**Natrinema soli** sp. nov., a novel halophilic archaeon isolated from a hypersaline wetland

Mehroon Rasooli,1 Ali Naghoni,1 Mohammad Ali Amoozegar,2,4 Leila Mirfeizi,1 Mahdi Moshtaghi Nikou,1 Seyed Abolhassan Shahzadeh Fazeli,1,3 Hiroaki Minegishi4 and Antonio Ventosa5

**Abstract**

An extremely halophilic archaeon, designated strain 5-3T, was isolated from a soil sample of Meighan wetland in Iran. Strain 5-3T was strictly aerobic, catalase-positive and oxidase-negative. Cells were Gram-stain-negative, non-motile and ovoid. Colonies of strain 5-3T were cream-coloured. The isolate showed optimum growth at 4.0 M NaCl, 40 °C and pH 7.0. The major polar lipids of the strain were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two unknown phospholipids and three glycolipids (including one that was chromatographically identical to S2-DGD). The major respiratory quinone was menaquinone MK-8. The G+C content of the genomic DNA was 61.5 mol%. The closest relative was *Natrinema salaciae* JCM 17869T with 97.3 % similarity in the orthologous 16S rRNA gene sequence. Analysis of 16S rRNA and *rpoB* gene sequences indicated that strain 5-3T is a member of the genus *Natrinema* in the family *Natrialbaceae* and forms a distinct cluster. On the basis of phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, a novel species of the family *Natrialbaceae, Natrinema soli* sp. nov., is proposed. The type strain is 5-3T (=IBRC-M 11063T=LMG 29247T).

Extremely halophilic archaea, included within the class *Halobacteria*, are a group of prokaryotes that are widely distributed in various hypersaline environments [1–3]. There are several saline and hypersaline lakes in Iran, both athalassohaline and thalassohaline, that have not been described from a microbiological point of view [4]. The genera *Natrinema* and *Haloterrigena* comprise several species that seem to be intermixed phylogenetically and may represent only one genus, as has been suggested by Tindall [5] and Minegishi et al. [6].

The genus *Natrinema* was proposed by McGinity et al. [7] to accommodate *Natrinema pellirubrum* (formerly *Halobacterium salinarum* NCIMB 786T) and *Natrinema pallidum* (formerly *Halobacterium halobium* NCIMB 777T) and comprises seven species at the time of writing. The genus *Haloterrigena* was proposed by Ventosa et al. [8] to accommodate *Haloterrigena turkmenica* (formerly *Halococcus turkmenicus* VKM B-1734) and comprises nine species. Recently, the genus *Natrinema* was classified within the family *Natrialbaceae*, order *Natriales*, in the class *Halobacteria* [2].

Strain 5-3T was isolated from Meighan wetland, which is located in north-eastern Arak, an industrial city in Iran. Meighan wetland is one of the most important hypersaline playas in Iran. Salinity reaches saturation during the dry season [9]. This wetland (34° 11′ 44″ N 49° 50′ 48″ E) is the largest source of sodium sulfate in the centre of the country, and it lies at an altitude of about 1700 m above sea level and covers an area of about 2.5×10^4 ha where an arid–semi-arid continental climate dominates [10]. In this study, we isolated an extremely halophilic archaeon from this habitat, strain 5-3T, which phylogenetically and phenotypically may represent a novel species of the genus *Natrinema*.

Sampling from Meighan wetland was performed in June 2013. Soil samples were diluted in sterile 20 % (w/v) salts solution and spread onto NOM plates and incubated at 40 °C. The NOM medium (neutral oligotrophic medium) contained (per litre distilled water) 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, 0.05 g yeast extract, 0.25 g fish peptone and 0.25 g sodium pyruvate. The pH was adjusted to 7.3 and 2 % agar was used to solidify this medium [11].

**Author affiliations:** 1Microorganisms Bank, Iranian Biological Resource Centre (IBRC), ACECR, Tehran, Iran; 2Extremophiles Laboratory, Department of Microbiology, Faculty of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran; 3Department of Molecular and Cellular Biology, Faculty of Basic Sciences and Advanced Technologies in Biology, University of Science and Culture, Tehran, Iran; 4Department of Applied Chemistry, Faculty of Science and Engineering, Toyo University, Kawagoe-shi, Saitama, Japan; 5Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain.

**Correspondence:** Mohammad Ali Amoozegar, amoozegar@ut.ac.ir

**Keywords:** halophilic archaea; *Natrinema*, Meighan wetland; hypersaline wetland.

**Abbreviations:** ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB* gene sequences of strain 5-3T are KY381123 and LC206002, respectively. Four supplementary figures are available with the online Supplementary Material.
After 4 weeks of incubation colonies were picked up and purified by restreaking several times. Isolates were stored at −80 °C in NOM with 20% glycerol. Strain 5-3T was isolated in pure culture from a soil sample of Meighan wetland after subculturing several times.

Genomic DNA was extracted and purified as described by Lam [12] for haloarchaea. The orthologous 16S rRNA gene sequences were determined as described previously [13], and relevant sequences retrieved from the DNA Data Bank of Japan [14, 15] were aligned using the CLUSTAL X 2.1 software package [16]. Phylogenetic trees were reconstructed by the neighbour-joining (NJ) algorithm [17] and evaluated by bootstrap sampling, expressed as percentages of 1000 replicates [18]. Maximum-likelihood (ML) analysis and determination of support values were achieved with raxmlGUI ver 1.31 [19, 20]. Phylogenetic trees were also reconstructed with MEGA7 [21] using the maximum-parsimony (MP) algorithm [22] with 1000 randomly selected bootstrap replicates.

The orthologous 16S rRNA gene sequence of strain 5-3T (1473 bp) was related most closely to that of Natrinema salaciae JCM 17869T (GenBank accession number AB935413, 1474 bp), with 97.3% similarity, followed by the type strains of members of the genera Natrinema and Haloterrigena, with similarities of 96.6–95.9 and 96.1–95.1%, respectively. 16S rRNA gene sequence similarity between strain 5-3T and the most closely related species indicated that this strain can be proposed as representing a novel species [23], and comparisons also indicated that strain 5-3T has one type of 16S rRNA gene sequence. The phylogenetic position of the new isolate was determined in trees generated using the NJ (Fig. 1a), ML (Fig. S1), and MP (Fig. S2) algorithms. The results suggested that the strain represents a novel species, constituting a separate branch most closely related to Natrinema salaciae JCM 17869T. However, as previously reported, the species of the genera Natrinema and Haloterrigena do not form monophyletic groups [6].

Determination of the DNA-dependent RNA polymerase B’ gene sequence (rpoB’) and its analysis were performed according to Minegishi et al. [6]. The rpoB’ gene of strain 5-3T was sequenced and found to be 1830 bp in length. Based on the alignment of rpoB’ gene sequences, strain 5-3T showed 92% similarity to Natrinema salaciae JCM 17869T, followed by the type strains of members of the genera Natrinema and Haloterrigena, with similarities of 92–93 and 88–93%, respectively. The rpoB’ gene sequence similarity confirmed that strain 5-3T represents a novel species [6]. In the reconstructed phylogenetic tree based on rpoB’ gene sequences, strain 5-3T clustered closely with members of the genus Natrinema and formed a monophyletic group in the family Natrilbaceae (Fig. 1b).

The DNA G+C content of the total DNA of strain 5-3T, determined by HPLC as described by Mesbah et al. [24], was 61.5 mol%. This value is slightly lower than the range reported for the genus Natrinema, 62.9–65.6 mol% [7, 25–29], suggesting that strain 5-3T may belong to the genus Natrinema.

All phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the class Halobacteria [30]. Natrinema salaciae MDB25T and Natrinema ejinorense JCM 13890T were used as reference strains in all tests. Cell morphology and motility were determined by phase contrast microscopy with cultures at the exponential growth phase. Gram staining was performed according to Dussault [31]. To determine the salt requirements, the strains were cultured in NOM with different NaCl concentrations [1.0–5.0 M, at intervals of 0.5 M, and at 5.4 M (saturation)] or MgSO4·7H2O concentrations (0–1 M, at intervals of 0.1 M). The temperature range for growth was examined by incubation at 20–50 °C (at intervals of 5 °C). The pH range was examined from pH 5.0 to 9.0 (at intervals of 0.5 pH units) at 40 °C by using 50 mM MES, HEPES or CHES buffers. Catalase, oxidase and DNase activity and nitrate reduction were examined according to Smibert and Krieg [32]. Hydrolysis of starch, aesculin, casein and gelatin were tested as described by Zhu [33]. Esterase activity was examined as outlined by Gutiérrez and González [34]. Lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase tests were performed as recommended by Oren et al. [30]. Production of acid from carbohydrates was determined as described previously [30]. Utilization of single carbon sources was examined in NOM with 0.01% yeast extract and the carbon sources (2.0 g l⁻¹) (without Casamino acids and sodium pyruvate) [35]. Susceptibility to antibiotics was determined on NOM agar plates using antibiotic discs. Anaerobic growth was examined by using filled and stoppered tubes containing liquid medium supplemented with KNO3, L-arginine, DMSO, Na2S3O7 and Na2S2O7 (each at 5.0 g l⁻¹) [25]. Production of H2S was tested by growing the strain in NOM liquid medium supplemented with 0.5% (w/v) Na2S2O7 [30]. Tryptone water medium was used for detection of indole production [36].

The optimum growth temperature for strain 5-3T was 40 °C and growth was observed from 25 to 45 °C. The optimum pH for growth was 7.0 and growth was observed between pH 6.0 and 8.0. Growth occurred at NaCl concentrations of 3.0–4.5 M, with optimum growth at 4.0 M. Mg2+ was not required and growth occurred from 0 to 0.4 M MgSO4·7H2O, with optimum growth at 0.08–0.2 M MgSO4·7H2O. Cells of strain 5-3T were Gram-stain-negative, aerobic, non-motile and ovoid (Fig. S3). The organism was catalase-positive and oxidase-negative. Colonies of strain 5-3T were cream-coloured while those of Natrinema salaciae MDB25T and Natrinema ejinorense JCM 13890T were red-pigmented, as described by Albuquerque et al. [25] and Castillo et al. [26], respectively.

Nitrate reduction, hydrolysis of starch, casein and gelatin, and DNase activity were negative for strain 5-3T while hydrolysis of Tweens 20, 40 and 80 and aesculin was positive. No acid was produced by strain 5-3T from the...
sequence of more than 50% bootstrap support. The sequence of database (accession numbers in parentheses). Bootstrap values (%) are based on 1000 replicates and are shown for branches with members of the genus Natrinema. (a) NJ phylogenetic tree based on 16S rRNA gene sequence comparisons, showing the relationships between strain 5-3 and other closely related haloarchaea. The sequence data used were obtained from the GenBank database (accession numbers in parentheses). Bootstrap values higher than 50% are indicated. The Haloterrigena longa JCM 13562\(^\text{T}\) (AB477195) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position. (b) ML tree based on rpoB gene sequences showing the phylogenetic relationship between strain 5-3, members of the genus Natrinema and other closely related haloarchaea. Bootstrap values higher than 50% are indicated. The sequence of Halobacterium salinarum JCM 8978\(^\text{T}\) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

---

Fig. 1. (a) NJ phylogenetic tree based on 16S rRNA gene sequence comparisons, showing the relationships between strain 5-3\(^\text{T}\), other members of the genus Natrinema and other closely related haloarchaea. The sequence data used were obtained from the GenBank database (accession numbers in parentheses). Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. The sequence of Halobacterium salinarum JCM 8978\(^\text{T}\) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. (b) ML tree based on rpoB gene sequences showing the phylogenetic relationship between strain 5-3\(^\text{T}\), members of the genus Natrinema and other closely related haloarchaea. Bootstrap values higher than 50% are indicated. The sequence of Halobacterium salinarum JCM 8978\(^\text{T}\) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.
carbohydrates tested. Anaerobic growth did not occur with sodium thiosulfate, potassium nitrate, sodium sulfate, l-arginine or DMSO. The strain was sensitive to novobiocin (30 µg), rifamycin B (5 µg) and nitrofurantoin (300 µg) but resistant to ampicillin (10 µg), penicillin G (10 IU), erythromycin (15 µg), chloramphenicol (30 µg), gentamicin (10 µg), bacitracin (0.04 IU), tetracycline (30 µg), neomycin (30 µg), kanamycin (30 µg) and nalidixic acid (30 µg). Other detailed characteristics of strain 5-3T are given in the species description and Table 1.

Cell biomass for polar lipid analyses was obtained by cultivation in NOM broth at 150 r.p.m. and 40 °C. Cells were harvested in the mid-exponential growth phase. Polar lipids were extracted from freeze-dried cells by using a chloroform/methanol system. They were separated by TLC using silica gel 60 F254 aluminium-backed thin-layer plates (Merck) [37]. Two-dimensional chromatography was performed by using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/glacial acetic acid/methanol/water (80:15:12:4, by vol.) in the second dimension and the second solvent system was also used for one-dimensional TLC. Total lipid material and specific functional groups were detected using molybdenum blue (for phospholipids) and α-naphthol-sulphuric acid (for glycolipids) [38]. The polar lipids of strain 5-3T consisted of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two unknown phospholipids and three glycolipids (one that was chromatographically identical to S2-DGD (mannose-2,6-dissulfate (1→2)-glucose glycerol diether) (Fig. S4). The polar lipid pattern of strain 5-3T was similar to that of the type species of the genus Natrinema, Natrinema pellirubrum CIP 106293T [7], except for the number of minor unknown phospholipids and also phosphatidylglycerolsulfate was not found in strain 5-3T. Respiratory lipoquinones were determined as previously reported by Wainø et al. [39]. Menaquinone MK-8 was the major respiratory lipoquinone present, which was similar to the respiratory quinone of Natrinema salaciae JCM 17869T [25], and that of Natrinema pellirubrum CIP 106293T [7].

The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain 5-3T represents a novel species in the genus Natrinema, within the family Natrialbaceae, for which the name Natrinema soli sp. nov. is proposed.

### Table 1. Differential characteristics between strain 5-3T and closely related species of the genus *Natrinema*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)*</td>
<td>1.2–1.6</td>
<td>0.8–1.5×1.0–3.0*</td>
<td>0.8–2.0×1.5–4.0*</td>
</tr>
<tr>
<td>NaCl concentration for optimum growth (M)</td>
<td>4.0</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Optimum MgCl2 requirement (M)</td>
<td>0.08–0.2</td>
<td>0.08–0.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>25–45</td>
<td>30–50</td>
<td>25–50</td>
</tr>
<tr>
<td>Temperature optimum for growth (°C)</td>
<td>40</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>6.0–8.0</td>
<td>6.5–9.0</td>
<td>6.0–8.5</td>
</tr>
<tr>
<td>pH optimum for growth</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Anaerobic growth in the presence of nitrate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casein</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Aesculin</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>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Proline</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)*</td>
<td>61.5</td>
<td>64.6a</td>
<td>64.7b</td>
</tr>
</tbody>
</table>

*Data taken from: a, Albuquerque et al. [25]; b, Castillo et al. [26].
DESCRIPTION OF NATRINEMA SOLI SP. NOV.

Natrinema soli (so´li. L. neut. gen. n. soli of soil, the source of the organism).

Strictly aerobic, Gram-stain-negative and non-motile. Cells form cream-coloured colonies (1.5 mm in diameter). Ovoid cells are 1.2–1.6 µm in size when grown in liquid NOM medium. Growth occurs at 25–45 °C (optimum 40 °C), at 3.0–4.5 M NaCl (optimum 4.0 M), at 0–0.4 M MgSO_4·7H_2O (optimum 0.08–0.2 M) and at pH 6.0–8.0 (optimum pH 7.0). Oxidase-negative and catalase-positive. Reduction of nitrate to nitrite is negative. Hydrolysis of casein, starch and gelatin is negative. DNA is not degraded; hydrolyses Tween 20, 60, 80 and ascinul. No growth occurs with DSMO, L-arginine, Na_2S_2O_3 or Na_2SO_4. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Indole is not produced from tryptophan. The following substrates are utilized as sole source of carbon and energy: L-arabinose, D-galactose, D-glucose, maltose, cellubiose, raffinose, proline, glutamine, L-lysine, threonine, D-glucuronate and D-glucose; utilization of the following substrates is negative: D-mannose, L-ribonose, D-ribose, D-xylose, D-arabinose, sucrose, lactose, trehalose, melibiose, xylitol, sorbitol, D-mannitol, myo-inositol, D-arabitol, L-histidine, L-phenylalanine, glutamate, L-alanine, L-asparagine, L-lysine, L-arginine, L-serine, L-ornithine, methionine, D-glucaronate, aspartate and glycine. Acid is not produced from any of the above-mentioned carbohydrates. H_2S is not produced from thiosulfate. The polar lipid pattern consists of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two unknown phospholipids and three glycolipids (one chromatographically identical to S_2-DGD). The major menaquinone is MK-8.

The type strain is 5·3^T (=IBRC-M 11063^T=LMG 29247^T), isolated from soil of Meighan wetland in Iran. The G+C content of the genomic DNA of the type strain is 61.5 mol%.


Funding information
This work was supported by grants from the Iranian Biological Resource Centre (IBRC) (MI-1391–151) (to M. A. Amoozegar), and from Spanish Ministerio de Economía y Competitividad (CGL2013-46941-P) with European funds (FEDER) (to A. Ventosa).

Acknowledgements
We thank Professor Milton S. da Costa for kindly providing Natrinema salaciea MDB25^T.

Conflicts of interest
The authors have no conflicts of interest relating to this study.

Ethical statement
No experimental work with animals or humans was done for this study.

References


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.