Haloferax larsenii sp. nov., an extremely halophilic archaeon from a solar saltern

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Three strains of Gram-negative, aerobic, neutrophilic, extremely halophilic archaea, designated ZJ206^T, ZJ203 and ZJ204, were isolated from a solar saltern in Zhe-Jiang Province, China. Phenotypically and on the basis of 16S rRNA gene sequences, the strains were very similar. Comparative 16S rRNA gene analysis revealed 96.4–97.4 % sequence similarity to members of the genus *Haloferax*. The major polar lipids were $C_{20}C_{20}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, diglycosyl glycerol diether and sulfated diglycosyl diether. The DNA G+C content of strain ZJ206^T was 62.2 mol%. The results of DNA–DNA hybridizations and physiological and biochemical tests allowed genotypic and phenotypic differentiation of the isolates from closely related species. Therefore the isolates should be classified as members of a novel species, for which the name *Haloferax larsenii* sp. nov. is proposed. The type strain is ZJ206^T (=CGMCC 1.5347^T=JCM 13917^T).

The genus *Haloferax* was originally proposed by Torreblanca *et al.* (1986) as a member of the family *Halobacteriaceae*. At present, the genus comprises seven species: *Haloferax volcanii* (Mullakhanbhai & Larsen, 1975), *Hfx. mediterranei* (Rodriguez-Valera *et al.*, 1983), *Hfx. denitrificans* (Tomlinson *et al.*, 1986; Tindall *et al.*, 1989), *Hfx. gibbonsii* (Juez *et al.*, 1986), *Hfx. alexandrinus* (Asker & Ohta, 2002), *Hfx. lucentense* (Gutierrez *et al.*, 2002) and *Hfx. sulfurifontis* (Elshahed *et al.*, 2004).

The current taxonomic classification of the *Halobacteriaceae* is mainly based on 16S rRNA gene sequence comparison, polar lipid composition and phenotypic characteristics (Oren *et al.*, 1997; Grant *et al.*, 2001; Castillo *et al.*, 2006). All *Haloferax* species described so far possess diphytanyl ether derivatives of sulfated diglycosyl diether but do not contain phosphatidylglycerol sulfate (Grant *et al.*, 2001). In phylogenetic trees based on 16S rRNA gene sequences, they form an independent cluster, the nearest taxon being *Halogeometricum borinquense*. Additionally, most members of the genus *Haloferax* are extremely pleomorphic and require relatively low salt levels for growth in comparison with other members of the *Halobacteriaceae*.

We have isolated three strains of extremely halophilic archaea from a mixture of mud and brine obtained from a solar saltern (122° 17′ N 29° 55′ E) located in the Zhoushan archipelago, Zhe-Jiang Province, China. The water sample (approx. 50 ml) was filtered through 0.45 and 0.22 μm filters in sequence and then the 0.22 μm membrane was added to DSMZ medium 823 and plated by using a 10-fold dilution-series method. The plates were incubated aerobically at 25 and 37 °C. After 1–2 weeks incubation, representative colonies were picked and maintained on S-G medium (Sehgal & Gibbons, 1960) at 37 °C. Strains were purified by repeated restreaking; purity was confirmed from the uniformity of the cell morphology. Cell morphology and motility were examined by using phase-contrast microscopy (BX40; Olympus/Axiovert 135TV; Zeiss) and transmission electron microscopy (H-600; Hitachi). The cells of all three isolates were very pleomorphic; in cultures grown at the highest salinities, elongated cells were common (see Supplementary Fig. S1 available in IJSEM Online). The cells showed motility, but we did not observe flagella by electron microscopy (Supplementary Fig. S2). The cultures

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains ZJ206^T, ZJ203 and ZJ204 are AY838278, DQ458847 and AY838279, respectively.

Phase-contrast photomicrographs and transmission electron photomicrographs of strain ZJ206^T are available as supplementary figures in IJSEM Online.

were pink in colour as a result of the presence of bacterioruberin carotenoids. Phenotypic characteristics were tested according to Xu *et al.* (2005), as mentioned previously in the minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Parallel tests were performed with the strains *Hfx. sulfurifontis* JCM 12327^T and *Hfx. volcanii* CGMCC 1.2350^T.

Total lipids were extracted by using the modified method of Kamekura & Kates (1988). Polar lipids were separated by two-dimensional silica-gel TLC. Phospholipids were detected with Zinzadze reagent. Glycolipids were analysed by spraying the plate with 0.5 % 1-naphthol in methanol/water (1 : 1) and then with sulfuric acid/ethanol (1 : 1) before heating it at 120 °C for 10 min (Xin *et al.*, 2000). The glycolipid analysis indicated the presence of sulfated diglycosyl diether and the absence of phosphatidylglycerol sulfate.

The 16S rRNA genes were analysed as described previously (Xu *et al.*, 2005). Phylogenetic trees were constructed by using the neighbour-joining method with the MEGA3 program package (Kumar *et al.*, 2004), after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). The 16S rRNA gene sequences of the three isolates showed 99.8–100 % similarity with respect to each other. Sequence similarity analysis by the FASTA network service revealed that the strains shared 96.4–97.4 % similarity with the type strains of previously described *Haloferax* species, and 92.3–92.5 % similarity with *Hgm. borinquense* PR3^T, its closest relative outside the genus *Haloferax*. Both the neighbour-joining tree (Fig. 1) and the maximum-parsimony tree (not shown) indicated that the isolates are separate from *Haloferax* species and cluster most closely with *Hfx. sulfurifontis* M6^T.

The DNA G+C content of strain ZJ206^T as determined by thermal denaturation (T_m) (Marmur & Doty, 1962) was found to be 62.2 mol%, which is within the range reported for *Haloferax* species (Grant *et al.*, 2001). DNA–DNA hybridizations were performed by using the thermal denaturation and renaturation method of De Ley *et al.* (1970), as modified by Huß *et al.* (1983), with a Beckman DU 800 spectrophotometer. DNA–DNA relatedness levels for strain ZJ206^T with respect to *Hfx. sulfurifontis* JCM 12327^T and *Hfx. volcanii* CGMCC 1.2350^T were 48 and 44 %, respectively. Comparisons of phenotypic properties (Table 1) also indicated differences (such as motility, hydrolysis of substrates and acid production from sugars) between strain ZJ206^T and *Haloferax* species.

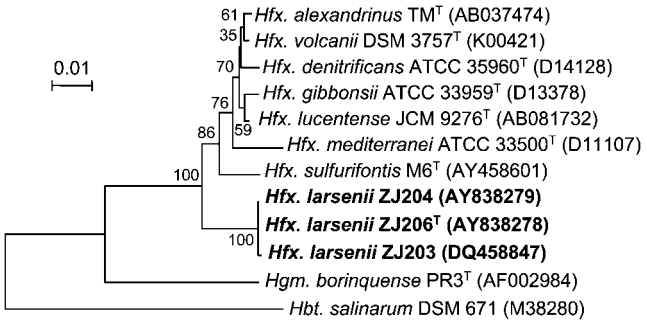

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain ZJ206^T and related taxa. Numbers at branching nodes are bootstrap values (percentages of 1000 resamplings). Bar, 0.01 substitutions per nucleotide position.

The phenotypic, phylogenetic and chemotaxonomic data suggest that strain ZJ206^T is a member of the genus *Haloferax*. Differences in phenotypic characteristics (Table 1) and 16S rRNA gene sequences, together with the DNA–DNA hybridization data, justify the creation of a novel species. We propose to name the novel species in honour of Professor Helge Larsen, who has contributed much to our understanding of halophilic micro-organisms, and who originally described the type species of the genus *Haloferax*. Thus, strain ZJ206^T represents a novel species within the genus *Haloferax*, for which the name *Haloferax larsenii* sp. nov. is proposed.

Description of *Haloferax larsenii* sp. nov.

Haloferax larsenii (lar.se′ni.i. N.L. gen. n. *larsenii* of Larsen, named in honour of Professor Helge Larsen, one of the pioneers of halophile research).

Gram-negative. Extremely pleomorphic, motile, occurring mainly as irregularly shaped cells (0.8–1.5 μm in diameter). Cells grown at the highest salinities are often elongated. Colonies on complex agar medium are 1–2 mm in diameter, smooth, circular, elevated and orange–red. Halophilic. Cells lyse immediately in distilled water after 2 h with 10–20 g NaCl l^{-1}. The NaCl concentration for growth is between 1 and 4.8 M, with an optimum at 2.2–3.4 M. Saturated NaCl inhibits growth in liquid medium. Growth occurs in media containing more than 5 mM Mg^{2+}, the optimum Mg^{2+} concentration being between 20 and 500 mM. The pH range for growth is 6.0–8.5, with an optimum at pH 6.5–7.0; the temperature range for growth is 25–55 °C, with an optimum at 42–45 °C. Chemoorganotrophic. Oxidase- and catalase-positive. Anaerobic growth with arginine or DMSO does not occur. Positive for indole formation. Nitrate is reduced to nitrite. Grows anaerobically on nitrate, with the production of gas. H_2S is produced from thiosulfate. Starch and Tweens 40 and 80 are hydrolysed. Gelatin is hydrolysed; casein is not hydrolysed. The following substrates are utilized for growth: glucose, glycerol, mannose, starch, maltose, sucrose, glutamate, alanine, ornithine, fumarate, malate, pyruvate, succinate and lactate. Arabinose, lactose, mannitol, rhamnose, sorbitol, galactose, ribose, xylose, arginine, lysine, aspartate, glycine, acetate, propionate and citrate are not utilized for growth. Acid is produced from glycerol and maltose and, to a lesser extent, on glucose, fructose and sucrose. Sensitive to novobiocin, bacitracin, anisomycin, aphidicolin and rifampicin, but not to

Table 1. Phenotypic characteristics that distinguish strain ZJ206^T from *Haloferax* species

Taxa: 1, strain ZJ206^T; 2, *Hfx. volcanii*; 3, *Hfx. denitrificans*; 4, *Hfx. gibbonsii*; 5, *Hfx. mediterranei*; 6, *Hfx. alexandrinus*; 7, *Hfx. lucentense*; 8, *Hfx. sulfurifontis*. The data are based on our comparative studies with *Hfx. volcanii* and *Hfx. sulfurifontis*, as well as on data derived from Asker & Ohta (2002), Elshahed *et al.* (2004), Grant *et al.* (2001), Gutierrez *et al.* (2002), Juez *et al.* (1986), Mullakhanbhai & Larsen (1975), Rodriguez-Valera *et al.* (1983), Tindall *et al.* (1989), Tomlinson *et al.* (1986) and Torreblanca *et al.* (1986). +, Positive; −, negative; W, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Motility	+	−	−	+	+	−	+	−
Colony colour	Orange–red	Red to orange	Orange–red	Orange–red	Pink	Red	Pink	Salmon pink
NaCl range (M)	1.0–4.8	1.0–4.5	1.5–4.5	1.5–5.2	1.3–4.7	1.7–5.2	1.8–5.1	1.0–5.2
NaCl optimum (M)	2.2–3.4	1.7–2.5	2.0–3.0	2.5–4.3	2.9	4.3	4.3	2.1–2.6
pH optimum	6.5–7.0	7.0	6.7	6.5–7.0	6.5	7.2	7.5	6.4–6.8
Temperature optimum (°C)	42–45	45	50	35–40	35	37	37	32–37
Anaerobic growth on nitrate	+	−	+	−	+	−	ND	−
Nitrate reduction	+	+	+	−	+	+	−	+
H_2S formation from thiosulfate	+	+	+	+	−	+	+	+
Hydrolysis of:								
Starch	+	−	−	−	+	−	−	−
Casein	−	−	−	+	+	−	−	−
Gelatin	+	−	+	+	+	+	−	+
Tween 80	+	−	−	+	+	+	ND	+
Acid production from:								
Mannose	−	−	ND	+	+	−	ND	−
Arabinose	−	+	ND	+	+	+	+	+
Galactose	−	+	ND	+	ND	−	−	+
Xylose	−	+	ND	+	+	+	+	+
Sucrose	W	+	ND	+	+	+	−	+
Resistance to rifampicin	−	−	ND	+	−	+	ND	+
DNA G+C content (mol%)	62.2±0.8	63.4±0.5	64.2	61.8	60.0	59.5±0.3	64.5	60.5

ampicillin, chloramphenicol, erythromycin, nalidixic acid, neomycin, nystatin, penicillin, tetracycline, streptomycin or kanamycin. The major polar lipids are the $C_{20}C_{20}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, diglycosyl glycerol diether and sulfated diglycosyl diether. The DNA G+C content of DNA of the type strain is 62.2±0.8 mol% (T_m).

The type strain, ZJ206^T (=CGMCC 1.5347^T=JCM 13917^T), was isolated from a solar saltern in Zhe-Jiang Province, China.

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