Haloarchaeobius iranensis gen. nov., sp. nov., an extremely halophilic archaeon isolated from a saline lake

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Strain EB21T was isolated from a brine sample from Aran-Bidgol salt lake, a saline playa in Iran. Strain EB21T was an orange–red-pigmented, motile rod and required at least 2 M NaCl but not MgCl2 for growth. Optimal growth was achieved at 3.5 M NaCl and 0.2 M MgCl2. The optimum pH and temperature for growth were pH 7.5 and 40 °C, while it was able to grow at pH 6.0–8.0 and 25–55 °C. Analysis of the 16S rRNA gene sequence revealed that strain EB21T is a member of the family Halobacteriaceae, showing low levels of similarity to other members of the family. The highest sequence similarities, 91.8, 91.7 and 91.5%, were obtained with the 16S rRNA gene sequences of the type strains of Halobiforma lacisalsi, Haloterrigena thermotolerans and Halalkalicoccus tibetensis, respectively. Polar lipid analyses revealed that strain EB21T contains phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and phosphatidylglycerol sulfate. Three unidentified glycolipids and one minor phospholipid were also observed. The only quinone present was MK-8(II-H2). The G+C content of its DNA was 67.7 mol%. On the basis of the data obtained, the new isolate could not be classified in any recognized genus. Strain EB21T is thus considered to represent a novel species in a new genus within the family Halobacteriaceae, order Halobacterales, for which the name Haloarchaeobius iranensis gen. nov., sp. nov. is proposed. The type strain of Haloarchaeobius iranensis is EB21T (=IBRC-M 10013T =KCTC 4048T).

The family Halobacteriaceae (order Halobacterales, domain Archaea) contains micro-organisms that need at least 1.5 M NaCl for growth. They are rods, cocci or pleomorphic cells including flat discs, triangles and squares that occur ubiquitously in nature where the salt concentration is high, i.e. in salt lakes, soda lakes, salterns, etc. (Grant et al., 2001). The classification of taxa belonging to this family is currently based on a polyphasic approach including phenotypic features, chemical data (polar lipid composition) and genotypic data (16S rRNA gene sequence analysis and DNA–DNA hybridization) (Oren et al., 1997). At the time of writing (February 2011), members of this family are classified within 32 different genera, with a large number of species (http://www.the-icsp.org/taxa/halobacterlist.htm).

Here we describe a halophilic archaeal strain, designated EB21T, which was isolated from a salt lake located in the central desert of Iran. Preliminary 16S rRNA gene sequence comparisons indicated that the isolate was a member of the family Halobacteriaceae. The aim of the present work was to determine the exact taxonomic position of strain EB21T by using a polyphasic taxonomic characterization that combined phenotypic, chemotaxonomic, phylogenetic and genotypic analyses. These results indicate that strain EB21T should be placed in a new genus as a representative of a novel species.

Sampling was carried out in November 2007 from Aran-Bidgol salt lake (34° 26’ N 51° 48’ E), a playa that can be considered as thalassohaline, with NaCl as the major salt. The lake water is neutral (about pH 7.0) and its salinity reaches saturation. For isolation, modified growth medium
(MGM) with 23 % total salt concentration, as described in the *Halohandbook* online protocol (Dyall-Smith, 2006), was used. This medium contains a 23 % salt mixture, prepared from 30 % stock solution, which consists of (g l$^{-1}$): NaCl, 240; MgSO$_4$·7H$_2$O, 35; MgCl$_2$·6H$_2$O, 30; KCl, 7; and CaCl$_2$·2H$_2$O, 1; supplemented with 1 % (w/v) peptone (Merck) and 0.2 % (w/v) yeast extract (Merck). Agar (1.5 % w/v) was used to solidify media if necessary. The medium was adjusted to pH 7.2–7.4 with 2 M Tris base (Merck). Samples were cultured in 23 % MGM solid medium after preparing the appropriate dilutions in the laboratory. Inoculated plates were incubated at 40 °C for up to 2 months. After successive cultivations, a pure isolate designated strain EB21$^T$ was obtained. The characterization of this strain was achieved by following the minimal standards recommended by Oren et al. (1997) for describing novel taxa of the order Halobacteriales. The type strains *Halofex volcanii* DSM 3754$^T$, *Halobiforma haloterestris* DSM 13078$^T$ and *Haloterrigena thermotolerans* DSM 11552$^T$ were selected as reference strains in the genus and species descriptions.

Cell morphology and motility were examined using an Olympus BX41 microscope equipped with phase-contrast optics. For photography, drops of exponentially growing liquid cultures were used directly without fixing. Colony morphology was observed on agar medium under optimal growth conditions after incubation at 40 °C for 14 days. The Gram reaction was determined by following the method outlined by Dussault (1955). Strain EB21$^T$ was motile and rod-shaped (Fig. S1, available in IJSEM Online) and stained Gram-negative. Colonies formed on agar plates were small (about 1.0–2.0 mm diameter), convex, round with an entire edge and orange–red-pigmented.

Physiological tests were conducted using liquid or solid (1.5 % agar) MGM medium unless stated otherwise. Liquid cultures were incubated at 40 °C on a shaking incubator at 200 r.p.m. Growth rates were determined by monitoring the increase in OD$_{660}$. The temperature range for growth was examined in liquid MGM medium at 20–60 °C at 5 °C intervals. Growth at pH 5.0–9.0 was tested; the buffers MES (pH 5–6.5), HEPES (pH 7–8) and CHES (pH 8.5–9) were added at a concentration of 50 mM. The requirements for NaCl and MgCl$_2$ for growth were determined in media containing 0–5 M NaCl (0.5 M increments) or 0–1 M MgCl$_2$ (0.05 M increments), respectively.

Strain EB21$^T$ grew at 25–55 °C (optimum 40 °C) and pH 6–8 (optimum pH 7.5). Routine cultivation was conducted at 40 °C and pH 7.5. Strain EB21$^T$ was capable of growing over a wide range of NaCl concentration: 2–5 M (12–30 %). It grew optimally in the presence of 3.5 M (20 %) NaCl, in the range that has been shown for most extremely halophilic archaea (Grant et al., 2001). MgCl$_2$ was not required for growth, but optimal growth occurred in the presence of 0.2 M MgCl$_2$.

Acid production from substrates was tested in unbuffered MGM medium and was determined by measuring the initial and final pH of the medium. The culture was considered positive for acid production if the pH decreased by at least 1 pH unit. To test for carbon-source utilization, peptone was omitted from the MGM medium and the concentration of yeast extract was reduced to 0.1 g l$^{-1}$ (Oren et al., 1997). The ability of strain EB21$^T$ to grow anaerobically in the presence of DMSO (5.0 g l$^{-1}$) and to ferment arginine (5.0 g l$^{-1}$) was tested in MGM medium prepared anaerobically in serum tubes according to the procedures described by Bryant (1972) and Balch & Wolfe (1976). Growth and gas formation with nitrate as electron acceptor were tested in 10 ml stoppered tubes, filled completely with liquid growth medium to which NaNO$_3$ (5 g l$^{-1}$) had been added, and containing an inverted Durham tube (Oren et al., 1997). Tween hydrolysis activity was detected as described by Gutiérrez & González (1972). Casein, gelatin and starch hydrolysis was determined as described by Oren et al. (1997). Tests for catalase and oxidase activities were performed as described by González et al. (1978). Production of H$_2$S was tested by growing strain EB21$^T$ in MGM liquid medium supplemented with 0.5 % (w/v) Na$_2$S$_2$O$_3$ (Oren et al., 1997). Tryptone water medium supplemented with 23 % total salts was used for detection of indole production (Smibert & Krieg, 1994). Susceptibility to antimicrobial compounds was determined by the disc diffusion method after spreading the strain on solid MGM medium (Oren et al., 1997).

Strain EB21$^T$ was catalase- and oxidase-positive. It could hydrolyse gelatin and Tweens 20 and 40, but Tweens 60 and 80 and starch could not be hydrolysed. Strain EB21$^T$ utilized D-fructose, D-galactose, D-glucose, maltose and lactose. Acid was produced from D-glucose and maltose but not from D-galactose, sucrose, D-fructose, D-xylene, D-ribose, D-mannitol, D-mannose, trehalose, L-arabinose or lactose. The detailed physiological and biochemical characteristics of strain EB21$^T$ are listed in Table 1 and in the genus and species descriptions.

Genomic DNA of strain EB21$^T$ was extracted as described by Wan Lam in the *Halohandbook* (Dyall-Smith, 2006) and the 16S rRNA gene was amplified using the archaeal universal primers 21F (5′-TTCGCGGGTGTATTGCYCCG-GGA-3′; DeLong, 1992) and 1492R (5′-GTTACCTTGGTACCTTGC-7AG-3′; Lane et al., 1985). PCR products were purified with a DNA purification kit (Roche), according to the manufacturer’s protocol. Purified PCR products were then electrophoresed on a 1 % agarose gel to check their quality. Ligation of the PCR products with the pGEM-T vector, transformation of *Escherichia coli* DH5α and selection of transformants were carried out with a pGEM-T TA cloning kit (Promega), used according to the manufacturer’s protocol. Multiple clones were picked randomly and then sequenced by the service of Macrogen (Seoul, South Korea) to determine whether the strain possessed multiple distinct 16S rRNA genes. Phylogenetic analysis was performed using the software package MEGA version 4 (Tamura et al., 2004) after obtaining multiple alignments of data available from public databases by
Table 1. Differential characteristics between strain EB21<sup>T</sup> and closely related genera within the order Halobacteriales

<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>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Rod</td>
<td>Coccus or rod</td>
<td>Coccus</td>
<td>Coccus</td>
<td>Coccobacillus or rod</td>
<td>Coccus</td>
<td>Small rod</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>1.5–2.0 × 3–7</td>
<td>1.25–2.0 (coccus) or 0.5–1.5 × 2–8 (rod)</td>
<td>1.5–2.0</td>
<td>1.0–1.5</td>
<td>0.5–0.7 × 1–3</td>
<td>1.0–2.0</td>
<td>0.5–1.0 × 1.0–6.0</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Orange–red</td>
<td>Red</td>
<td>Red</td>
<td>Orange</td>
<td>Not pigmented</td>
<td>Pale brown</td>
<td>Red</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lysis in distilled water</td>
<td>+</td>
<td>– (coccus) or + (rod)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>NaCl concentration for growth (M)</td>
<td>3.5</td>
<td>2.6–4.3</td>
<td>3–4</td>
<td>3.4</td>
<td>4</td>
<td>2.5–3.6</td>
<td>3.4–4.3</td>
</tr>
<tr>
<td>Optimum</td>
<td>40</td>
<td>42–45</td>
<td>45</td>
<td>40</td>
<td>30–40</td>
<td>35–45</td>
<td>50</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; requirement</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Optimum temperature (°C)</td>
<td>40</td>
<td>42–45</td>
<td>45</td>
<td>40</td>
<td>30–40</td>
<td>35–45</td>
<td>50</td>
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<td>Growth at pH 7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Hydrolysis of gelatin</td>
<td>+</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>Indole production</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Presence of PGS</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Minor phospholipid(s)</td>
<td>PL1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>PL1, PL2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>67.7</td>
<td>66.9</td>
<td>59.8</td>
<td>61.5</td>
<td>62.3</td>
<td>61.2</td>
<td>67.1</td>
</tr>
<tr>
<td>16S rRNA gene sequence similarity to EB21&lt;sup&gt;T&lt;/sup&gt; (%)</td>
<td>(100)</td>
<td>&lt;92.0</td>
<td>&lt;91.9</td>
<td>&lt;91.8</td>
<td>&lt;91.4</td>
<td>&lt;91.2</td>
<td>&lt;89.2</td>
</tr>
</tbody>
</table>

*Values for the type strain of the type species.

Taxa: 1, strain EB21<sup>T</sup> (Halouroarchaeobius iranensis gen. nov., sp. nov.); 2, Halobiforma; 3, Haloterrigena; 4, Halalkalicoccus; 5, Natrinalba; 6, Natronococcus; 7, Halobacterium. Data were derived from this study and Hezayen et al. (2002), Ventosa et al. (1999), Xue et al. (2005), Kamekura & Dyall-Smith (1995) and Grant et al. (2001).
CLUSTAL_X (Thompson et al., 1997). Clustering was performed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) methods. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985).

Fifteen almost-complete 16S rRNA gene sequences (1441 nt) were obtained from strain EB21T. Sequence comparisons indicated that the strain has two rRNA genes, rrnA and rmbB, which have 98.4 % sequence similarity to each other. 16S rRNA gene sequence analysis showed that strain EB21T is a member of the family Halobacteriaceae, but there were low similarities to type species of other members of this family; the most closely related haloarchaeal taxa were Halobiforma lacisali AI5T (92.0 % similarity), Haloterrigena thermotolerans PR5T (91.9 %) and Halalkalicoccus tibetensis DS12T (91.8 %). Phylogenetic analysis using the neighbour-joining algorithm revealed that strain EB21T clustered in a separate clade (Fig. 1); this phylogenetic position was also confirmed by trees generated using the minimum-evolution and maximum-parsimony algorithms (Figs S2 and S3).

The DNA G+C content was determined by the HPLC method (Mesbah et al., 1989). The DNA G+C content of strain EB21T was 67.7 mol%, which is within the range described for the family Halobacteriaceae (Grant et al., 2001).

The polar lipid composition and respiratory quinones were determined using the services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and Dr Brian Tindall (DSMZ). Polar lipids were separated by two-dimensional silica gel TLC. Methods including solvents and detection reagents were described by Hezayen et al. (2001). Phosphatidylglycerol, phosphatidylglycerol sulfate, three glycolipids yet to be identified and one minor phospholipid were also observed. (Fig. S4). The presence of PGS in this novel archaeon allows it to be differentiated from phylogenetically related members of the family Halobacteriaceae: Halobiforma, Haloterrigena, Halalkalicoccus, Natribia and Natronococcus, which do not contain PGS (Hezayen et al., 2002; Ventosa et al., 1999; Xue et al., 2005; Kamekura & Dyall-Smith, 1995; Grant et al., 2001). Respiratory lipoquinones were determined as described previously (Wainø et al., 2000); MK-8(II-H2) was the only quinone present.

In conclusion, the morphological and physiological properties of the new isolate, the low levels of 16S rRNA gene sequence similarity with other genera within the family Halobacteriaceae and distinctive polar lipids suggest that strain EB21T represents a novel species of a new genus within the family Halobacteriaceae, for which the name Haloarchaeobius iranensis is proposed. In conclusion, the morphological and physiological properties of the new isolate, the low levels of 16S rRNA gene sequence similarity with other genera within the family Halobacteriaceae and distinctive polar lipids suggest that strain EB21T represents a novel species of a new genus within the family Halobacteriaceae, for which the name Haloarchaeobius iranensis is proposed. Neighbor-joining phylogenetic tree showing the relationship between strain EB21T and close relatives within the family Halobacteriaceae. Accession numbers are given in parentheses.

**Description of Haloarchaeobius gen. nov.**

Haloarchaeobius [Halo.ar.chae.o’bi.us. Gr. n. hals, halos salt; N.L. adj. archaeos from Gr. adj. archais ancient; N.L. masc. n. Bios from Gr. masc. n. bios life; N.L. masc. n. Haloarchaeobius halophilic ancient (archaeal) life].

Cells are motile rods. Gram-negative. Colonies are pigmented orange–red. Strictly aerobic; oxygen is used as the terminal electron acceptor. Growth occurs at pH 6.0–8.0, 25–50 °C and 2.0–5.0 M (12–30 %) NaCl. MgCl2 is not required. Optimal growth occurs at pH 7.5, 40 °C, 3.5 M (20 %) NaCl and 0.2 M MgCl2. Extremely halophilic. Polar lipids include phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, three unidentified glycolipids and one minor phospholipid. MK-8(II-H2) is the only respiratory lipoquinone present. The DNA G+C content of the type strain of the only species in the genus is 67.7 mol% (HPLC method). Affiliated phylogenetically to the Halobacteriaceae. The type species is Haloarchaeobius iranensis. Recommended three-letter abbreviation: Hab.

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain EB21T and close relatives within the family Halobacteriaceae. Accession numbers are given in parentheses. The sequence of the methanogenic archaeon Methanospirillum hungatei JF-1T was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Bar, 0.02 substitutions per nucleotide position.
Description of Haloarchaeobius iranensis sp. nov.

_Haloarchaeobius iranensis_ (i.ra.nen.’sis. N.L. masc. adj. _iranensis_ of or belonging to Iran, referring to the isolation of the type strain).

Exhibits the following properties in addition to those given of or belonging to Iran, referring to the isolation _iranensis_ following antimicrobial compounds: anisomycin (35 \( \mu \)g), D-galactose, maltose, lactose, L-asparagine, L-glycine, L-lactate, L-lysine, L-methionine, L-aspartic acid, L-cysteine and L-valine are not. Susceptible to the following antimicrobial compounds: ampicillin (10 \( \mu \)g), chloramphenicol (30 \( \mu \)g), kanamycin (10 \( \mu \)g), neomycin (30 \( \mu \)g), gentamicin (5 \( \mu \)g), tetracycline (30 \( \mu \)g), tobramycin (10 \( \mu \)g). Indole is not produced from _D_-arabinose, _D_-fructose, _D_-fructose, _D_-mannitol, _D_-galactose, sucrose or _D_-ribose. _H_2_5_S is not produced from thiosulfate. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Methyl red and Voges–Proskauer tests are negative. _D_-Fructose, _D_-glucose, _D_-galactose, maltose, lactose, _L_-asparagine, _L_-glycine, _L_-proline, _L_-phenylalanine and _L_-tyrosine are utilized as sole source of carbon and energy, while _L_-arabinose, _D_-fructose, sucrose, _D_-mannitol, _D_-ribose, _L_-arginine, _L_-methionine, _L_-aspartic acid, _L_-cysteine and _L_-valine are not. Susceptible to the following antimicrobial compounds: anisomycin (35 \( \mu \)g), bacitracin (10 \( \mu \)g), nitrofurantoin (300 \( \mu \)g), novobiocin (5 \( \mu \)g) and rifampicin (5 \( \mu \)g). Resistant to amoxicillin (25 \( \mu \)g), ampicillin (10 \( \mu \)g), cephalotin (30 \( \mu \)g), carbenicillin (100 \( \mu \)g), cefoxitin (30 \( \mu \)g), chloramphenicol (30 \( \mu \)g), erythromycin (5 \( \mu \)g), gentamicin (10 \( \mu \)g), kanamycin (5 \( \mu \)g), nalidixic acid (30 \( \mu \)g), neomycin (30 \( \mu \)g), penicillin _G_ (10 \( \mu \)g), polymyxin _B_ (100 \( \mu \)g), streptomycin (10 \( \mu \)g), tetracycline (30 \( \mu \)g) and tobramycin (10 \( \mu \)g).

The type strain, EB21\(^T\) (=IBRC-M 10013\(^T\) =KCTC 4048\(^T\)), was isolated from Aran-Bidgol salt lake, Iran.

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References


