Haloterrigena hispanica sp. nov., an extremely halophilic archaeon from Fuente de Piedra, southern Spain

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An extremely halophilic archaeon belonging to the order *Halobacteriales* was isolated from Fuente de Piedra salt lake, Spain. This strain, designated FP1^T, was a pleomorphic coccoid, neutrophilic and required at least 15 % (w/v) NaCl for growth. Strain FP1^T grew at 37–60 °C, with optimal growth at 50 °C. Mg²⁺ was not required, but growth was observed with up to 10 % (w/v) MgSO₄. Polar lipid analysis revealed the presence of mannose-6-sulfate(1-2)-glucose glycerol diether as a major glycolipid. Both C₂₀C₂₀ and C₂₀C₂₅ core lipids were present. The genomic DNA G + C content was 62.0 mol%. Phylogenetic analysis based on comparison of 16S rRNA gene sequences demonstrated that the isolate was most closely related to species of the genus *Haloterrigena*. DNA–DNA reassociation values between strain FP1^T and the most closely related species of the genus *Haloterrigena* (*Haloterrigena thermotolerans*, *Haloterrigena saccharevitans* and *Haloterrigena limicola*) were lower than 29 %. It is therefore considered that strain FP1^T represents a novel species of the genus *Haloterrigena*, for which the name *Haloterrigena hispanica* sp. nov. is proposed. The type strain is FP1^T (=DSM 18328^T =ATCC BAA-1310^T).

Halophilic *Archaea* belonging to the order *Halobacteriales* are found in large numbers in the crystallizer ponds of solar salterns worldwide (Kamekura & Dyll-Smith, 1995; Oren *et al.*, 1997; Grant, 2004; Fendrihan *et al.*, 2006). Several saline and hypersaline environments in Spain have been studied and many halophiles have been isolated from them. A few studies have described halophilic bacteria from the Fuente de Piedra saline lake in the province of Malaga, southern Spain (Martinez-Canovas *et al.*, 2004; Martinez-Checa *et al.*, 2005; Mata *et al.*, 2006), but no extremely halophilic archaea have been described from this lake.

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PHB, poly- β -hydroxybutyric acid; S-DGD, mannose-6-sulfate(1-2)-glucose glycerol diether; S₂-DGD, mannose-2,6-disulfate(1-2)-glucose glycerol diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FP1^T is AM285297.

A thin-layer chromatogram of total polar lipids and a table giving ¹³C NMR assignments for polar lipids from strain FP1^T and related species are available with the online version of this paper.

In the present study, a novel member of the genus *Haloterrigena* is proposed on the basis of conventional physiological, biochemical and chemical characteristics and through phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization results. At the time of writing, the genus *Haloterrigena* comprises five species of extremely halophilic archaea, *Haloterrigena turkmenica* (Ventosa *et al.*, 1999), *Haloterrigena thermotolerans* (Montalvo-Rodriguez *et al.*, 2000), *Haloterrigena saccharevitans* (Xu *et al.*, 2005a), *Haloterrigena longa* and *Haloterrigena limicola* (Cui *et al.*, 2006). Owing to taxonomic problems arising within the genera *Haloterrigena* and *Natrinema* (Montalvo-Rodriguez *et al.*, 2000; Xin *et al.*, 2000; Tindall, 2003; Xu *et al.*, 2005b; Wright, 2006; Castillo *et al.*, 2006), a combination of other morphological and chemotaxonomic characters such as lipid analyses have been used to distinguish between species of these two genera.

Strain FP1^T was isolated from samples collected during summer 2005 from Fuente de Piedra saline lake, Malaga province, southern Spain (37° 6' N 4° 44' W).

Strain FP1^T was isolated from a saltern crystallizer pond by the dilution-plating technique. This strain represented the predominant organism in the enrichment and was the only colony-forming organism at the highest dilutions. Enrichment medium (medium 1; DSM 372) contained the following components (per litre): 5.0 g yeast extract (Oxoid), 5.0 g Casamino acids (Oxoid), 3.0 g trisodium citrate (Applichem), 2.0 g KCl (Applichem), 20 g MgSO₄·7H₂O (Carlo Erba), 200 g NaCl (Applichem), 0.36 µg MnCl₂·4H₂O (J. T. Baker) and 0.05 g FeSO₄·7H₂O (Carlo Erba). The pH of medium 1 was 7.0. Growth on single carbon sources (medium 2) was tested on liquid media containing (per litre) 200 g NaCl (Applichem), 2.0 g KCl (Applichem), 1 g MgSO₄·7H₂O (Carlo Erba), 16.4 g MgCl₂·6H₂O (Riedel-de Haën), 0.2 g NaHCO₃ (J. T. Baker), 2.29 g CaCl₂·2H₂O (J. T. Baker), 152 mg NH₄Cl (Applichem), 33 mg K₂HPO₄ (Applichem), 0.26 mg FeCl₂·4H₂O (J. T. Baker) and 10.0 g of the test compound. Solid media were prepared by the addition of 1.8 % (w/v) agar.

Haloterrigena turkmenica JCM 9191^T, *Haloterrigena thermotolerans* DSM 11552^T, *Haloterrigena saccharovitans* JCM 12889^T, *Haloterrigena limicola* JCM 13563^T and *Natrinema pellirubrum* JCM 10476^T (McGenity *et al.*, 1998), obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ) and from the Japan Collection of Microorganisms (JCM), were grown in the media suggested by the culture collections (DSMZ medium no. 372 for *Htg. turkmenica* and *Htg. thermotolerans*; JCM medium no. 168 for *Htg. saccharovitans*, *Htg. limicola* and *N. pellirubrum*). *Haloferax mediterranei* CCM 3361^T was grown as described by Lanzotti *et al.* (1988).

Cell morphology was determined by phase-contrast microscopy (Zeiss). Colony morphology was analysed on solid medium via a stereomicroscope (M8; Leica). Tolerance to NaCl and MgSO₄ and growth at various temperatures and pH were tested in medium 1. All growth tests were performed at the optimal growth temperature (50 °C) for 3 days. Sensitivity of the strain to antibiotics was tested by using medium 1 with agar (1.8 %, w/v) and Sensi-discs (6 mm; Oxoid) (Romano *et al.*, 1993). Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa of the order *Halobacteriales* (Oren *et al.*, 1997). Gelatin hydrolysis was determined as described by Oren *et al.* (2002). Casein hydrolysis and oxidase, tyrosinase, aminopeptidase (Bactident-Merck) and catalase activities were tested in medium 1 as described by Oren *et al.* (1997). For nitrate reduction, medium 1 plus 0.1 % (w/v) KNO₃ was employed. Hydrolysis of hippurate was tested in medium 1 plus hippurate (1 %, w/v) (Poli *et al.*, 2006). For the indole test, the novel strain was grown at pH 7.0 in medium 1. Gram-staining was performed according to Dussault (1955). The KOH test was performed according to Halebian *et al.* (1981). Hydrolysis of *N*-benzoyl-arginine-*p*-nitroaniline (BAPNA) stereoisomers was tested as described by Oren & Galinski (1994). Intracellular solutes and poly-hydroxybutyric acid (PHB) were

extracted according to Motta *et al.* (2004) and Sykes (1971), respectively. Cell mass for quinone and lipid analyses was obtained from cultures of test strains grown under their optimal growth conditions for 24 h. Lipid analysis, lipid hydrolysis and identification of core lipids were performed as reported by Nicolaus *et al.* (2001). Quinones were analysed by LC/MS on a reversed-phase column by Electron Impact MS (EI/MS) and ¹H NMR spectra. Phospholipids and glycolipids were separated by TLC on silica gel plates (10 × 10 cm; 10 × 25 cm; 0.25 mm, F₂₅₄; Merck) and were analysed according to Nicolaus *et al.* (2001). Complex lipids of *Hfx. mediterranei* CCM 3361^T were used as reference (Lanzotti *et al.*, 1988).

The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) and the values given are the mean of three independent analyses of the same DNA sample. DNA was hydrolysed and the resultant nucleotides were analysed by HPLC. The DNA was isolated as described by Poli *et al.* (2006).

The 16S rRNA gene sequence was determined by direct sequencing of the PCR product. Genomic DNA extraction, amplification of the 16S rRNA gene and purification of the PCR products were carried out as described by Reed *et al.* (2006) with the primers 5'-CTGGTTGATCCTGCCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3'. Purified PCR products were sequenced by the DSMZ with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's recommendations. Sequence reactions were electrophoresed by using the Applied Biosystems 373A DNA sequencer.

The sequence of the novel strain was compared with closely related sequences of reference organisms from the FASTA network service. Sequence data were aligned with the CLUSTAL W 1.8 program (Chenna *et al.*, 2003). Phylogenetic analysis was performed by using the PHYLIP package, version 3.6 (Felsenstein, 2004) and with the neighbour-joining method within the MEGA3 program package (Kumar *et al.*, 2004). DNA-DNA hybridizations were performed at 48.5 °C according to Ezaki *et al.* (1989) and the levels of DNA-DNA relatedness were calculated according to Goris *et al.* (1998). Hybridizations were carried out between strain FP1^T and related species (*Htg. thermotolerans* DSM 11552^T, *Htg. saccharovitans* JCM 12889^T and *Htg. limicola* JCM 13563^T).

Cells of strain FP1^T were coccoid, Gram-negative and able to grow over a restricted range of salinities (2.2–4.0 M NaCl). Colonies on agar medium were light red-pigmented. Strain FP1^T did not require magnesium for growth and was unable to assimilate sugars (glucose, sucrose or maltose). Detailed results of morphological analyses, antibiotic sensitivity tests and biochemical tests for strain FP1^T are given in the species description below.

In a phylogenetic tree based on 16S rRNA gene sequences, strain FP1^T clustered with recognized species of the genus *Haloterrigena* (Fig. 1). 16S rRNA gene sequence similarities

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain FP1^T and related taxa. Bootstrap values (%) are based on 1000 replicates and are shown at each node. Bar, 0.01 expected changes per site.

between strain FP1^T and *Htg. limicola* JCM 13563^T, *Htg. thermotolerans* DSM 11552^T, *Htg. saccharevitans* JCM 12889^T, *Htg. turkmenica* JCM 9101^T and *Htg. longa* JCM 13562^T were 98.9, 96.2, 95.6, 95.5 and 94.6 %, respectively, but the novel strain was also related to *Natrinema versiforme* JCM 10478^T and *Natrinema altunense* JCM 12890^T (96.1 and 95.3 % sequence similarity, respectively). The position of *Natrinema ejinorens* in this phylogenetic tree should be resolved in the near future (Fig. 1).

The polar lipid profile of strain FP1^T, which comprised C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and mannose-6-sulfate(1-2)-glucose glycerol diether (S-DGD), was consistent with that of species of the genus *Haloterrigena* but differed with regard to the glycolipid pattern. The type strains of recognized species of the genus *Haloterrigena* possess mannose-2,6-disulfate(1-2)-glucose glycerol diether (S₂-DGD) (Ventosa *et al.*, 1999; Montalvo-Rodriguez *et al.*, 2000; Xu *et al.*, 2005a; Cui *et al.*, 2006), whereas strain FP1^T lacks this component. Species of the genus *Natrinema* have been shown to possess unknown glycolipids and sulfated glycolipids (McGenity *et al.*, 1998; Xin *et al.*, 2000; Xu *et al.*, 2005b) (see Supplementary Fig. S1 in IJSEM Online). Polar lipids were identified by ¹H and ¹³C NMR spectra (see Supplementary Table S1) (De Rosa *et al.*, 1988; Lanzotti *et al.*, 1988, 1989). LC/MS as well as EI/MS analyses of the quinone content of strain FP1^T revealed the presence of two menaquinones, MK8 and MK8(H₂). Strain FP1^T

accumulated PHB under optimal growth conditions. The above results indicated that strain FP1^T was a member of the genus *Haloterrigena*. However, it could be distinguished from recognized species of the genus *Haloterrigena* on the basis of several phenotypic characteristics (Table 1).

The DNA G + C content of strain FP1^T was determined to be 62.0 mol%. DNA–DNA relatedness values for strain FP1^T with respect to *Htg. thermotolerans* DSM 11552^T, *Htg. saccharevitans* JCM 12889^T and *Htg. limicola* JCM 13563^T were 22.85, 28.35 and 23.40 %, respectively.

On the basis of the phylogenetic, genotypic and chemotaxonomic data presented here, we suggest that strain FP1^T should be classified as the type strain of a novel species within the genus *Haloterrigena*, for which the name *Haloterrigena hispanica* sp. nov. is proposed.

Description of *Haloterrigena hispanica* sp. nov.

Haloterrigena hispanica (his.pa'ni.ca. L. fem. adj. *hispanica* of Hispania, from where the organism was originally isolated).

Cells are Gram-negative, coccoid (1.5–2.0 µm in diameter) and become oval in stationary cultures. Colonies on complex agar medium with 3.4 M NaCl are light red, elevated and circular. Growth occurs at NaCl concentrations of 2.2–4.0 M, at Mg²⁺ concentrations of 0–0.4 M, at pH values in the range 6.5–8.5 and at temperatures of 37–60 °C. The

Table 1. Characteristics that distinguish strain FP1^T from species of the genus *Haloterrigena* and from *Natrinema altunense*

Taxa: 1, strain FP1^T; 2, *Htg. saccharevitans* AB14^T (data from Xu *et al.*, 2005a); 3, *Htg. thermotolerans* DSM 11552^T (data from Montalvo-Rodriguez *et al.*, 2000); 4, *Htg. turkmenica* VKM B-1734^T (data from Ventosa *et al.*, 1999); 5, *Htg. limicola* AX-7^T (data from Cui *et al.*, 2006); 6, *Htg. longa* ABH32^T (data from Cui *et al.*, 2006); 7, *N. altunense* AJ2^T (data from Xu *et al.*, 2005b). +, Positive; –, negative; w, weak; NR, data not reported; UK, unknown; S-DGD, mannose-6-sulfate(1-2)-glucose glycerol diether; S₂-DGD, mannose-2,6-disulfate(1-2)-glucose glycerol diether.

Characteristic	1	2	3	4	5	6	7
Cell morphology	Coccoid	Rods/coccoid	Rods	Coccoid	Rods	Rods	Rods
NaCl range (M)	>2.2	>1.7	>2.0	>2.0	>1.7	>1.7	>1.7
DNA G+C content (mol%)	62.0	66.6	63.3	59.8	61.9	63.2	65.6
Cell size (µm)	1.5–2.0	0.8–7.0	4–13	1.5–2.0	0.7–2.7	0.5–0.6 × 2.8–11	1.0–5.0
Lysis in distilled water	+	NR	+	–	NR	NR	+
Temperature optimum (°C)	50	42–45	50	45	40–50	41–45	37
Use of sugars as carbon source	–	–	–	+	–	+	+
Gelatin liquefaction	–	–	w	–	–	–	+
Hydrolysis of Tween 20	–	NR	NR	NR	+	+	+
Major glycolipid	S-DGD	S ₂ -DGD	S ₂ -DGD	S ₂ -DGD	S ₂ -DGD	S ₂ -DGD	One major glycolipid: UK
Sensitivity to tetracycline	–	+	–	NR	–	–	NR
Motility	–	+	–	+	+	–	+

optimal NaCl concentration, Mg²⁺ concentration, pH and temperature for growth are 3.4 M, 0.2 M, pH 7.0 and 50 °C, respectively. Chemo-organotrophic and aerobic. Oxidase- and catalase-positive. Indole formation is positive. Nitrate reduction is observed but nitrite reduction is not. Gelatin, starch, casein, and Tweens 20, 40 and 60 are not hydrolysed. The following substrates are utilized for growth: glycerol, sodium acetate, sodium propionate and sodium citrate. Glucose, mannose, rhamnose, fructose, sucrose, galactose, trehalose, ribose, xylose, cellobiose and arabinose are not utilized for growth. Sensitive to (µg per disc) fusidic acid (10), bacitracin (10) and novobiocin (30). Resistant to the following antibiotics (µg per disc): streptomycin (25), tetracycline (50), nystatin (100), vancomycin (30), kanamycin (30), lincomycin (10), neomycin (30), erythromycin (30), ampicillin (25), chloramphenicol (50) and penicillin (10 U). Major polar lipids are C₂₀C₂₀ and C₂₀C₂₅ derivatives of PG, PGP-Me and S-DGD. The DNA G+C content is 62.0 mol% (T_m).

The type strain, FP1^T (=DSM 18328^T=ATCC BAA-1310^T), was isolated from Fuente de Piedra salt lake, southern Spain.

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