**Halorientalis persicus** sp. nov., an extremely halophilic archaeon isolated from a salt lake and emended description of the genus *Halorientalis*

Mohammad Ali Amoozegar,1,2 Ali Makhdoumi-Kakhki,3 Maliheh Mehrshad,1,2 Seyed Abolhassan Shahzadeh Fazeli,2 Cathrin Spröer4 and Antonio Ventosa5

Correspondence
Mohammad Ali Amoozegar
amoozegar@ut.ac.ir or amoozegar@ibrc.ir

1Extremophiles Laboratory, Department of Microbiology, Faculty of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran
2Microorganisms Bank, Iranian Biological Resource Centre (IBRC), ACECR Tehran-Iran
3Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
4Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany
5Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

An extremely halophilic archaeon, strain D108T, was isolated from a brine sample of Aran-Bidgol salt lake in Iran. The novel strain was cream-pigmented, motile, pleomorphic rod-shaped and required at least 2.5 M NaCl but not MgCl₂ for growth. Optimal growth was achieved with 4.3 M NaCl and 0.1 M MgCl₂. The optimum pH and temperature for growth were pH 7.5 and 40 °C, respectively, and the strain was able to grow over a pH range of 6.5 to 9.0, and a temperature range of 30 to 50 °C. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain D108T clustered with the type strain of the sole species of the genus *Halorientalis*, *Halorientalis regularis* TNN28T, with a sequence similarity of 98.8 %. The polar lipid pattern of strain D108T consisted of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, one phosphoglycolipid and two glycolipids. The only quinone present was MK-8(II-H₂). The DNA G+C content of strain D108T was 62.8 mol%. DNA–DNA hybridization studies (45 % with *Halorientalis regularis* IBRC-M 10760T), as well as biochemical and physiological characterization, allowed strain D108T to be differentiated from *Halorientalis regularis*. A novel species of the genus *Halorientalis*, *Halorientalis persicus* sp. nov., is therefore proposed to accommodate this strain. The type strain is D108T (=IBRC-M 10043T=CECT 8375T). An emended description of the genus *Halorientalis* is also proposed.

The genus *Halorientalis* was proposed by Cui et al. (2011) to accommodate an extremely halophilic archaeon isolated from an artificial marine solar saltern in Eastern China. The only species of this genus, *Halorientalis regularis*, is red-pigmented, motile and pleomorphic rod-shaped (Cui et al., 2011). During a study of the microbial population in Aran-Bidgol salt lake, a thalasohaline lake in Iran (35° 70’ N 51° 39’ E), a cream-pigmented, extremely halophilic archaeal strain, designated D108T, was isolated.

On the basis of 16S rRNA gene sequence analysis, strain D108T is closely related to the sole member of the genus *Halorientalis*. The aim of the present work was to determine the exact taxonomic position of strain D108T by using a polyphasic approach that combined phenotypic, chemotaxonomic, phylogenetic and genotypic analyses. These results indicate that strain D108T represents a novel species of the genus *Halorientalis*.

Strain D108T was isolated from a water sample (pH 7.1, salinity 31 % w/v) obtained from the Aran-Bidgol salt lake in Iran. The environmental sample was inoculated in modified growth medium (MGM) with 23 % (w/v) total salt concentration (Dyall-Smith, 2008). It contains a 23 % (w/v) salt mixture prepared from 30 % (w/v) stock.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain D108T is KF313134.

Two supplementary figures are available with the online version of this paper.
Haloritalis persicus sp. nov.

The genomic DNA of the isolate was extracted as described by Lam in the Halohandbook (Dyall-Smith, 2008) for halarchaea and the 16S rRNA gene was amplified using the archaeal universal primers: 21F (5'-TTCCGAGTT-GATCCCGCCGA-3') (DeLong, 1992) and 1492R (5'-GGTACCTGTAGACCTT-3') (Lane et al., 1985). The PCR conditions were: 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 DH5α and selection of transformants were carried out with the pGEM-T TA cloning kit (Promega), according to the manufacturer’s protocol. Several clones were randomly picked and sequencing was conducted on an ABI 3730XL DNA sequencer at Macrogen, to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. 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).

Fifteen almost-complete 16S rRNA gene sequences (1445 nt each) of strain D108T were obtained. Sequence comparisons indicated that this strain had one distinct 16S rRNA gene. 16S rRNA gene sequencing revealed that strain D108T is a member of the family Halobacteriaceae, and showed a high degree of similarity (98.8%) to the type strain of the type species of the genus Haloritalis, Haloritalis regularis TNN28T, followed by similarities of 93.9% to Halomicrobium zhouii TBN51T, 93.5% to Halorhabdus utahensis DSM 12940T and 93.5% to Halosimplex carlsbadense 2-9-1T. Phylogenetic analysis using the neighbour-joining algorithm revealed that the novel strain clustered with Haloritalis regularis TNN28T (Fig. 1). The phylogenetic position was also confirmed in trees generated using the minimum-evolution and maximum-parsimony algorithms.

Physiological tests were conducted using liquid or solid (1.5% agar) MGM, as mentioned previously, 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. The temperature range for growth was examined in liquid MGM at temperatures from 20°C to 55°C in 5°C intervals. For growth experiments at different pH, the range of pH 5.0–9.5 was tested; the buffers MES (for pH 5–6.5), HEPES (pH 7–8) and CHES (pH 8.5–9.5) were added at a concentration of 50 mM. NaCl and MgCl2 requirements 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 D108T grew over a temperature range of 30–50°C (optimum 40°C) and a pH range of 6.5–9.0 (optimum pH 7.5). Routine cultivation was conducted at 40°C and pH 7.0. Strain D108T was capable of growing over a wide range of NaCl concentrations: from 2.5 M (15%) to 5.1 M (30%); it grew optimally in the presence of 4.3 M (25%) NaCl. MgCl2 was not required for growth, but optimal growth occurred in the presence of 0.1 M MgCl2. Acid production from carbohydrates was tested in buffered MGM 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 and yeast extract concentration was reduced to 0.1 g l⁻¹ (Oren et al., 1997). The ability of strain D108T 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 prepared anaerobically in serum tubes, according to the procedures described by Bryant (1972) and Balch & Wolfe (1976). Growth and gas formation with nitrate as an electron acceptor were tested in 10 ml stoppered tubes, completely filled with liquid growth medium to which NaNO3 (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 Gutierrez & Gonzalez (1972). Casein, gelatin and starch were used in substrate medium to which NaNO3 (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 Oren et al.
Tests for catalase and oxidase activities were performed as described by González et al. (1978). Production of H₂S was tested by growing strain D108ᵀ in liquid MGM supplemented with 0.5 % (w/v) Na₂S₂O₃ (Oren et al., 1997). Tryptone water medium supplemented with 23 % (w/v) total salts was used to detect indole production (Smibert & Krieg, 1994). Susceptibility to antimicrobials was determined by the disc diffusion method after spreading the strain on solid MGM (Oren et al., 1997).

Strain D108ᵀ was catalase- and oxidase-positive. It could hydrolyse Tween 80 but not aesculin, casein, DNA, gelatin, starch or Tweens 20, 40 and 60. Strain D108ᵀ utilized D-fructose, D-glucose, lactose, maltose and sucrose as sole carbon sources. Strain D108ᵀ was susceptible to the following antimicrobial compounds: anisomycin (35 µg), bacitracin (10 U), nitrofurantoin (300 µg), novobiocin (5 µg) and rifampicin (5 µg), but resistant to amoxicillin (25 µg), ampicillin (10 µg), cephalexin (30 µg), carbencillin (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 D108ᵀ are listed in Table 1 and in the species description.

The DNA G+C content was determined by the HPLC method (Mesbah et al., 1989). DNA–DNA hybridization experiments (as described by De Ley et al., 1970 and modified by Huß et al., 1983) were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The DNA G+C content of strain D108ᵀ was 62.8 mol%. This value is slightly higher than that reported for Halorientalis regularis (61.9 mol%), the type species of the genus Halorientalis (Cui et al., 2011). DNA–DNA hybridization experiments between strain D108ᵀ and Halorientalis regularis IBRC-M 10760ᵀ produced a DNA–DNA relatedness value of 45 %. According to the 70 % threshold proposed by Wayne et al. (1987) for the delineation of species using DNA–DNA relatedness, the results confirmed that the isolate constituted a novel species.

Polar lipid composition and respiratory quinones were determined using the services of the DSMZ. Polar lipids were separated by two-dimensional silica gel TLC. Methods including solvents in each direction and detection reagents were as described by Hezayen et al. (2001). Phosphatidylglycerol and phosphatidylglycerol phosphate methyl

### Table 1. Characteristics that distinguish strain D108ᵀ from Halorientalis regularis IBRC-M 10760ᵀ

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.7–1 x 1–2</td>
<td>0.5–1 x 1–6*</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Cream</td>
<td>Red</td>
</tr>
<tr>
<td>Optimum NaCl concentration (M)</td>
<td>4.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Mg²⁺ requirement</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Optimum MgCl₂ concentration(M)</td>
<td>0.1</td>
<td>0.01–0.3</td>
</tr>
<tr>
<td>H₂S production</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>62.8</td>
<td>61.9*</td>
</tr>
</tbody>
</table>

*Data from Cui et al. (2011).
ester were detected in strain D108\textsuperscript{T}. One phosphoglycolipid and two glycolipids yet to be identified were also observed (Fig. S2). This polar lipid pattern was similar to the type species of the genus except with respect to the number of glycolipids (Cui et al., 2011). Respiratory lipoquinones were analysed as previously described by Wainø et al. (2000). MK-8(II-H\textsubscript{2}) was the only respiratory lipoquinone present in the novel strain and also in the type strain of the type species of the genus, Halorientalis regularis IBRC-M 10760\textsuperscript{T}.

In conclusion, on the basis of the results of the phylogenetic, genotypic, chemotaxonomic and phenotypic analyses, strain D108\textsuperscript{T} represents a novel species of the genus Halorientalis, for which we propose the name Halorientalis persicus sp. nov. On the basis of the data obtained in this study, an emended description of the genus Halorientalis is also proposed.

**Emended description of the genus Halorientalis Cui et al. 2011**

The description of the genus Halorientalis is as given by Cui et al. (2011) with the following amendment. The only quinone present is MK-8(II-H\textsubscript{2}).

**Description of Halorientalis persicus sp. nov.**

*Halorientalis persicus* (per’si cus. L. masc. adj. persicus of Persia).

Cells are Gram-stain-negative, motile, pleomorphic rods with a width of 0.7–1.0 \( \mu \)m and a length of 1.0–2.0 \( \mu \)m. Colonies are circular, entire, smooth and cream-pigmented with a diameter of 2.0 mm on 23 % MGM agar after 14 days of incubation at 40 °C. Extremely halophilic. Growth occurs over a wide range of NaCl concentrations from 2.5–5.0 M (15–30 % w/v) NaCl. MgCl\textsubscript{2} is not required for growth. Optimal growth occurs at pH 7.5 (range, pH 6.5–9.0), 40 °C (range, 30–50 °C), 4.3 M NaCl and 0.1 M MgCl\textsubscript{2}. Catalase- and oxidase-positive. Tween 80 is hydrolysed but aesculin, DNA, gelatin, Tewsens 20, 40 and 60, casein and starch are not hydrolysed. Anaerobic growth does not occur in the presence of arginine, nitrate or DMSO. Nitrate is reduced without the production of gas. Indole and H\textsubscript{2}S are not produced. Acid is produced from D-glucose, but not from D-fructose, D-mannitol, D-galactose, lactose, sucrose, maltose, trehalose, D-mannose or D-sylxose. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Methyl red test is positive but Voges–Proskauer test is negative. The following compounds are utilized as a sole source of carbon and energy: D-fructose, D-glucose, lactose, maltose and sucrose. The following compounds are not utilized as a sole source of carbon and energy: D-galactose, D-mannitol, D-ribose and glycerol. Polar lipids include phosphatidylglycerol, phosphatidyglycerol phosphate methyl ester, one phosphoglycolipid and two glycolipids. MK-8(II-H\textsubscript{2}) is the only respiratory lipoquinone present.

The type strain is D108\textsuperscript{T} (=IBRC-M 10043\textsuperscript{T}=CECT 8375\textsuperscript{T}), which was isolated from Aran-Bidgol salt lake, Iran. The DNA G+C content of the type strain is 62.8 mol% (HPLC method).

**References**


