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Taxonomic characterization of *Haloferax* sp. ("*H. alicantei*") strain Aa 2.2: description of *Haloferax lucentensis* sp. nov.

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Abstract An extremely halophilic archaeon, previously named as *Haloferax* sp. strain Aa 2.2 or "*Haloferax alicantei*" that has been extensively used for genetic studies with halobacteria, was taxonomically characterized by using phenotypic tests (including morphological, physiological, biochemical and nutritional features), DNA-DNA hybridization and 16S rRNA sequence phylogenetic analysis. This organism was isolated in 1986 by Torreblanca et al. from a pond of a Spanish saltern located in Alicante. The cells were pleomorphic, Gram negative and grew optimally at 25% NaCl. The polar lipid composition was similar to that of species of the genus *Haloferax*. The DNA G + C content of this strain was 64.5 mol%. Phylogenetic analysis based on 16S rRNA sequence comparison confirmed that this archaeon is a member of the genus *Haloferax* and was most closely related to *Haloferax volcanii*. DNA-DNA hybridization between strain Aa 2.2 and the type strain of all named species of the genus *Haloferax* revealed low levels of relatedness (25–2%), supporting the placement of this organism in a new species. On the basis of the phenotypic characteristics, molecular data and phylogenetic analysis we propose to name strain Aa 2.2 as a new species, *Haloferax lucentensis* sp. nov. The type strain is Aa 2.2 (= JCM 9276 = NCIMB 13854 = CIP 107410 = DSM 14919 = CECT 5871 = CCM 7023).

Key words *Haloferax lucentensis* · Halobacteria · 16S rRNA · Extremely halophilic archaea

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Introduction

Halobacteria are considered among the most extremely halophilic micro-organisms which inhabit hypersaline environments. They require at least 1.5 M NaCl for growth and grow optimally at 3.5–4.5 M NaCl (Grant et al. 2001). Currently, they are classified in the Class *Halobacteria*, Order *Halobacteriales*, Family *Halobacteriaceae*, and the different species are included in the 15 recognized genera (Grant et al. 2001; Wainø et al. 2000). The genus *Haloferax* includes species characterized by their extremely pleomorphic cells, an optimum salt concentration for growth of 3.5–4 M NaCl, a high Mg^{2+} requirement (40–80 mM), a characteristic polar lipid composition and typical signature 16S rDNA sequences (Grant et al. 2001; Ventosa 2001). The four species currently recognized within this genus are *H. volcanii, H. denitrificans*, *H. gibbonsii* and *H. mediterranei* (Ventosa 2001). Halobacterial taxonomy has been largely based on polar lipid composition and these data are consistent with current phylogenetic data. Recent studies based on 16S rRNA sequence comparison have shown that the species of the genus *Haloferax* constitute a phylogenetically coherent genus (Grant et al. 2001; Ventosa 2001).

The genus *Haloferax* was proposed by Torreblanca et al. (1986) based on a study of a large number of non-alkaliphilic strains isolated from different hypersaline environments and culture collection strains that were characterized by numerical taxonomy techniques and polar lipid composition. Some of the 13 phenons defined in this study were not assigned to any named species. Strain Aa 2.2, isolated from a saltern located in Alicante, Spain, was a representative of phenon K (Torreblanca et al. 1986), closely related to *H. volcanii* and other *Haloferax* species, with a polar lipid composition typical of that reported for the genus *Haloferax*. However, since 1986 it has not been used in other taxonomic studies or characterized in more detail.

This organism is able to grow rapidly in media with simple substrates. It is β-galactosidase positive, and it could thus be used as a source of a reporter gene (*bgaH*) for genetic studies on halobacteria (Gregor and Pfeifer 2001; Patenge et al. 2000). It is also the host of a novel halovirus, HF1 (Nuttall and Dyall-Smith 1993). Thus, this micro-organism has been extensively used for genetic purposes, such as the analysis of halobacterial plasmids and the construction of cloning and shuttle vectors (Holmes and Dyall-Smith 1990, 1991, 1998, 2000; Holmes et al. 1991, 1995, 1997). In these publications strain Aa 2.2 is cited as "*Haloferax alicantei*", *Haloferax* sp. or *Haloferax* phenon K, but up to date a detailed taxonomic description has not yet been given. In this study we characterize this organism, and we show that it represents a new species of the genus *Haloferax*, for which we propose the name *Haloferax lucentensis* sp. nov.

Materials and methods

Bacterial strains and cultivation

Strain Aa 2.2 was isolated from water of a multi-pond saltern located in Alicante, Spain. The isolation medium and methodology followed was described in detail by Torreblanca et al. (1986). The culture collection strains used in this study as reference strains are showed in Table 1. They were cultured in a medium with a final concentration of ca. 25% salts and 0.5% yeast extract (Difco). The pH was adjusted to 7.5 with 1 N KOH (Torreblanca et al. 1986). Incubation was at 37°C in an orbital shaker at 200 strokes per minute.

Phenotypic characterization

For the phenotypic characterization of strain Aa 2.2 we followed the Minimal Standards for Description of New Taxa in the Order *Halobacteriales* (Oren et al. 1997). The tests performed are included in the species description. The methodology used has been described previously (Torreblanca et al. 1986). Unless otherwise indicated, the tests were carried out in media containing 25% salts, at pH 7.5 and incubated at 37°C.

Lipid extraction and TLC

Polar lipids were extracted with chloroform/methanol as described (Kamekura 1993). Thin layer chromatrography was done using Merck HPTLC plates silica gel 60 (art. 5641) in a solvent system, chloroform: methanol: acetic acid: water (85 : 22.5 : 10 : 4, by volume). Glycolipids were detected as purple spots by spraying 0.5% α-naphtol in methanol-water (1 : 1) and then with sulfuric acid-ethanol (1 : 1), followed by slight heating at 160°C. Phosphatidylglycerophosphate-methyl ester and phosphatidylglycerol were detected as brown spots after prolonged heating.

Table 1. Levels of DNA-DNA relatedness between strain Aa 2.2 and related strains

Micro-organism	Percentage relatedness with ^{3}H-labelled DNA from strain Aa 2.2
Strain Aa 2.2	100
Haloferax volcanii NCIMB 2012^{T}	19
Haloferax denitrificans DSM 4425^{T}	2
Haloferax gibbonsii ATCC 33959^{T}	25
Haloferax mediterranei CCM 3361^{T}	12
Haloarcula vallismortis ATCC 29715^{T}	3
Haloarcula hispanica ATCC 33960^{T}	7
Haloarcula japonica JCM 7785^{T}	11
Haloarcula marismortui ATCC 43049^{T}	9
Haloarcula mukohataei DSM 11483^{T}	30
Haloarcula quadrata DSM 11927^{T}	4
Halobacterium salinarum DSM 3754^{T}	18
Halorubrum saccharovorum ATCC 29252^{T}	15
Halorubrum sodomense ATCC 33755^{T}	5
Halorubrum lacusprofundi DSM 5036^{T}	4
Halobaculum gomorrense DSM 9297^{T}	6
Haloterrigena turkmenica JCM 9743^{T}	13
Haloterrigena thermotolerans DSM 11552^{T}	6
Natrialba asiatica JCM 9576^{T}	1

Extraction of genomic DNA and determination of DNA base composition

The cells were harvested, washed, and suspended in 0.15 M NaCl, 0.1 M EDTA buffer (pH 8.0). Lysis was accomplished at 60°C for 10 min adding sodium dodecyl sulphate at a final concentration of 2% (w/v). The DNA was extracted and purified by the method of Marmur (1961). The purity was assessed from the A_{260}/A_{280} and A_{230}/A_{260} extinction ratios (Johnson 1994). The G + C content of the DNA was determined from the mid-point value (*Tm*) of the thermal denaturation profile (Marmur and Doty 1962) obtained with a Perkin-Elmer UV-Vis 551S spectrophotometer at 260 nm. This instrument was programmed for temperature increases of 1.0°C/min. The *Tm* was determined by a graphic method described by Ferragut and Leclerc (1976), and the G + C content was calculated from this temperature by using the equation of Owen and Hill (1979), in 0.1 × SSC buffer (0.15 M NaCl buffered with 0.015 M trisodium citrate, pH 7.0). The *Tm* value of reference DNA from *Escherichia coli* NCTC 9001 was taken as 74.6°C in 0.1 × SSC (Owen and Pitcher 1985).

Preparation of labelled DNA and DNA-DNA hybridization experiments

DNA was labelled by the multiprime system with a commercial kit (RPN 1601Y, Amersham International, Amersham, UK) with deoxy[1′,2′,5-^{3}H]cytidine 5′-triphosphate (Amersham). The average specific activity obtained with this procedure was 8.4×10^{6} cpm/mg DNA. The labelled DNA was denatured before hybridization by heating at 100°C for 5 min and then placed on ice. DNA-

DNA hybridization studies were performed by the competition procedure of the membrane method described by Johnson (1994). Competitor DNAs were sonicated (Braun Melsungen, Melsungen, Germany) at 50 W for two 15-s time intervals. Membrane filters (HAHY, Millipore, Bedford, Mass., USA) containing reference DNA (25 μg/cm^2) were placed in 5-ml screw cap vials that contained the labelled, sheared, denatured DNA and the denatured, sheared competitor DNA. The ratio of the concentrations of competitor to labelled DNA was at least 150 : 1. The final volume and concentration were adjusted to 140 ml, 2 × SSC, and 30% formamide. The hybridization temperature was 57°C, which is within the limits of validity for the filter method (De Ley and Tijtgat 1970). The vials were shaken slightly for 18 h in a water bath (Grant Instruments, Cambridge, UK); these procedures were done in triplicate. After hybridization the filters were washed in 2 × SSC at the optimal renaturation temperature (57°C). The radioactivity bound to the filters was measured in a liquid scintillation counter (Beckman Instruments, Palo Alto, Calif., USA), and the percentage of relatedness was calculated according to Johnson (1994). At least two independent determinations were carried out for each experiment and the reported results are the mean values.

Phylogenetic studies based on 16S rRNA sequence comparison

The 16S rRNA genes were amplified by PCR with a primer set (5′-ATT CCG GTT GAT CCT GCC GG, positions 6–25 according to *E. coli* numbering, and 5′-AGG AGG TGA TCC AGC CGC AG, positions 1540–1521) using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR products were cloned into *Sma*I site of pUC119 and sequencing was carried out with a Beckman capillary DNA sequencer SEQ2000XL. The DDBJ/GenBank/EMBL accession number of the sequence (1473 nucleotides) of strain Aa 2.2 is AB081732. Aligned 16S rRNA sequences were obtained from the Ribosome Database Project II (Michigan State University, USA; http://rdp.cme.msu.edu/html). The aligned sequences were manipulated in the ae2 sequence editor at ANGIS (Australian National Genomic Information System; http://mell.angis.usyd.edu.au), and trimmed to remove ragged ends. Then positions with uncertain bases or indels were removed. Tree reconstructions were performed using a distance matrix method and the Apple Macintosh program PAUP 4.0b8 (Sinauer Associates, Mass., USA; Swofford 2001).

Results and discussion

Strain Aa 2.2 is a pleomorphic, motile, Gram-negative organism, and able to grow in a wide range of salinities, from 10% to 30% salts (optimal growth at 25% salts). It lyses in distilled water. Colonies on solid media are pink pigmented. The results of the phenotypic tests, and the nutritional features of strain Aa 2.2 are included in the species description. This micro-organism was previously studied by numerical taxonomy techniques and polar lipid composition and it was classified as a member of the genus *Haloferax*, closely related to *H. volcanii* and other *Haloferax* species (Torreblanca et al. 1986). However, there are several phenotypic features which differentiate strain Aa 2.2 from the species of the genus *Haloferax* that have been validly described (Table 2).

The glycolipid of strain Aa 2.2 was S-DGD-1 (Fig. 1), which has been shown to occur in all species of the genus *Haloferax* (Ventosa 2001).

The G + C content of the DNA, determined by the *Tm* method, of strain Aa 2.2 was 64.5 mol%. This result is within the G + C content reported for the genus *Haloferax* (59.1 to 65.5 mol%; Ventosa 2001). The results of the DNA-DNA hybridization experiments are shown in Table 1. Low levels of relatedness were found between strain Aa 2.2 and all type strains tested as representatives of other species of different halobacterial genera. In fact, the levels of DNA relatedness with species of the genus *Haloferax* are between 25% and 2%, lower than 70% that is generally accepted for the delineation of species in prokaryotes.

The 16S rRNA gene sequence of strain Aa 2.2 was most closely related to that of *H. volcanii*. There were only eight nucleotide differences, and all the signature bases specific for the genus *Haloferax* (Grant et al. 2001; Kamekura et al. 2002) were completely conserved. DNA-DNA hybridization data are determinative in order to define the species status (Stackebrandt and Goebel 1994). The DNA relatedness between strain Aa 2.2 and *H. volcanii* NCIMB 2012^T is

Table 2. Differential characteristics of *Haloferax lucentensis* sp. nov. and other species of the genus *Haloferax*. Data from this study and from Gutierrez et al. (1989), Juez et al. (1986), Mullakhanbhai and Larsen (1975), Rodriguez-Valera et al. (1983) and Tindall et al. (1989) (+ positive, – negative, *ND* not determined)

Characteristic	*H. lucentensis*	*H. volcanii*	*H. denitrificans*	*H. gibbonsii*	*H. mediterranei*
Nitrate reduction	–	+	+	–	+
H_2S production	+	+	+	+	–
Gelatin hydrolysis	–	–	+	–	+
Starch hydrolysis	–	–	–	–	+
Indole production	+	+	–	+	+
Acid production from sucrose	–	ND	+	+	+
G + C content (mol%)	64.5	63.4	64.2	61.8	60

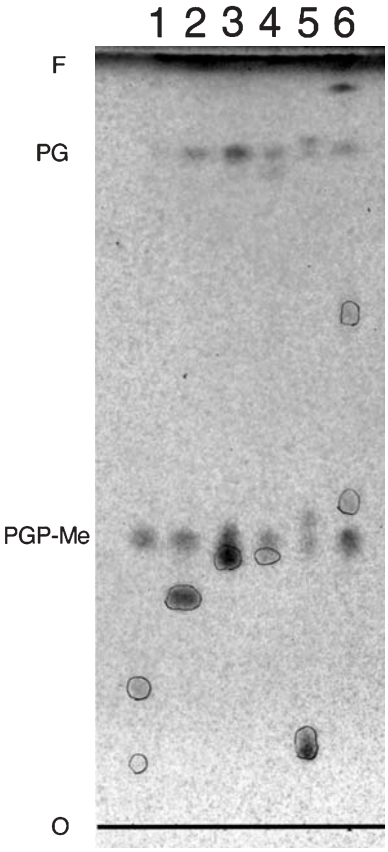

Fig. 1. Thin layer chromatogram of polar lipids of strain Aa 2.2 and other reference halobacteria. *Lane 1 Halobacterium salinarum* CCM 2090; *lane 2 Halorubrum sodomense* ATCC 33755; *lane 3 Haloferax mediterranei* ATCC 33500; *lane 4* strain Aa 2.2; *lane 5 Natrialba asiatica* JCM 9576; *lane 6 Haloarcula hispanica* ATCC 33960. *PG* Phosphatidylglycerol; *PGP-Me* phosphatidylglycerorophosphate-methl ester. *Circled* Glycolipids detected as purple spots

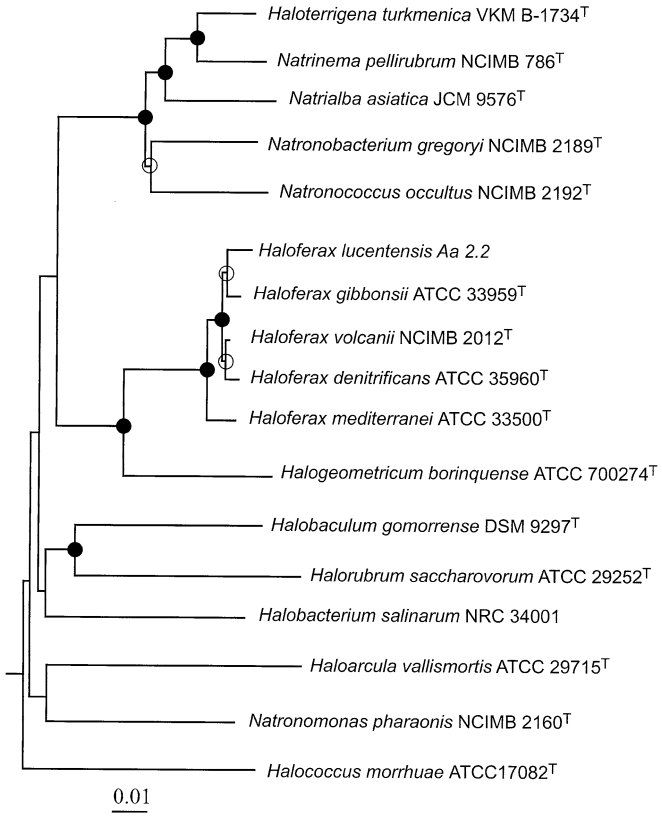

Fig. 2. Phylogenetic tree, based on the 16S rRNA sequence comparison, showing the relationship between strain Aa 2.2 and species of the genus *Haloferax* and other related halobacterial genera. *Scale bar* 0.01 expected nucleotide substitutions per site. Bootstrap values were derived (100 replicates) and significant nodes (>75% or 65–69% bootstrap values) on the consensus tree are indicated by *filled* or *open circles*, respectively, at the appropriate branch points. The tree was rooted with the 16S rRNA sequence of *Methanothrix soehngenii* DSM 2139^T as outgroup (not shown)

19%, which clearly indicates that they constitute different species. Phylogenetic tree reconstructions showed the closest relationship of strain Aa 2.2 to *H. volcanii* (Fig. 2) and also showed its relationship to other species of the genus *Haloferax*.

Overall, our results show that strain Aa 2.2 is significantly different from other previously named species of the genus *Haloferax*, and on the basis of phenotypic features, as well as DNA-DNA hybridization data, we propose to place this organism in a new species, with the name *H. lucentensis* sp. nov.

Description of *Haloferax lucentensis* sp. nov.

H. lucentensis [lu.cen.ten'sis L. adj., pertaining to the city of Lucentum, Alicante, Spain, where the strain was isolated). Cells are pleomorphic rods (0.6 × 2.5 μm). Gram negative. Motile. Colonies are pink, circular, opaque convex with an entire margin. Growth occurs in media containing 10–30% NaCl, with an optimum at 25% NaCl. Grows between 10 and 45°C (optimum at 37°C) and pH 5–9 (optimum at 7.5). Chemoorganotrophic. Aerobic. Acid is produced from arabinose, fructose, glucose, maltose, xylose and glycerol but not from galactose, lactose, sucrose or trehalose. Oxidase and catalase positive. Nitrate is not reduced to nitrite. Indole is produced from tryptophan. Starch, gelatin and casein are not hydrolyzed. H_2S is produced. Methyl red test is positive. Voges-Proskauer negative. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. The following compounds are used as sole carbon and energy sources: arabinose, cellobiose, esculin, fructose, glucose, glucosamine, rhamnose, sucrose, trehalose, xylose, dulcitol, glycerol, acetate, α-aminobutyrate, butyrate, fumarate or glycerate. The following compounds are not used as sole carbon and energy sources: fucose, galactose, gluconolactone, inulin, lactose, maltose, mannose, melibiose, raffinose, ribose, adonitol, erythritol, ethanol, mannitol, mesoinositol, propanol, sorbitol, aconitate, α-aminovalerate, caprilate, α-ketoglutarate, citrate, glutamate, malate, malonate, oxalate, propionate, saccharate or tartrate. The following compounds are used as sole carbon, nitrogen and

energy sources: L-alanine, L-glutamine, L-methionine, L-proline, L-serine or L-valine. The following compounds are not used as sole carbon, nitrogen and energy sources: L-arginine, L-asparagine, betaine, creatine, glycine, L-histidine, L-lysine, L-ornithine, putrescine, sarcosine or L-threonine.

The polar lipids are $C_{20}C_{20}$ derivatives of PG, PGP-Me, DGD-1 and S-DGD-1. The G + C content of the DNA is 64.5 mol% (*Tm* method). Isolated from salterns in Alicante, Spain. Type strain is Aa 2.2^T. (= JCM 9276 = NCIMB 13854 = CIP 107410 = DSM 14919 = CECT 5871 = CCM 7023).

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