Haloferax sulfurifontis sp. nov., a halophilic archaeon isolated from a sulfide- and sulfur-rich spring

Mostafa S. Elshahed,[1] Kristen N. Savage,[1] Aharon Oren,[2] M. Carmen Gutierrez,[3] Antonio Ventosa[3] and Lee R. Krumholz[1]

Correspondence
Mostafa S. Elshahed
mostafa@ou.edu

[1]Department of Botany and Microbiology, and Institute of Energy and the Environment, University of Oklahoma, Norman, OK 73019, USA

[2]The Institute of Life Sciences and the Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

[3]Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain

A pleomorphic, extremely halophilic archaeon (strain M6^T) was isolated from a sulfide- and sulfur-rich spring in south-western Oklahoma (USA). It formed small (0·8–1·0 mm), salmon pink, elevated colonies on agar medium. The strain grew in a wide range of NaCl concentrations (6 % to saturation) and required at least 1 mM Mg^{2+} for growth. Strain M6^T was able to reduce sulfur to sulfide anaerobically. 16S rRNA gene sequence analysis indicated that strain M6^T belongs to the family *Halobacteriaceae*, genus *Haloferax*; it showed 96·7–98·0 % similarity to other members of the genus with validly published names and 89 % similarity to *Halogeometricum borinquense*, its closest relative outside the genus *Haloferax*. Polar lipid analysis and DNA G+C content further supported placement of strain M6^T in the genus *Haloferax*. DNA–DNA hybridization values, as well as biochemical and physiological characterization, allowed strain M6^T to be differentiated from other members of the genus *Haloferax*. A novel species, *Haloferax sulfurifontis* sp. nov., is therefore proposed to accommodate the strain. The type strain is M6^T (=JCM 12327^T=CCM 7217^T=DSM 16227^T=CIP 108334^T).

Members of the family *Halobacteriaceae* are the dominant heterotrophic micro-organisms in hypersaline ecosystems (NaCl concentrations greater than 25 %), including saltern crystallizer ponds, the Dead Sea and other hypersaline lakes (Grant *et al.*, 2001; Oren, 2002). A shared character of all members of the family is the absolute requirement for high concentrations of NaCl.

The genus *Haloferax* was first described by Torreblanca *et al.* (1986) and currently comprises six species with validly published names: *Haloferax volcanii* (Mullakhanbhai & Larsen, 1975), *Haloferax denitrificans* (Tomlinson *et al.*, 1986), *Haloferax gibbonsii* (Juez *et al.*, 1986), *Haloferax mediterranei* (Rodriguez-Valera *et al.*, 1983), *Haloferax alexandrinus* (Asker & Ohta, 2002) and *Haloferax lucentense* (Gutierrez *et al.*, 2002). Members of the genus *Haloferax* are characterized by extreme pleomorphism and a relatively low salt requirement compared with other genera of the *Halobacteriaceae*.

In spite of the fact that members of the *Halobacteriaceae* are generally considered to have an absolute requirement for at least 1·5 M NaCl for growth, a few studies have demonstrated that members of the family may be isolated from a variety of low-salt environments. Rodriguez-Valera *et al.* (1979) isolated *Halococcus* species from sea water. Also, *Natrinema* isolates have been recovered from low-salt saltern ponds (McGenity *et al.*, 1998). In addition, several culture-independent analyses have detected 16S rRNA gene sequences suggestive of the presence of *Halobacteriaceae* species in other low-salt environments, including coastal marshes (Munson *et al.*, 1997) and deep-sea vent black smoker chimney structures (Takai *et al.*, 2001). In the course of our studies of microbial diversity in the sulfide- and sulfur-rich Zodletone spring (Oklahoma, USA), 16S rRNA genes were cloned from microbial mats (Elshahed *et al.*, 2004). Clone libraries were observed to contain many clones related to the family *Halobacteriaceae*, even though the spring has a stream-water salinity of only 1 %. Field

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M6^T is AY458601.

Scanning electron micrographs showing cells of strain M6^T are available as supplementary material in IJSEM Online.

measurements indicated the presence of relatively high salt habitats near the shallow stream. These habitats presumably provide a suitable environment for members of the *Halobacteriaceae* to survive and grow (Elshahed *et al.*, 2004). Eighteen halophilic strains were isolated from the microbial mats and mineral crusts that form near the stream using high-salt medium supplemented with antibiotics to suppress the growth of halophilic or halotolerant bacteria (Elshahed *et al.*, 2004). Six of these strains were studied and a preliminary investigation (colony and cell morphology, salt tolerance profile, lipid pattern and partial 16S rRNA gene sequence) suggested that all six isolates belong to a single species. In this paper, one of the isolates, strain M6^T, has been characterized in detail; it is proposed that this strain represents a novel species of the genus *Haloferax*.

The isolation procedure was described previously in detail (Elshahed *et al.*, 2004). Characterization of strain M6^T followed the guidelines outlined by Oren *et al.* (1997) for describing novel species of the *Halobacteriaceae*. Physiological, biochemical and nutritional tests were performed in halophilic medium (HM), pH 7·0, adapted from Oren (2002), containing (g l^{-1}): NaCl (150), $MgCl_2.7H_2O$ (20), K_2SO_4 (5), $CaCl_2.2H_2O$ (0·1) and yeast extract (5). Cultures were incubated at 37 °C with shaking (200 r.p.m.) unless otherwise specified. Growth was monitored by measuring the increase in OD_{600}. Growth was evaluated at NaCl concentrations of 0–37 % (saturation), Mg^{2+} concentrations of 0–200 mM, a temperature range of 4–60 °C and at pH 3–11. Suitable organic buffers were included in the pH range and pH optimum determination experiments (25 mM MES, MOPS, HEPES or TES) to prevent changes in pH due to acid production during growth. Substrate utilization was tested by lowering the yeast extract concentration to 0·1 g l^{-1}, including the substrate at a concentration of 0·5 g l^{-1} and adding 25 mM MOPS as a buffer. Acid production from a variety of substrates was tested in a similar, but unbuffered HM medium, with 0·5 g substrate l^{-1} and phenol red (0·004 g l^{-1}) as an indicator. Tests for the ability of strain M6^T to grow utilizing S^0, $S_2O_3^{2-}$, SO_4^{2-}, NO_3^-, DMSO and TMAO as electron acceptors, as well as its ability to ferment arginine, were performed in HM prepared anaerobically in serum tubes (Balch & Wolfe, 1976; Bryant, 1972). SO_4^{2-}, $S_2O_3^{2-}$ and NO_3^- were added at a concentration of 30 mM and DMSO, TMAO and arginine were added at a concentration of 5 g l^{-1}. SO_4^{2-} and $S_2O_3^{2-}$ reduction under anaerobic conditions was determined by quantification of the electron acceptors using a Dionex ion chromatography system. Aerobic thiosulfate reduction was determined by the formation of black sulfide precipitate in HM medium amended with 0·5 % sodium thiosulfate. Biochemical tests were performed according to the methods outlined by Gerhardt *et al.* (1994). *Haloferax volcanii* DSM 33755^T was used as a control in all tests. 16S rRNA genes were amplified using the primers Arch21f and Arch1492r (Reysenbach *et al.*, 2000), cloned using a TOPO-TA cloning kit (Invitrogen) and sequenced at the Oklahoma Medical Research Foundation Core Sequencing Facility. Sequence alignment was performed using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analysis involved evaluation of the evolutionary distance with a neighbour-joining algorithm and Jukes–Cantor corrections using PAUP 4.01b10 (Sinauer Associates). Samples were fixed for EM on a poly(lysine)-coated cover-slip using glutaraldehyde, coated with gold/palladium and examined using a JSM-880 SEM. G+C content of the total cellular DNA and DNA–DNA hybridization values were determined according to methods outlined previously (Gutierrez *et al.*, 2002).

Strain M6^T formed small (0·8–1·0 mm), salmon pink, transparent and elevated punctiform colonies with undulate margins. Cells were extremely pleomorphic and stained Gram-negative. Rods, irregular cells and flattened disc shapes were observed using phase-contrast microscopy and SEM. Rod-shaped cells (single or in pairs) were observed more frequently during the exponential growth phase, whereas irregularly shaped cells were common during the stationary phase or from colonies on agar plates. Scanning electron micrographs of strain M6^T are available as supplementary material in IJSEM Online. Strain M6^T grew in HM medium in a wide range of NaCl concentrations (from 60 g l^{-1} to saturation) with an optimum between 125 and 150 g l^{-1}. It required at least 1 mM Mg^{2+} and grew best with 30 mM or more. Cells lysed if suspended in distilled water or in NaCl concentrations below 30 g l^{-1}, but they retained their viability for prolonged incubations in NaCl solutions of 40 g l^{-1} and above (Elshahed *et al.*, 2004). Strain M6^T did not grow anaerobically with nitrate, sulfate, thiosulfate, DMSO or TMAO as electron acceptors, nor was it able to ferment arginine. It was, however, capable of reducing elemental sulfur to sulfide (Elshahed *et al.*, 2004). Control experiments with *Haloferax volcanii* DSM 33755^T indicated that this species could also reduce elemental sulfur to sulfide, albeit at a much slower rate (50 μM sulfide formed after 3 months compared with 0·4 mM in 2 weeks for strain M6^T). These results suggest that strain M6^T may be capable of surviving under the anaerobic conditions of the Zodletone spring by reducing elemental sulfur to sulfide. It is likely that elemental sulfur reduction is a common capability within the *Halobacteriaceae*, as suggested by Grant & Ross (1986) and Tindall & Trüper (1986).

Detailed results of nutritional experiments, antibiotic sensitivity and the physiological description are given in the species description. In general, strain M6^T was similar to other members of the genus *Haloferax* in being oxidase- and catalase-positive and able to grow on a single carbon source. It is also similar in that all species are unable to grow anaerobically on DMSO or TMAO and they are unable to ferment arginine or to decarboxylate lysine and ornithine. Differences between M6^T and other members of the genus *Haloferax* are highlighted in Table 1.

16S rRNA gene sequence analysis indicated that strain M6^T clusters within the genus *Haloferax* (Fig. 1). Sequence

Table 1. Characteristics that distinguish strain M6^T from other members of the genus *Haloferax*

Strains/species: 1, strain M6^T; 2, *H. volcanii*; 3, *H. gibbonsii*; 4, *H. denitrificans*; 5, *H. mediterranei*; 6, *H. alexandrinus*; 7, *H. lucentense*. Data obtained from Asker & Ohta (2002), Mullakhanbhai & Larsen (1975), Rodriguez-Valera *et al.* (1983), Tomlinson *et al.* (1986), Juez *et al.* (1986), Gutierrez *et al.* (2002), Oren (2000) and Grant *et al.* (2001), as well as from our comparative studies with *H. volcanii* DSM 33755^T. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Growth temperature (°C)							
Optimum	32–37	40	35–40	50	40	37	37
Range	18–50	ND	25–55	30–55	20–55	20–55	10–45
Growth in NaCl:							
Optimum concentration (M)	2·1–2·6	1·7–2·5	2·5–4·3	2·0–3·0	2·9	4·3	4·3
Concentration range (M)	1·0–5·2	1·0–4·5	1·5–5·2	1·5–4·5	1·3–4·7	1·8–5·1	1·8–5·1
Cell stability (M NaCl)*	0·5	0·5	0·5–0·7	1·5	0·5	1·7	ND
Motility	−	−	−	−	+	−	+
Optimum pH for growth	6·4–6·8	7·0	6·5–7·0	6·0–7·0	6·5	7·2	7·5
Hydrolysis of:							
Gelatin	+	−	+	+	+	+	−
Starch	−	−	−	−	+	−	−
Tween 80	+	−	+	−	+	+	ND
Casein	−	−	+	−	+	−	−
Anaerobic nitrate reduction	−	−	−	+	+	−	−
Indole production	+	+	+	−	+	+	+
H_2S production from thiosulfate	+	+	+	+	−	+	+
DNA G+C content (mol%)	60·5	63·4	61·8	64·2	59·1–62·2	59·5	64·5
Resistance to rifampicin	+	−	−	−	−	+	ND
DNA–DNA hybridization values†	100	21	24	1	4	ND	3

*Cell stability is defined as the lowest concentration of NaCl below which the cells would lyse.
†Degree of relatedness (%) with ^{3}H-labelled DNA from strain M6^T.

similarity calculations using neighbour-joining analysis indicated that the sequence of strain M6^T has between 96·7 % (*Haloferax mediterranei*) and 98·0 % (*Haloferax lucentense*) similarity to sequences of other members of the genus *Haloferax*. The closest relative to strain M6^T was *Haloferax* sp. strain D1227, isolated from soil contaminated with highly saline oil brine (Emerson *et al.*, 1994). The closest relative to strain M6^T outside the genus *Haloferax* was *Halogeometricum borinquense*, with a sequence similarity of only 89 %. Phospholipid analysis indicated the presence of S-DGD-1 and the absence of phosphatidylglycerosulfate, a pattern characteristic of members of the genus *Haloferax* (Oren, 2000). The DNA G+C content of strain M6^T is 60·5 mol%, a value within the designated range of the genus (Grant *et al.*, 2001). Results of DNA–DNA hybridization experiments (Table 1) showed hybridization values between strain M6^T and other species of the genus *Haloferax* ranging between 1 and 24 %, thus indicating that this strain is clearly a novel species of the genus *Haloferax*.

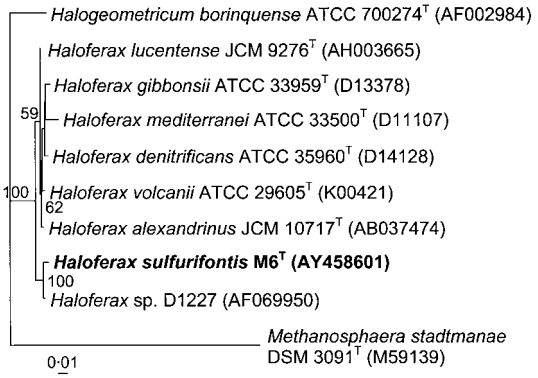

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolate M6^T, other members of the genus *Haloferax* and other selected genera of the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50 % bootstrap support. Bar, 0·01 substitutions per site.

The physiological, biochemical and genotypic tests described above suggest that strain M6^T is a member of the genus *Haloferax*. Differences outlined in Table 1, as well as 16S rRNA gene sequence analysis and DNA–DNA hybridization data justify the placement of strain M6^T within a novel species of the genus *Haloferax*, for which the name *Haloferax sulfurifontis* sp. nov. is proposed.

Description of *Haloferax sulfurifontis* sp. nov.

Haloferax sulfurifontis (sul.fu.ri.fon′tis. L. masc. n. *fons*, *fontis* spring; L. neut. n. *sulfur* sulfur; N.L. gen. n. *sulfurifontis* of a sulfurous spring).

Cells are non-motile, extremely pleomorphic, occurring mainly as irregularly shaped cells (1·0–1·5 μm in diameter), particularly during the stationary phase, and as rods (1·5–1·7 × 0·5–0·6 μm), especially during the exponential growth phase. Occurs mostly as single cells, sometimes in pairs and clusters. Gram-negative. Colonies on agar medium with 150 g NaCl l^{-1} are small (0·8–1·0 mm), salmon pink, transparent and elevated with undulate margin. Halophilic. Cells lyse immediately in distilled water, within 24 h in 10–20 g NaCl l^{-1} and within 72 h in 30 g NaCl l^{-1}. Cells survive prolonged incubation in 40–50 g NaCl l^{-1}. Grows in a wide range of NaCl concentrations (60 g l^{-1} to saturation). Requires at least 1 mM Mg^{2+} for growth and grows best at 30 mM Mg^{2+} and above. The optimum pH and temperature values for growth are pH 6·4–6·8 and 32–37 °C. The type strain, M6^T, grows at 18–50 °C and at pH 4·5–9·0. Cannot utilize SO_4^{2-}, $S_2O_3^{2-}$, NO_3^-, DMSO or TMAO as electron acceptors. Reduces elemental sulfur to sulfide under strictly anaerobic conditions. Chemoorganotrophic. Grows on complex media with yeast extract, Casamino acids and peptone as carbon sources. Capable of growing in defined media. The following substrates are utilized as carbon sources: acetate, benzoate, citrate, fumarate, L-glutamate, malate, succinate, glycerol, maltose, glucose, fructose, sucrose, arabinose, galactose and xylose. Alanine, aspartate, arginine, glycine, lactose, mannitol, sorbitol, ribose and starch are not utilized as carbon sources. Acid is produced in unbuffered medium from the following compounds: glycerol, xylose, maltose, sucrose, arabinose, fructose, glucose and galactose. Acid is not produced from mannitol, arabinose, lactate or sorbitol. Catalase- and oxidase-positive. Reduces thiosulfate aerobically to sulfide. Capable of aerobic nitrate reduction. Indole is formed from tryptophan. Gelatin is hydrolysed, whereas starch, casein and urea are not. Produces polyhydroxyalkanoates. Phosphatase and β-galactosidase tests are negative. Ornithine and lysine are not decarboxylated. Resistant to ampicillin, erythromycin, chloramphenicol, carbenicillin, gentamicin, ceftriaxone, ciprofloxacin, doxycycline, cefaclor, kanamycin, nalidixic acid, oxytetracycline, penicillin G, rifampicin and bacitracin up to 100 μg ml^{-1}. Sensitive to aphidicolin, anisomycin and novobiocin, and to high concentrations of bacitracin. The major polar lipids in the membrane are the diphytanyl ether derivatives of phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate, and S-DGD-1 as the main glycolipid. Phosphatidylglycerosulfate is absent.

The type strain is M6^T (=JCM 12327^T=CCM 7217^T=DSM 16227^T=CIP 108334^T). The DNA G+C content of M6^T is 60·5 mol%. Isolated from the Zodletone spring in south-western Oklahoma, USA.

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