Halovivax limisalsi sp. nov., an extremely halophilic archaeon from hypersaline mud

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A Gram-stain-negative, cream-pigmented, motile, extremely halophilic archaeon, designated strain IC38T, was isolated from a saline mud sample taken from a hypersaline lake, Aran-Bidgol, in Iran. The strain required at least 2.5 M NaCl for growth. However, MgCl2 was not required. Optimal growth occurred with 4.3 M NaCl and 0.2 M MgCl2. The optimum pH and temperature for growth were pH 7.0 and 35 °C, respectively, and strain IC38T was able to grow over a pH range of 6.5–9.0, and a temperature range of 25–45 °C. Analysis of the 16S rRNA gene sequence revealed that strain IC38T clustered with the two species of the genus Halovivax, Halovivax asiaticus EJ-46T and Halovivax ruber XH-70T, with sequence similarities of 96.4 % and 96.1 %, respectively. The similarities between the rpoB gene of the novel strain and Halovivax asiaticus and Halovivax ruber were 90.7 % and 90.3 %, respectively. The polar lipid pattern of strain IC38T consisted of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Three unidentified glycolipids and two minor phospholipids were also observed. The DNA G+C content of strain IC38T was 62.6 mol%. On the basis of the phylogenetic analysis, as well as the biochemical and physiological characteristics, the new isolate is suggested to be a representative of a novel species of the genus Halovivax, for which the name Halovivax limisalsi sp. nov. is proposed. The type strain of Halovivax limisalsi is IC38T (=IBRC-M 10022T=KCTC 4051T).

The genus Halovivax was proposed by Castillo et al. (2006) to accommodate an extremely halophilic archaeon, which was isolated from a saline lake, Ejinor, in Inner Mongolia, PR China. At the time of writing, the genus comprises two species with validly published names, Halovivax asiaticus (the type species of the genus) and Halovivax ruber (Castillo et al., 2007), which are pale-pink and red strains, respectively. Members of this genus are extremely halophilic, strictly aerobic and they do not contain phosphatidylglycerol sulphate among their polar lipids (Castillo et al., 2006, 2007). Here we describe the isolation and polyphasic characterization of a novel, cream-pigmented, extremely halophilic archaeon isolated from a mud sample taken from a hypersaline lake, Aran-Bidgol, in Iran and propose it to represent a novel species of the genus Halovivax.

Strain IC38T was isolated from a mud sample [pH 7.5; salinity 22 % (w/v)] taken from Aran-Bidgol, a hypersaline lake in Iran (35° 70’ N 51° 39’ E). We sampled the hypersaline mud (up to 40 cm in depth), collecting it in sterile plastic containers and then kept samples in the dark at an environmental temperature for four hours until it was analysed in the laboratory. The environmental sample was inoculated in modified growth medium (MGM) with 23 % (w/v) total salt concentration (Dyall-Smith, 2009), after preparing the appropriate dilutions in sterile 20 % (w/v) salt solution in the laboratory. MGM contained a 23 % (w/v) salt mixture prepared from a 30 % (w/v) stock solution comprising (g l−1): 240 NaCl, 35 MgSO4, 7H2O, 30 MgCl2.6H2O, 7 KCl and 1 CaCl2.2H2O; this was supplemented with 1 % (w/v) peptone (Merck) and 0.2 % (w/v) yeast extract (Merck). If necessary, 1.5 % (w/v) agar was used for solid media. The pH of the medium was...
adjusted to pH 7.2–7.4 with 2 M Tris-base (Merck). Inoculated plates were incubated at 40 °C for up to two months. After successive cultivating, a pure isolate, designated strain IC38T, was obtained. Characterization of this strain was achieved by following the minimal standards, as recommended by Oren et al. (1997), for describing novel taxa of the order Halobacteriales. The type strains, Halovivax asiaticus CECT 7098T and Halovivax ruber IBRC-M 10339T, were used as reference strains and for comparison with isolate IC38T.

The genomic DNA of the isolate was extracted as described by Dyall-Smith (2009) for halarchaea and the 16S rRNA gene was amplified using archael universal primers: 21F (5’-TTCGTTGATCCYGCAGGA-3’) (DeLong, 1992) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) (Lane et al., 1985). The PCR conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 51 °C for 30 s and 72 °C for 60 s, with a final 7 min extension at 72 °C. PCR products were purified with a DNA purification kit (Roche), according to the manufacturer’s protocol. Ligation of the PCR products into the pGEM-T vector, transformation of Escherichia coli DH5α and selection of the transformants were all carried out with the pGEM-T TA cloning kit (Promega), according to the manufacturer’s instructions. Several clones were randomly selected and sequencing was conducted on an ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea), to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. The amplification and sequencing of the rpoB’ gene was performed as described by Minegishi et al. (2010). Phylogenetic analysis was performed using the software package MEGA5 (Tamura et al., 2011) after obtaining multiple alignments of data that were available from public databases using 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 (1403 nt, each) of strain IC38T were obtained. Sequence comparison indicated that this strain has one kind of 16S RNA gene. 16S rRNA gene sequencing revealed that strain IC38T is a member of the family Halobacteriaceae, with the highest degree of sequence similarity to type strains of species of the genus Halovivax, Halovivax asiaticus EJ-46T (96.4 % similarity) and Halovivax ruber XH-70T (96.1 % similarity) followed by Natronorubrum sediminis CG-6T (94.5 %), Haloterrigena daqingensis JX133T (94.3 %) and Natrinema salaciae MDB25T (94.3 %). These results support strain IC38T representing a novel species of the genus Halovivax (Stackebrandt & Goebel, 1994; Rossello-Mora & Amann, 2001). Phylogenetic analysis using the neighbour-joining algorithm revealed that the novel strain clustered with species of the genus Halovivax, but in a separate clade (Fig. 1a). The phylogenetic position was also confirmed in trees generated using the minimum-evolution and maximum-parsimony algorithms (Fig. S1 and S2, available in the online Supplementary Material).

Physiological tests were conducted using liquid or solid (1.5% agar) MGM medium as described above, unless stated otherwise. Several liquid cultures were incubated at 40 °C on a shaking incubator at 200 r.p.m. The growth rate was determined by measuring the increase in OD550. The temperature range for growth was examined in liquid MGM medium at temperatures ranging from 20 °C to 55 °C at 5 °C intervals. Growth at different pH values ranging from 5.0–9.5 was tested; the buffers MES (pH 5.0–6.5), HEPES (pH 7.0–8) and CHES (pH 8.5–9.5) were added at a concentration of 50 mM. The requirement for NaCl and MgCl2 for growth was determined in media containing 0–5 M NaCl (0.5 M increments) or 0–1 M MgCl2 (0.05 M increments), respectively.

Strain IC38T grew over a temperature range of 25–45 °C (optimum 35 °C) and a pH range of 6.5–9.0 (optimum pH 7.0). Routine cultivation was conducted at 35 °C and pH 7.0. Strain IC38T was capable of growing over a range of NaCl concentrations from 2.5 M [15 % (w/v)] to 5.1 M [30 % (w/v)]. It grew optimally in the presence of 4.3 M NaCl. MgCl2 was not required for growth, but optimal growth occurred at 0.2 M MgCl2. Acid production from carbohydrates 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 one pH unit. To test for carbon source utilization peptone was omitted from MGM medium and the yeast extract concentration was reduced to 0.1 g l−1 (Oren et al., 1997). The ability of strain IC38T 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, which had been filled with liquid growth medium with NaNO₃ (5 g l⁻¹) added, and contained an inverted Durham tube (Oren et al., 1997). The ability to hydrolyse Tweens 40, 60 and 80 was determined as described by Gutierrez & Gonzalez (1972). Casein, gelatin and starch hydrolysis were determined as described by Oren et al. (1997). Tests for catalase and oxidase activities were performed as described by Oren et al. (1997).

Fig. 1. Neighbour-joining phylogenetic trees based on (a) 16S rRNA gene and (b) rpoB gene sequences. The position of strain IC38ᵀ relative to Halovivax asiaticus and Halovivax ruber and some other related species within the family Halobacteriaceae is shown. Accession numbers of the sequences are given in parentheses. The sequences of the methanogenic archaean Methanospirillum hungatei JF-1ᵀ (CP000254) was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Bars, substitutions per nucleotide position.
described by González et al. (1978). Production of H₂S was tested by growing strain IC₃⁸ᵀ in liquid MGM medium supplemented with 0.5% (w/v) Na₂S₂O₃ (Oren et al., 1997). Tryptone water medium supplemented with 23% (w/v) total salts was used for the detection of indole production (Smibert & Krieg, 1994). Susceptibility to antimicrobials was determined by the disc diffusion method after spreading the strain on solid MGM medium (Oren et al., 1997).

Strain IC₃⁸ᵀ was catalase- and oxidase-positive, strictly aerobic, and extremely halophilic. It was susceptible to the following antimicrobial compounds: anisomycin (35 mg), bacitracin (10 U), novobiocin (5 µg) and rifampicin (30 µg). It was resistant to amoxicillin (25 mg), ampicillin (10 mg), cefalotin (30 mg), carbenicillin (100 µg), chloramphenicol (30 µg), erythromycin (5 µg), gentamicin (10 µg), kanamycin (5 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U), polymyxin B (100 U), streptomycin (10 µg), tetracycline (30 µg) and tobramycin (10 µg). The detailed physiological and biochemical characteristics of strain IC₃⁸ᵀ are listed in Table 1 and in the species description.

The DNA G+C content was determined by HPLC (Mesbah et al., 1989). The DNA G+C content of strain IC₃⁸ᵀ was 62.6 mol%. This value is in the range reported for Halovivax asiaticus (60.3 mol%), and Halovivax ruber (65.0 mol%), the two species of the genus Halovivax with validly published names (Castillo et al., 2006, 2007).

Polar lipids were separated by two-dimensional silica gel TLC. Methods used, including the use of solvents in each direction and reagents for detection, were described by Hezayen et al. (2001). Phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester were present. Three unidentified glycolipids and two minor phospholipids were also observed (Fig. S4). The polar lipid pattern was similar to that of other species of the genus (Castillo et al., 2006, 2007), except with respect to the minor phospholipids observed in the novel strain.

In conclusion, on the basis of the results of phylogenetic, genotypic, chemotaxonomic and phenotypic studies, it is proposed that strain IC₃⁸ᵀ should be classified as the type strain of a novel species within the genus Halovivax, for which we propose the name Halovivax limisalsi sp. nov.

**Description of Halovivax limisalsi sp. nov.**

*Halovivax limisalsi* (li.mi.sal’si. L. gen. n. limisalsi of a salted mud).

Cells are Gram-stain-negative, motile, pleomorphic, 0.9–1.3 × 1–2 µm. Colonies are circular, entire, smooth, cream and have a diameter of 1–2 mm on 23% MGM agar.

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**Table 1.** Physiological and biochemical characteristics distinguishing strain IC₃⁸ᵀ from the two other members of the genus Halovivax

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.9–1.3 × 1–2</td>
<td>0.4–0.5 × 4–5</td>
<td>0.6–0.8 × 3.5–4.5</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Cream</td>
<td>Pale pink</td>
<td>Red</td>
</tr>
<tr>
<td>Optimum NaCl (M)</td>
<td>4.3</td>
<td>3.4</td>
<td>3.4</td>
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<tr>
<td>Mg²⁺ requirement</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Methylish-red test</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>H₂S production</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Acid from D-xylose</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Utilization of:</td>
<td></td>
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<tr>
<td>D-Glucose</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>D-Fructose</td>
<td>+</td>
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<td>−</td>
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<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Sucrose</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Susceptibility to:</td>
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<tr>
<td>Ampicillin (10 µg)</td>
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<td>Gentamicin (10 µg)</td>
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<tr>
<td>Neomycin (30 µg)</td>
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<td>+</td>
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<tr>
<td>Rifampicin (30 µg)</td>
<td>+</td>
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<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>62.6</td>
<td>60.3*</td>
<td>65.0†</td>
</tr>
</tbody>
</table>

*Data from Castillo et al. (2006).
†Data from Castillo et al. (2007).
medium after 14 days of incubation at 40 °C. Extremely halophilic; growth occurs over a range of NaCl concentrations from 2.5–5.1 M [15–30 % (w/v)]. Optimal growth occurs at pH 7.0 (and over a range of pH 6.5–9.0), 35 °C (over a range of 25–45 °C), 4.3 M NaCl and 0.2 M MgCl₂. Catalase- and oxidase-positive. Gelatin, skimmed milk and Tween 80 are hydrolysed, while aesculin, DNA, Tween 40, Tween 60 and starch are not. Anaerobic growth does not occur in the presence of arginine, DMSO or nitrate. Nitrate and nitrite are not reduced and the production of gas from nitrate is negative. Indole is produced. H₂S is not produced. Acid is produced from some carbohydrate sources including D-glucose, lactose and d-mannitol. Acid is not produced from D-fructose, D-galactose, maltose, D-mannose, sucrose, trehalose or D-xylose. Negative for arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. The methyl-red test is positive, but the Voges–Proskauer test is negative. Citrate is not utilized. The following compounds are utilized as sole sources of carbon and energy: D-glucose, D-fructose, lactose, d-mannitol, starch and sucrose. The following compounds are not utilized as sole sources of carbon: D-galactose, maltose, D-ribose and glycerol. Polar lipids include phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, three unidentified glycolipids and two minor phospholipids.

The type strain is IC38T (=IBRC-M 10022T = KCTC 4051T); it was isolated from the saline lake, Aran-Bidgol, in Iran. The DNA G+C content of the type strain is 62.6 mol%.

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References


