Halovivax cerinus sp. nov., an extremely halophilic archaeon from a hypersaline lake

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An extremely halophilic archaeon, strain IC35T, was isolated from a mud sample of the Aran-Bidgol salt lake in Iran. The novel strain was cream, non-motile, rod-shaped and required at least 2.5 M NaCl, but not MgCl2, for growth. Optimal growth was achieved with 3.4 M NaCl and 0.1 M MgCl2. The optimum pH and temperature for growth were pH 7.0 (grew over a pH range of 6.5–9.0) and 40 °C (grew over a temperature range of 30–50 °C), respectively. Analysis of 16S rRNA gene sequences revealed that strain IC35T clustered with species of the genus Halovivax, with sequence similarities of 97.3 %, 96.6 % and 96.3 %, respectively, to Halovivax limisalsi IC38T, Halovivax asiaticus EJ-46T and Halovivax ruber XH-70T. The rpoB gene similarities between the novel strain and Halovivax limisalsi IBRC-M 10022T, Halovivax ruber JCM 13892T and Halovivax asiaticus JCM 14624T were 90.2 %, 90.2 % and 89.9 %, respectively. The polar lipid pattern of strain IC35T consisted of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester; six unknown glycolipids and two minor phospholipids were also observed. The only quinone present was MK-8 (II-H2). The G+C content of the genomic DNA was 63.2 mol%. DNA–DNA hybridization studies (29 % hybridization with Halovivax limisalsi) as well as biochemical and physiological characterization, allowed strain IC35T to be differentiated from other species of the genus Halovivax. A novel species, Halovivax cerinus sp. nov., is therefore proposed to accommodate this strain. The type strain is IC35T (=IBRC-M 10256T=KCTC 4050T).

Haloarchaea in the order Halobacterales, family Halobacteriaceae are extreme halophiles requiring at least 1.5 M NaCl for growth (Grant et al., 2001). These microorganisms have a salt-in strategy in order to meet the osmotic challenges associated with life in hypersaline environments (Madigan & Oren 1999; Grant et al., 2001). Accordingly, they have enzymes that are active in up to 5 M, or in more concentrated, NaCl, or in 4 M KCl (Danson & Hough 1997). As most industrial processes are carried out under very harsh physico-chemical conditions, haloarchaeal hydrolytic enzymes seem to be very good candidates for industrial applications. These enzymes are not only active at high salt concentrations, but may also have excellent activities at high temperatures, low water activity and high pH (van den Burg 2003; Oren 2010). These potential applications of halophilic archaea in biotechnology have encouraged the study of the microbial diversity of hypersaline environments and permitted the isolation and characterization of novel haloarchaeal taxa during the few past decades. The family Halobacterales

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene and rpoB sequences of strain IC35T are KJ406208 and KM111181, respectively.

Two supplementary figures are available with the online Supplementary Material.
contains 47 genera with validly published names, as of July 2014 (www.bacterio.net; Euzéby, 1997; Parte, 2014). The genus *Halovivax* was proposed by Castillo *et al.* (2006) to accommodate an extremely halophilic archaeon, which was isolated from the saline Lake Ejinor in Inner Mongolia, China. Currently, the genus comprises three species, *Halovivax asiaticus* (type species of the genus), *Halovivax ruber* (Castillo *et al.*, 2007) and *Halovivax limisalsi* (Amoozegar *et al.*, 2014). Members of this genus are extremely halophilic and they do not contain phosphatidylglycerol sulphate in their polar lipid patterns (Castillo *et al.*, 2006, 2007; Amoozegar *et al.*, 2014). The aim of this study was to determine the taxonomic status of a novel strain isolated from a mud sample of the Aran-Bidgol salt lake, Iran and we propose this strain to be a representative of a novel species of the genus *Halovivax*.

Strain IC35\(^T\) was isolated from a mud sample (pH 7.5, 22 % total salts) of the hypersaline lake Aran-Bidgol, the largest playa in the centre of Iran (34° 26′ N, 51° 48′ E). The strain was isolated by diluting the sample in sterile 20 % (w/v) salt solution, plating on modified growth medium (MGM) with 23 % (w/v) total salt concentration (Dyall-Smith, 2009) and incubating at 40 °C aerobically. The 23 % MGM medium was prepared from 30 % (w/v) salt 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); 1.5 % (w/v) agar was used for solid media if necessary. The pH of the medium was adjusted to pH 7.2–7.4 with 2 M Tris-base (Merck). After successive cultivations a pure isolate, designated strain IC35\(^T\), was obtained and routinely grown on 23 % MGM solid medium at 40 °C. The strain was maintained on the same 23 % MGM slants at 4 °C and by lyophilization for short- and long-term preservation, respectively.

The characterization of this strain was achieved by following a polyphasic approach, according to the minimal standards recommended by Oren *et al.* (1997) for describing novel taxa of the order *Halobacterales*, including conventional phenotypic features, chemotaxonomic data (polar lipid and quinone composition) and molecular analysis (16S rRNA gene sequence similarity and DNA–DNA relatedness).

The type strains *Halovivax asiaticus* CECT 7098\(^T\), *Halovivax ruber* IBRC-M 10339\(^T\) and *Halovivax limisalsi* IBRC-M 10022\(^T\) were used as reference strains for positive and negative testing and for comparison with isolate IC35\(^T\).

The genomic DNA of the isolate was extracted with a DNA extraction kit (High Pure PCR Template Preparation kit, Roche) according to the manufacturer's protocol, and the 16S rRNA gene was amplified using the archaean universal primers: 21F (5′'-TTCCGTTGATCCTGCGGaA-3′') (DeLong, 1992) and 1492R (5′'-GGTACCTTGTACGACTT-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 with the pGEM-T vector, transformation of *Escherichia coli* DH5\(\alpha\) and selection of the transformants were carried out with the pGEM-T TA cloning kit (Promega), according to the manufacturer's protocol. Clones were randomly picked and sequencing was conducted on an ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Amplification and sequencing of the rpoB' gene was performed as described by Minegishi *et al.* (2010). Phylogenetic analysis was performed using the software package MEGA version 5 (Tamura *et al.*, 2011) after obtaining multiple alignments of data, 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).

Ten almost complete 16S rRNA gene sequences of strain IC35\(^T\) (1402 nt) were obtained. Sequence comparisons indicated that this strain has a single type of 16S rRNA gene. 16S rRNA gene sequence analysis revealed that strain IC35\(^T\) is a member of the genus *Halovivax*. The closest relative of strain IC35\(^T\) was *Halovivax limisalsi* IC38\(^T\), with a 16S rRNA gene sequence similarity of 97.3 %. The sequence similarities of the novel strain to other members of this genus were lower than 97 %, including *Halovivax asiaticus* EJ-46\(^T\) and *Halovivax ruber* XH-70\(^T\), which showed similarities of 96.6 % and 96.3 %, respectively. Phylogenetic analysis using the neighbour-joining algorithm revealed that the novel strain clustered with members of this genus, although in a separate clade (Fig. 1a). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1. The phylogenetic position was also confirmed in trees generated using the maximum-parsimony and maximum-likelihood algorithms (data not shown).

The rpoB' gene of strain IC35\(^T\) was sequenced and found to be 1833 bp in length. The nucleotide sequence was similar to the corresponding genes of *Halovivax limisalsi* IBRC-M 10022\(^T\), *Halovivax asiaticus* JCM 14624\(^T\) and *Halovivax ruber* JCM 13892\(^T\) (90.2 %, 90.2 % and 89.9 % similarity, respectively). The lowest similarity of the rpoB' gene sequence amongst species within this genus (89.9 % similarity) was higher than similarities with members of the genera: *Halococcus* (86.0 %), *Natrialba* (88.5 %), *Halorubrum* (88.5 %) or *Halalkalicoccus* (89.1 %) and also those with members of the genera *Haloterrigena* (90.1 %), *Natronococcus* (91.3 %), *Halobacterium* (91.4 %), *Halobiforma* (92.1 %), *Halofexa* (92.1 %), *Natrinema* (92.2 %) and *Haloarcula* (94.4 %) (Minegishi *et al.*, 2010). Phylogenetic tree reconstructions using the neighbour-joining algorithm supported strain IC35\(^T\) belonging to the genus *Halovivax* (Fig. 1b).
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 the method described by Dussault (1955). Strain IC35T was non-motile, rod-shaped and Gram-stain-negative (Fig. S1, available in the online Supplementary Material). Colonies formed on agar plates were circular, entire, smooth and cream.

Physiological tests were conducted using liquid or solid (1.5% agar) MGM medium as mentioned above, 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 OD600.

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene (a) and rpoB gene (b) sequences showing the position of strain IC35T among species of the genus *Halovivax* and some other related species within the family Halobacteriaceae. Accession numbers of sequences are given in parentheses. The sequence of the methanogenic archaeon *Methanospirillum hungatei* JF-1T (CP000254) was used as the outgroup. Bootstrap values (%), shown at nodes, are based on 1000 replicates. Bar, substitutions expected per nucleotide position.
temperature range for growth was examined in liquid 23% MGM medium at temperatures from 20 °C to 55 °C with 5 °C intervals. For experiments at different pH values a pH range of 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 requirements for NaCl and MgCl₂ for growth were determined in media containing 0–5 M NaCl (in 0.5 M increments) or 0–1 M MgCl₂ (in 0.05 M increments), respectively. Strain IC35ᵀ was capable of growing over a range of NaCl concentrations from 2.5–5.1 M (15–30%). MgCl₂ was not necessary for growth. Optimal growth occurred at pH 7.0 (range, 6.5–9.0), 40 °C (range, 30–50 °C), 3.4 M NaCl and 0.1 M MgCl₂.

Acid production from carbohydrates was tested in unbuffered 23% MGM liquid 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 23% MGM liquid medium and yeast extract concentration was reduced to 0.1 g l⁻¹. The requirement for NaCl and MgCl₂ for growth were determined in media containing 0–5 M NaCl (in 0.5 M increments) or 0–1 M MgCl₂ (in 0.05 M increments), respectively. Strain IC35ᵀ was capable of growing over a range of NaCl concentrations from 2.5–5.1 M (15–30%). MgCl₂ was not necessary for growth. Optimal growth occurred at pH 7.0 (range, 6.5–9.0), 40 °C (range, 30–50 °C), 3.4 M NaCl and 0.1 M MgCl₂.

The detailed physiological and biochemical characteristics of strain IC35ᵀ are listed in Table 1 and in the species description.

For determination of the DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). The DNA G+C content was determined by the HPLC method (Mesbah et al., 1989). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), incorporating the modifications described by Huss et al. (1983) using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multichannel changer and a temperature controller with an in situ temperature probe (Varian).

The G+C content of the DNA of strain IC35ᵀ was 63.2 mol%. This value is in the range reported for the genus Halovivax. The percentage of DNA–DNA reassociation of strain IC35ᵀ and its closest relative Halovivax limisalsi IBRC-M 10022ᵀ was 29% (31%, result of measurements in duplicate). According to the 70% threshold proposed by Wayne et al. (1987) for the discrimination of species using DNA–DNA relatedness, this result confirmed that the new isolate is a representative of a novel species.

The cell biomass for polar lipids and quinone analyses was obtained by cultivation in 23% MGM broth at 150 r.p.m. and 40 °C. Cells were harvested in the mid-exponential growth phase. Polar lipids were separated by two-dimensional silica gel TLC. Methods, including the solvents used in each direction and the detection reagents have been described by Hezayen et al. (2001). Respiratory lipoquinones were determined as previously described by Waino et al. (2000). The polar lipids of the novel isolate were phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Six unknown glycolipids and two minor phospholipids were also observed (Fig. S2). The polar lipids of the novel isolate were determined as previously described by Castillo et al., 2006, 2007; Amoozegar et al., 2014), except with respect to the number of glycolipids and minor phospholipids. The only quinone present in strain IC35ᵀ was MK-8 (II-H₂).

On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, it is proposed that strain IC35ᵀ should be classified as the type strain of a novel species within the genus Halovivax, for which we propose the name Halovivax cerinus sp. nov.

**Description of Halovivax cerinus sp. nov.**

*Halovivax cerinus* (ce.r'i′nus. L. masc. adj. *cerinus* wax-coloured, yellow-like wax, referring to the pigmentation of the colonies).

Cells are Gram-stain-negative, non-motile and rod-shaped, 0.5–0.7 × 1.5–2 μm. Colonies are circular, entire, smooth, cream and have a diameter of 1–2 mm on 23% MGM agar medium after 14 days of incubation at 40 °C. Extremely
halophilic. Optimal growth occurs at 3.4 M NaCl (range, 2.5–5.1 M), pH 7.0 (range, 6.5–9.0), 40 °C (range, 30–50 °C) and 0.1 M MgCl₂ (range, 0–1 M). Catalase-positive, but oxidase-negative. Skimmed milk is hydrolysed, while aesculin, casein, DNA, gelatin, Tweens 40, 60 and 80, and starch are not. Anaerobic growth occurs in the presence of arginine, DMSO and nitrate. Nitrate and nitrite are reduced, but the production of gas from nitrate is negative. Indole is produced, H₂S is not. Acid is produced from D-glucose, but not from other carbohydrate sources including D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, sucrose, trehalose and D-xylose. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Methyl red test is positive but 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, six unknown glycolipids and two minor phospholipids.

The type strain is IC35T (=IBRC-M 10256T=KCTC 4050T), which was isolated from Aran-Bidgol salt lake, Iran. The DNA G+C content of the only strain of the species is 63.2 mol% (HPLC method).

**Table 1. Differential characteristics between strain IC35T and phylogenetically related species within the genus Halovivax**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain IC35T</th>
<th>Strain IC35T (Halovivax cerinus sp. nov.); 2, Halovivax limisalsi IBRC-M 10022T (data from the present study); 3, Halovivax asiaticus CECT 7098T; 4, Halovivax ruber IBRC-M 10339T. All data from the present study, except where indicated. +, Positive; −, negative.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.5–0.7 × 1.5–2</td>
<td>0.9–1.3 × 1-2</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Cream</td>
<td>Cream</td>
</tr>
<tr>
<td>Optimum NaCl concn (M)</td>
<td>3.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>40.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Oxidase</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Anaerobic growth in the presence of: Arginine</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolysis of: Gelatin</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acid from mannitol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Production of: H₂S</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Susceptibility to: Amoxicillin (10 μg)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tetracycline (30 μg)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>63.2</td>
<td>62.6</td>
</tr>
</tbody>
</table>

*Data from Castillo et al. (2006).
†Data from Castillo et al. (2007).

Acknowledgements

This work was supported by grants from the Research Council, University of Tehran and Iranian Biological Resource Centre (IBRC) (MI-1388-01) (M. A. A.), from Spanish Ministerio de Economı́a y Competitividad that includes European Funds (FEDER) (CGL2013-46941-P) and Junta de Andalucı́a (P10-CVI-6226) (A. V.).

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