Halovenus aranensis gen. nov., sp. nov., an extremely halophilic archaeon from Aran-Bidgol salt lake

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A novel red-pigmented halophilic archaeon, strain EB27^T, was isolated from Aran-Bidgol salt lake, a hypersaline playa in Iran. Cells of strain EB27^T were non-motile and pleomorphic (rods to triangular or disc-shaped). Strain EB27^T required at least 2.5 M NaCl and 0.1 M $MgCl_2$ for growth. Optimal growth was achieved at 4 M NaCl and 0.5 M $MgCl_2$. The optimum pH and temperature for growth were pH 7.5 and 40 °C; it was able to grow at pH 6.0–8.0 and 25–50 °C. 16S rRNA gene sequence analysis showed that strain EB27^T is a member of the family *Halobacteriaceae*; however, levels of 16S rRNA gene sequence similarity were as low as 90.0, 89.3 and 89.1 % to the most closely related haloarchaeal taxa, namely *Halalkalicoccus tibetensis* DS12^T, *Halosimplex carlsbadense* 2-9-1^T and *Halorhabdus utahensis* AX-2^T, respectively. The DNA G+C content of strain EB27^T was 61 mol%. Strain EB27^T contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, common phospholipids found in haloarchaea, together with two minor phospholipids. The only quinone present was MK-8(II-H_2). Physiological, biochemical and phylogenetic differences between strain EB27^T and recognized genera of extremely halophilic archaea suggest that this strain represents a novel species in a new genus within the family *Halobacteriaceae*, for which the name *Halovenus aranensis* gen. nov., sp. nov. is proposed. The type strain of *Halovenus aranensis*, the type species of the new genus, is strain EB27^T (=IBRC-M 10015^T=CGMCC 1.11001^T).

Halophilic archaea which need at least 1.5 M NaCl for growth are classified within the family *Halobacteriaceae* in the order *Halobacteriales* (Grant *et al.*, 2001). The characteristic pink to red pigmentation of haloarchaea permitted their detection early in the 20th century, especially in salted foods (Clayton & Gibbs, 1927; Harrison & Kennedy, 1922; Lochhead, 1934). Hypersaline environments, which are widespread worldwide, are a common source for haloarchaea. Advances in molecular techniques and conventional culturing approaches have led to numerous diversity studies in these extreme habitats. In recent decades, many new haloarchaea have been characterized from these environments (Oren, 2008). There are several hypersaline lakes in Iran, both thalassohaline and athalassohaline, as well as artificial crystallizer ponds, which have not been characterized from a microbiological standpoint. During a study of the microbial population in one of these salt lakes, Aran-Bidgol, a large number of extremely halophilic archaea were isolated. Here we present the isolation and polyphasic characterization of a novel halophilic archaeal strain that is considered to represent a novel species of a new genus in the family *Halobacteriaceae*.

Aran-Bidgol salt lake is located in the central desert of Iran at an altitude of 800 m and 1000 km from the coast (35° 70′ 47′′ N 51° 39′ 62′′ E). This playa was formed as a result of deposition of halite sediments over different geological periods. Sediments are dissolved by rainfall during the winter and salt is produced as a result of evaporation during the dry season and subsequently harvested commercially. The predominant salts in the lake are NaCl, Na_2SO_4, $MgCl_2$ and $MgSO_4$ with trace carbonates, and it can be considered as a thalassohaline lake. The pH of the brine in the lake is neutral (about pH 7.0) and salinity reaches saturation during the dry season. The water temperature was 38 °C at the time of sampling. Sampling was carried out in the dry season (July–November 2007)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EB27^T is HQ197980.

Three supplementary figures are available with the online version of this paper.

and water and sediment samples were collected in sterile plastic containers. Modified growth medium (MGM) with 23 % total salt concentration was used for isolation of micro-organisms from the lake (Dyall-Smith, 2006). This medium contains a 23 % salt mixture prepared from 30 % stock solution, which consists of (per litre): 240 g NaCl, 35 g $MgSO_4 . 7H_2O$, 30 g $MgCl_2 . 6H_2O$, 7 g KCl and 1 g $CaCl_2 . 2H_2O$, supplemented with 1 % (w/v) peptone (Merck) and 0.2 % (w/v) yeast extract (Merck); 1.5 % (w/v) agar was used for solidified media if necessary. The pH of media was adjusted to 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 cultivation, a pure isolate, designated strain EB27^T, was obtained. Characterization of this strain was achieved following the minimal standards recommended by Oren *et al.* (1997) for describing novel taxa of the order *Halobacteriales*. *Haloferax volcanii* DSM 3754^T was used as a reference strain in subsequent testing in this regard.

Cell morphology and motility were examined with 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 according to the method outlined by Dussault (1955). Physiological tests were conducted by 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 OD_{600}. Growth temperature range was examined in liquid MGM medium from 20 to 60 °C at 5 °C intervals. Growth was tested at pH 5.0–9.0; MES (pH 5–6.5), HEPES (pH 7–8) and CHES (pH 8.5–9) buffers 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 intervals) or 0–1 M $MgCl_2$ (0.05 M intervals), respectively.

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 unit. To test for carbon source utilization, peptone was omitted from MGM medium and yeast extract concentration was reduced to 0.1 g l^{-1} (Oren *et al.*, 1997). The ability of strain EB27^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, completely filled 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). Hydrolysis of casein, gelatin and starch 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_2S was tested by growing strain EB27^T in MGM liquid medium supplemented with 0.5 % (w/v) $Na_2S_2O_3$ (Oren *et al.*, 1997). Tryptone water medium was used for detection of indole production (Smibert & Krieg, 1994). Antimicrobial sensitivity was determined by the disc diffusion method after spreading the strain on solid MGM medium (Oren *et al.*, 1997).

Cells of strain EB27^T were non-motile and pleomorphic (rods to triangles, squares or disc-shaped; Fig. 1) and stained Gram-negative. Colonies formed on solid MGM medium were small (about 1.0 mm), convex, round, with an entire edge, shiny and intensely red-pigmented. Strain EB27^T was able to grow over a range of NaCl concentrations from 2.5 to 5 M, with optimal growth at 4 M NaCl. Magnesium was required for growth within the range 0.1–1 M (optimum growth at 0.5 M). The growth pH range was 6.0–8.0 (optimum growth at pH 7.5) and the isolate grew at 25–50 °C (optimum growth at 40 °C). Strain EB27^T was catalase- and oxidase-positive. It did not hydrolyse gelatin, starch or Tweens 20, 40, 60 and 80. Acid was produced from D-glucose but not from D-galactose, sucrose, D-fructose, D-xylose, D-mannitol, D-mannose, trehalose, D-arabinose or lactose. Strain EB27^T utilized D-glucose and D-galactose as single carbon source but not lactose, L-glycine, L-proline or L-cysteine. The detailed physiological and biochemical characteristics of strain EB27^T are given in Table 1 as well as in the genus and species descriptions below.

The genomic DNA of the new isolate was extracted as described by Lam in the Halohandbook (Dyall-Smith, 2006) for haloarchaea and the 16S rRNA gene was amplified by using archaeal universal primers 21F (DeLong, 1992) (5′-TTCCGGTTGATCCYGCCGGA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane *et al.*, 1985). PCR products were purified with the DNA purification kit

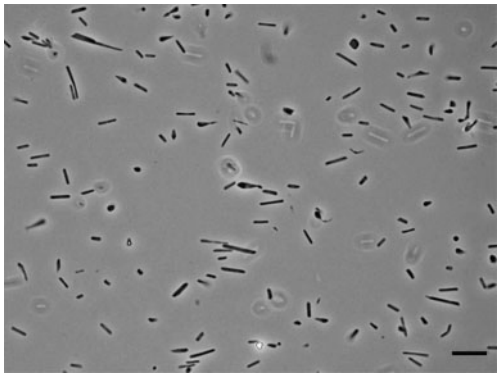

Fig. 1. Phase-contrast photomicrograph of cells of strain EB27^T. Bar, 10 μm.

Table 1. Differential characteristics of strain EB27^T and strains of type species of closely related genera within the order *Halobacteriales*

Taxa: 1, strain EB27^T (data from this study); 2, *Halalkalicoccus tibetensis* DS12^T (Xue *et al.*, 2005); 3, *Halosimplex carlsbadense* 2-9-1^T (Vreeland *et al.*, 2002); 4, *Halorhabdus utahensis* AX-2^T (Wainø *et al.*, 2000); 5, *Haloterrigena turkmenica* VKM B-1734^T (Ventosa *et al.*, 1999); and 6, *Halobacterium salinarum* DSM 3754^T (Grant *et al.*, 2001).

Character	1	2	3	4	5	6
Cell shape	Pleomorphic/rods	Cocci	Rods	Rods	Cocci	Small rods
Cell size (μm)	0.6–1.3 × 4.9–10.7	1–1.5	0.95 × 5	0.5–1 × 2–10	1.5–2.0	0.5–1.0 × 1.0–6.0
Motility	−	−	+	+	−	+
Pigmentation	Red	Orange	Pink to red	Red	Red	Red
Optimum NaCl concentration (M)	4	3.4	4.3	4.6	3–4	3.4–4.3
Mg^{2+} requirement	+	−	+	+	+	+
Lysis in distilled water	+	−	+	+	−	+
Optimum temperature (°C)	40	40	37–40	50	30–40	50
Growth at:						
pH 7	+	−	+	+	+	+
pH 10	−	+	−	−	−	−
Nitrate reduction to nitrite	−	+	−	+	+	+
Acid from D-glucose	+	−	−	+	+	−
Hydrolysis of gelatin	−	−	−	−	−	+
Indole production	−	−	−	−	−	+
Presence of glycolipids	−	−	+	+	+	+
DNA G+C content (mol%)	61.0	61.5	64.4	64.0	59.8	67.1
16S rRNA gene sequence similarity to strain EB27^T (%)	100	90.0	89.3	89.1	88.7	87.3

(Roche) according to the manufacturer's protocol. The 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 the transformants were carried out with the pGEM-T TA cloning kit (Promega) according to the manufacturer's protocol. Several clones were randomly picked and then sequenced by the service of Macrogen Company, South Korea, to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. Phylogenetic analysis was performed by using the software package MEGA version 4 (Tamura *et al.*, 2007) after obtaining multiple alignments of the data available from public databases via CLUSTAL X (Thompson *et al.*, 1997). Clustering was performed with 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 based on 1000 resamplings (Felsenstein, 1985).

Fifteen almost-complete 16S rRNA gene sequences (each 1440 nt) of strain EB27^T were obtained. Comparisons indicated that this strain has one type of 16S rRNA gene sequence. 16S rRNA gene sequencing showed that strain EB27^T is a member of the family *Halobacteriaceae*; levels of similarity were as low as 90, 89.3 and 89.1 % to its most closely related haloarchaeal taxa, namely *Halalkalicoccus tibetensis* DS12^T, *Halosimplex carlsbadense* 2-9-1^T and *Halorhabdus utahensis* AX-2^T, respectively. Phylogenetic analysis by using the neighbour-joining algorithm revealed that the strain clustered in a separate clade (Fig. 2). This phylogenetic position was also confirmed in the trees generated with the minimum-evolution and maximum-parsimony algorithms (see Figs S1 and S2 in IJSEM Online).

The DNA G+C content was determined by the HPLC method (Mesbah *et al.*, 1989). The DNA G+C content of strain EB27^T was 61.0 mol%, which is lower than the values reported for *Halosimplex* and *Halorhabdus* but similar to that for *Halalkalicoccus* (Table 1).

Polar lipid composition and respiratory quinones were determined/identified by using the services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen and Dr Brian Tindall (Braunschweig, Germany). 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). Strain EB27^T contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two minor phospholipids (Fig. S3). Polar lipid compositions are one of the important differential features in haloarchaeal taxonomy. Non-alkaliphilic haloarchaea contain a variety of glycolipids, whereas haloalkaliphilic archaea have a comparatively simple polar lipid pattern and do not contain any glycolipids (Torreblanca *et al.*, 1986; Kamekura & Dyall-Smith, 1995). Strain EB27^T, a non-alkaliphilic haloarchaeon, showed a simple pattern of polar lipids and did not contain any glycolipid derivatives. The minor phospholipids associated with this novel strain

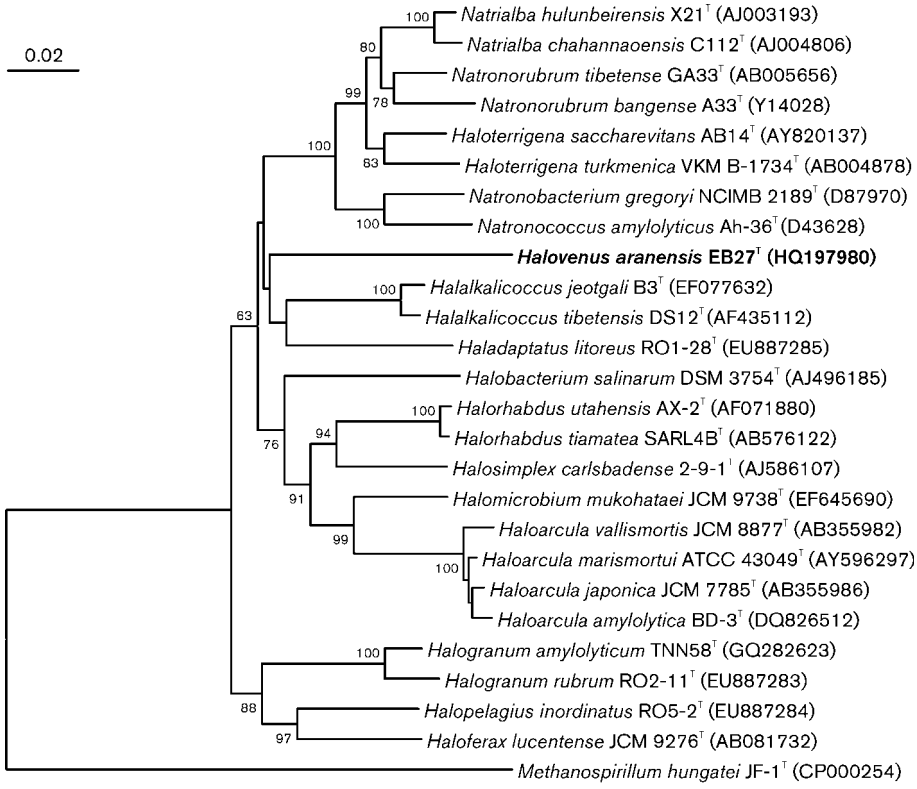

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain EB27^T and close relatives within the family *Halobacteriaceae*. Accession numbers of the sequences are given in parentheses. The sequence of the methanogenic archaeon *Methanospirillum hungatei* JF-1^T (CP000254) was used as an outgroup. Numbers at nodes are bootstrap values (percentages of 1000 replicates). Bar, 0.02 substitutions per nucleotide position.

were not detected in the haloalkaliphilic genus *Halalkalicoccus*, to which it is closely related phylogenetically (Xue *et al.*, 2005). This result indicates a difference between strain EB27^T and its closest relatives in the family *Halobacteriaceae*. Respiratory lipoquinones were analysed as described by Wainø *et al.* (2000). MK-8(II-H_2) was the only respiratory lipoquinone present.

In conclusion, the morphological and physiological properties of the novel isolate, low levels of 16S rRNA gene sequence similarity with members of other genera within the family *Halobacteriaceae* and the distinctive pattern of polar lipids suggest that strain EB27^T represents a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halovenus aranensis* gen. nov., sp. nov. is proposed.

Description of *Halovenus* gen. nov.

Halovenus (Ha.lo.ve′nus. Gr. n. *hals halos* salt; L. fem. n. *venus* beauty, grace, elegance; N.L. fem. n. *Halovenus* a salt-loving beauty, reflecting the attractive appearance of colonies).

Cells are non-motile and pleomorphic (rods to triangles, squares or disc-shaped). Gram-stain-negative, strictly aerobic and extremely halophilic. Cells lyse in distilled water. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Strictly aerobic; oxygen is used as the terminal electron acceptor. Cells contain phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two minor phospholipids. MK-8(II-H_2) is the only lipoquinone present. Phylogenetically related to the genera *Halalkalicoccus*, *Halosimplex* and *Halorhabdus* in the family *Halobacteriaceae*. The type species is *Halovenus aranensis*. *Hvn.* is proposed as the three-letter abbreviation.

Description of *Halovenus aranensis* sp. nov.

Halovenus aranensis (a.ra.nen′sis. N.L. fem. adj. *aranensis* belonging to Aran-Bidgol salt lake, from where the type strain was isolated).

Has the following properties in addition to those given for the genus. Cells are 0.6–1.3 × 4.9–10.7 μm when grown in MGM liquid medium at optimum conditions. Colonies are small (about 1.0 mm in diameter), shiny and intensely red-pigmented. Growth occurs at NaCl concentrations of 2.5–5.0 M (optimum 4.0 M), at Mg^{2+} concentrations of 0.2–1.0 M (optimum 0.5 M), at pH 6.0–8.0 (optimum pH 7.5)

and at 25–50 °C (optimum 40 °C). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite and gas formation from nitrate are not observed. Does not hydrolyse Tweens 20, 40, 60 or 80, casein, gelatin or starch. Utilizes D-glucose and D-galactose as carbon sources for growth but not lactose, L-glycine, L-proline or L-cysteine. Produces acid from D-glucose, but not from D-galactose, sucrose, D-fructose, D-xylose, D-mannitol, D-mannose, trehalose, D-arabinose or lactose. Indole is not produced. H_2S is not produced from thiosulfate. Sensitive to (μg per disc unless otherwise noted) bacitracin (10 U), nitrofurantoin (300), novobiocin (30), polymixin B (100 U), rifampicin (5), streptomycin (10) and anisomycin (30), but resistant to amikacin (30), amoxicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (30), kanamycin (30), tetracycline (30), tobramycin (10), nalidixic acid (30), cephalothin (30), penicillin G (10 U), ampicillin (10) and neomycin (10).

The type strain, EB27^T (=IBRC-M 10015^T=CGMCC 1.11001^T), was isolated from Aran-Bidgol salt lake, Iran. The DNA G+C content of the type strain is 61.0 mol% (as determined by HPLC).

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