and colonies were red-pigmented. Strain GX48T was able to grow at 25–50 °C (optimum 37 °C), in the presence of 1.4–4.8 M NaCl (optimum 2.6 M NaCl), with 0–1.0 M MgCl2 (optimum 0.05 M MgCl2) and at pH 5.5–9.5 (optimum pH 6.5–7.0). The cells of strain GX48T lysed in distilled water and the minimum NaCl concentration that prevented cell lysis was 8% (w/v). Strain GX48T produced H2S from sodium thiosulfate and hydrolysed starch, but did not produce indole from tryptophan and did not hydrolyse gelatin, casein or Tween 80. Strain GX48T was sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), rifampicin (5) and nitrofurantoin (300); and resistant to mycostatin (100), trimethoprim (5), erythromycin (15), penicillin G (10 IU per disc), ampicillin (10), chloramphenicol (30), neomycin (30), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The remarkable phenotypic characteristics differentiating strain GX48T from N. mannanilyticum YSM-123T and N. philippinense 294-194-5T were: optimum NaCl, optimum pH, utilization of specific substrates for growth, indole formation and H2S formation (Table 1). More detailed results of phenotypic features of strain GX48T are given in the species description.

Table 1. Characteristics that distinguish strain GX48T from N. mannanilyticum YSM-123T and N. philippinense 294-194-5T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Optimum NaCl (M)</td>
<td>2.6</td>
<td>3.4–4.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.5–7.0</td>
<td>8.5–9.0</td>
<td>8.0</td>
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<tr>
<td>Utilization of:</td>
<td></td>
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<tr>
<td>D-Galactose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maltoose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glycero</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DL-Lactate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Indole formation</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66.2</td>
<td>63.0</td>
<td>63.0</td>
</tr>
</tbody>
</table>

The major polar lipids of strain GX48\textsuperscript{T} were PG, PGP-Me and two major glycolipids (GL1, GL2), in a pattern chromatographically identical to the polar lipid profiles of *N. mannanylyticum* YSM-123\textsuperscript{T} and *N. philippinense* 294-194-5\textsuperscript{T} (Fig. S2). One of the glycolipids (GL1) was chromatographically identical to S\textsubscript{2}-DGD and the other glycolipid (GL2) was unidentified. The major polar lipid composition supported classification of strain GX48\textsuperscript{T} in the genus *Natronoarchaeum*.

Strain GX48\textsuperscript{T} had two dissimilar 16S rRNA gene sequences, *rrnA* and *rrnB*. The *rrnA* sequence (1471 nt, GenBank accession no. JF421970) showed 97.8% similarity to the *rrnB* sequence (1471 nt, GU951432). Strain GX48\textsuperscript{T} was closely related to *N. mannanylyticum* YSM-123\textsuperscript{T} (96.6–98.3%) and *N. philippinense* 294-194-5\textsuperscript{T} (96.2–96.6%). Phylogenetic tree reconstruction using the neighbour-joining algorithm revealed that strain GX48\textsuperscript{T} tightly clustered with the current two members of the genus.*

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**Fig. 1.** Neighbour-joining phylogenetic tree reconstructions based on 16S rRNA gene (a) and *rpoB* (b) sequences, showing the relationships between strain GX48\textsuperscript{T} and related members of the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with >50% bootstrap support. Bars, 0.02 (a) and 0.05 (b) changes per site.
**Natronoarchaeum** (Fig. 1a). This phylogenetic position was also confirmed in other trees generated using the maximum-parsimony and maximum-likelihood algorithms (data not shown).

The rpoB gene of strain GX48<sup>T</sup> was sequenced and found to be 1833 bp in length. It was very similar to the corresponding gene of *N. mannanilyticum* YSM-123<sup>T</sup> (96.0 %) and *N. philippinense* 294-194-5<sup>T</sup> (94.7 %). In the reconstructed phylogenetic tree based on rpoB gene sequences, strain GX48<sup>T</sup> tightly clustered with members of the genus *Natronoarchaeum* and formed a monophyletic group separated from other genera of the family *Halobacteriaceae* (Fig. 1b). This phylogenetic position was also confirmed in the trees generated using the maximum-parsimony and maximum-likelihood algorithms (data not shown).

The 16S rRNA gene-based and rpoB gene-based phylogenetic analysis results supported the placement of strain GX48<sup>T</sup> in the genus *Natronoarchaeum*.

The DNA G+C content of strain GX48<sup>T</sup> was 66.2 mol%. This value was higher than those of *N. mannanilyticum* YSM-123<sup>T</sup> (63.0 %) and *N. philippinense* 294-194-5<sup>T</sup> (63.0 %). The DNA–DNA hybridization values among strain GX48<sup>T</sup> and *Natronoarchaeum mannanilyticum* YSM-123<sup>T</sup> and *Natronoarchaeum philippinense* 294-194-5<sup>T</sup> were 49 % and 45 %, respectively, much lower than the accepted threshold value (70 %) to separate two species (Stackebrandt & Goebel, 1994).

The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain GX48<sup>T</sup> represents a novel species of the genus *Natronoarchaeum*, for which the name *Natronoarchaeum rubrum* sp. nov. is proposed.

**Emended description of the genus *Natronoarchaeum* Shimane et al. 2010**

Cells are rods or pleomorphic under optimal growth conditions and stain Gram-negative. Cells lyse in distilled water. Anaerobic heterotrophs and colonies on agar are small and are red-pigmented. Chemo-organotrophic, aerobic, halophilic and slightly alkaliphilic. The major polar lipids are PG, PGP-Me and two major glycolipids, one of them chromatographically identical to S<sub>2</sub>-DGD, the remaining glycolipid is unidentified. The genomic DNA G+C contents are between 63 mol% and 66.2 mol%. The type species is *Natronoarchaeum mannanilyticum*.

**Description of *Natronoarchaeum rubrum* sp. nov.**

*Natronoarchaeum rubrum* (ru’brum. L. neut. adj. rubrum red).

Cells are motile, rod-shaped (0.4–0.5 μm × 1.0–5.0 μm) under optimal growth conditions and Gram-stain-negative. Colonies on agar plates containing 2.6 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 25–50 °C (optimum 37 °C), in the presence of 1.4–4.8 M NaCl (optimum 2.6 M NaCl), with 0–1.0 M MgCl<sub>2</sub> (optimum 0.05 M MgCl<sub>2</sub>) and at pH 5.5–9.5 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 8 % (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite and gas formation from nitrate are not observed. H<sub>2</sub>S is produced from sodium thiosulfate. Indole formation is negative. Hydrolyses starch but not gelatin, casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, sucrose, lactose, starch, glycerol, acetate, pyruvate and DL-lactate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-glutamate and L-ornithine. No growth occurs on D-galactose, D-fructose, L-sorbose, D-ribose, D-xylene, maltose, D-mannitol, D-sorbitol, succinate, L-malate, fumarate, citrate, glycine, L-alanine, L-arginine, L-aspartate or L-lysine. The major polar lipids are PG, PGP-Me, S<sub>2</sub>-DGD and an unknown glycolipid.

The type strain, GX48<sup>T</sup> (=CGMCC 1.10388<sup>T</sup> = JCM 17119<sup>T</sup>), was isolated from the Gangxi marine solar saltern near Weihai city in Shandong Province, China. The DNA G+C content of the type strain is 66.2 mol% (T<sub>m</sub> method).

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**References**


