Haloterrigena jeotgali sp. nov., an extremely halophilic archaeon from salt-fermented food

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A novel red-pigmented halophilic archaeon, strain A29^T, was isolated from shrimp jeotgal, a traditional salt-fermented food from Korea. This strain grows in the ranges 10–30 % (w/v) NaCl, 17–50 °C and pH 6.5–8.5, with optimal growth occurring at 15–20 % NaCl, 37–45 °C and pH 7.0–7.5. The isolate is Gram-negative and non-motile. Phylogenetic analysis, based on 16S rRNA gene sequences, showed that strain A29^T is associated with the genus *Haloterrigena* and closely related to the species *Haloterrigena thermotolerans* (99.0 % similarity). However, DNA–DNA hybridization experiments revealed that the level of hybridization between strain A29^T and related strains of *Haloterrigena* is less than 70 %. The polar lipid fraction consists of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and mannose-2,6-disulfate(1-2)-glucose glycerol diether (S_2-DGD). The G+C content of genomic DNA of the type strain is 62.3 mol%. On the basis of this polyphasic taxonomic study, strain A29^T should be placed in the genus *Haloterrigena* as a novel species, for which the name *Haloterrigena jeotgali* sp. nov. is proposed. The type strain of the new species is A29^T (=KCTC 4020^T=DSM 18794^T=JCM 14585^T=CECT 7218^T).

Although high salinity is toxic to most cells, extreme halophiles are well adapted to their hypersaline environment (Grant, 2004). Since the establishment of the genus *Haloterrigena* within the family *Halobacteriaceae* in 1999 (Ventosa *et al.*, 1999), at the time of writing seven species belonging to it have been described: *Haloterrigena turkmenica* (Ventosa *et al.*, 1999), *Haloterrigena thermotolerans* (Montalvo-Rodríguez *et al.*, 2000), *Haloterrigena saccharevitans* (Xu *et al.*, 2005a), *Haloterrigena longa* and *Haloterrigena limicola* (Cui *et al.*, 2006), *Haloterrigena hispanica* (Romano *et al.*, 2007) and *Haloterrigena salina* (Gutiérrez *et al.*, 2008). To further increase our understanding of the family *Halobacteriaceae*, we searched for novel strains of this family in shrimp jeotgal, a traditional fermented seafood from Korea that comprises small shrimps and rock salt. Shrimp jeotgal has a liquid component with a salt concentration close to or at saturation (approx. 35 %, w/v), and it is known to be a reservoir of halophilic bacteria, such as *Jeotgalicoccus halotolerans* (Yoon *et al.*, 2003b), *Salinicoccus jeotgali* (Aslam *et al.*, 2007b), *Bacillus jeotgali* (Yoon *et al.*, 2001), *Nesterenkonia jeotgali* (Yoon *et al.*, 2006), *Methylobacterium jeotgali* (Aslam *et al.*, 2007a) and *Psychrobacter jeotgali* (Yoon *et al.*, 2003a), and halophilic archaea such as *Natronococcus jeotgali* (Roh *et al.*, 2007a) and *Halalkalicoccus jeotgali* (Roh *et al.*, 2007b). A novel strain, designated A29^T, was isolated from shrimp jeotgal on a medium designed for halophilic archaea. On the basis of 16S rRNA gene sequence analysis, strain A29^T was determined to belong to the genus *Haloterrigena*. In this paper, we describe the taxonomic position of this strain through phenotypic, genotypic and chemotaxonomic analyses.

Strain A29^T was isolated on a complex medium (DSM medium 954) containing (g l^{-1}) Casamino acids (5; Difco), yeast extract (5; Difco), $MgCl_2.6H_2O$ (20), KCl (2), Tris (12), $CaCl_2.2H_2O$ (0.2) and NaCl (200) in the presence of antimicrobial compounds (penicillin G, erythromycin and cycloheximide, 100 μg ml^{-1}) that are known to inhibit bacteria and eukaryotes but not archaea (Purdy *et al.*,

Abbreviations: S-DGD, mannose-6-sulfate(1-2)-glucose glycerol diether; S_2-DGD, mannose-2,6-disulfate(1-2)-glucose glycerol diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A29^T is EF077633.

Phylogenetic trees based on 16S rRNA gene sequences generated by the minimum-evolution and maximum-parsimony methods, and a thin-layer chromatogram of the polar lipids of strain A29^T and a reference strain (*H. thermotolerans* PR5^T) are available as supplementary figures in IJSEM Online.

2004). The pH was adjusted to 7.5 and incubation was at 37 °C for approximately 7 days. A pure culture was obtained by repeated restreaking. Phenotypic tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell morphology was examined using light (Eclipse 80*i*; Nikon) and electron microscopy (JEM 1010; JEOL). Gram staining was performed as described by Dussault (1955) and motility was examined on semi-solid agar. Optimal conditions for growth were determined in medium 954 containing 0–30 % (w/v) NaCl, and the pH range for growth was assayed from pH 5.0 to 11.0 at intervals of 0.5, and at different temperatures (4, 10, 15, 17, 21, 25, 30, 37, 45, 50 and 60 °C). The Mg^{2+} requirement for growth was also determined using medium 954 without $MgCl_2 . 6H_2O$ and yeast extract. Standard phenotypic tests for indole formation, nitrate reduction, hydrolysis of casein, starch and urea, and activity of oxidase and catalase were conducted as described by Gerhardt *et al.* (1994). Hydrolysis of gelatin and Tween 80 were tested simultaneously through the procedure adapted for haloarchaea by Gutiérrez & González (1972). Cell lysis in distilled water was detected by microscopic examination. DNase activity was detected on T-Hv agar medium as described by González *et al.* (1978). Utilization of sole carbon and energy sources, and acid production from sugars were determined as described by Montalvo-Rodríguez *et al.* (2000). Anaerobic growth in the presence of nitrate, sulfate, thiosulfate, DMSO and TMAO was tested in filled, stoppered tubes with Sehgal–Gibbons (SG) medium (Sehgal & Gibbons, 1960) for 1 month.

The isolate is Gram-negative and non-motile. This strain grows in the ranges 10–30 % NaCl, 17–50 °C and pH 6.5–8.5, with optimal growth occurring at 15–20 % NaCl, 37–45 °C and pH 7.0–7.5. Phenotypic differences between strain A29^T and type strains of other species of the genus are shown in Table 1. As shown in Table 1, the new isolate can be differentiated from other closely related species by several phenotypic properties.

Chromosomal DNA was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR using PCR Pre-Mix (iNtRON Biotechnology) and two universal primers, as described by Baker *et al.* (2003). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described previously (Roh *et al.*, 2008). Phylogenetic relationships between closely related species were determined using MEGA4 (Tamura *et al.*, 2007) and used to elaborate dendrograms by the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Kluge & Farris, 1969) methods. A bootstrap analysis investigating the stability of the trees was performed by obtaining a consensus tree based on 1000 randomly generated trees. Strain A29^T showed the highest level of 16S rRNA gene similarity to *H. thermotolerans* PR5^T (99.0 %), *H. saccharevitans* AB14^T (98.3 %), *H. limicola* AX-7^T (97.1 %), *H. turkmenica* 4k^T (96.4 %), *H. salina* XH-65^T (96.3 %), *H. hispanica* FP1^T (96.1 %) and *H. longa* ABH32^T (94.8 %). Phylogenetic trees based on 16S rRNA gene sequences indicated that the genus *Haloterrigena* can be divided into four groups, strain A29^T forming a separate clade with *H. thermotolerans* and *H. saccharevitans*, adjacent to the genus *Natrinema*, regardless of tree-making algorithms used, with high bootstrap values of 98, 99 and 92 based on the neighbour-joining, minimum-evolution and maximum-parsimony methods, respectively (Fig. 1; Supplementary

Table 1. Characteristics that differentiate *H. jeotgali* sp. nov. from its closest phylogenetic relatives

Species: 1, *H. jeotgali* sp. nov.; 2, *H. limicola* (data from Cui *et al.*, 2006); 3, *H. longa* (Cui *et al.*, 2006); 4, *H. saccharevitans* (Xu *et al.*, 2005a); 5, *H. thermotolerans* (Montalvo-Rodríguez *et al.*, 2000); 6, *H. turkmenica* (Ventosa *et al.*, 1999); 7, *H. hispanica* (Romano *et al.*, 2007); 8, *H. salina* (Gutiérrez *et al.*, 2008). +, Positive; −, negative; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Rods	Rods	Rods	Rods/coccoid	Rods	Coccoid	Coccoid	Coccoid
Motility	−	+	−	+	−	−	−	−
Growth at 20 °C	+	−	−	−	−	NR	−	−
Nitrate reduction	−	+	−	+	+	−	+	−
Mg^{2+} requirement	−	+	−	−	−	+	−	−
Indole formation	+	−	+	−	−	−	+	−
Oxidase	−	+	+	+	+	NR	+	+
Utilization of:								
Glucose	−	−	+	−	−	+	−	+
Sucrose	−	−	+	−	−	+	−	−
Fructose	+	−	−	−	−	+	−	+
Presence of S-DGD	−	−	−	−	−	−	+	−
Presence of S_2-DGD	+	+	+	+	+	+	−	+
G+C content (mol%)	62.3	61.9	63.2	66.6	63.3	59.8	62.0	67.0

Fig. S1, available in IJSEM Online). DNA–DNA hybridization was performed by using the fluorometric method of Ezaki *et al.* (1989). The DNA–DNA relatedness between strain A29^T and the related strains *H. thermotolerans* PR5^T, *H. saccharevitans* AB14^T and *H. limicola* AX-7^T is 23.2, 22.0 and 17.9 %, respectively. The 16S rRNA gene sequence similarity data and a DNA–DNA relatedness value of less than 70 % (Wayne *et al.*, 1987) indicate that strain A29^T could be a distinct genospecies.

The G+C content was determined by a fluorimetric method using SYBR Green and a real-time PCR thermocycler (González & Saiz-Jimenez, 2002). The G+C content of genomic DNA of strain A29^T is 62.3 mol%, which is in the range of those of the validly published species of the genus *Haloterrigena*. Total lipids were extracted by the modified method of Xin *et al.* (2000), separated by one-dimensional thin-layer chromatogram on a Merck silica gel 60 F254 aluminium-backed plate with the solvent chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.), and detected by spraying the plate with specific reagents, as described by Tindall (1990). The designation of all spots was compared with reference strain *H. thermotolerans* PR5^T. The polar lipid fraction contains phosphatidylglyerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), and mannose-2,6-disulfate(1-2)-glucose glycerol diether (S_2-DGD) (Supplementary Fig. S2, available in IJSEM Online). The polar lipid profile of strain A29^T resembles that of *Haloterrigena* species, containing PG, DPG-Me and S_2-DGD (Cui *et al.*, 2006; Gutiérrez *et al.*, 2008; Montalvo-Rodríguez *et al.*, 2000; Ventosa *et al.*, 1999; Xu *et al.*, 2005a).

It is known that the genera *Haloterrigena* and *Natrinema* overlap phylogenetically to some extent, and detailed taxonomic reassessment studies have been carried out (Montalvo-Rodríguez *et al.*, 2000; Oren & Ventosa, 2002). Currently, it is reported that *Haloterrigena* species contain the glycolipids S_2-DGD (Cui *et al.*, 2006; Gutiérrez *et al.*, 2008; Montalvo-Rodríguez *et al.*, 2000; Ventosa *et al.*, 1999; Xu *et al.*, 2005a) or mannose-6-sulfate(1-2)-glucose glycerol diether (S-DGD) (Romano *et al.*, 2007), whereas most *Natrinema* species have phosphatidylglycerol sulfate (PGS) and unidentified glycolipids (McGenity *et al.*, 1998; Tapingkae *et al.*, 2008; Xin *et al.*, 2000; Xu *et al.*, 2005b). Even though strain A29^T is phylogenetically related to members of both *Haloterrigena* and *Natrinema*, it is reasonable that strain A29^T can be classified as a member of the genus *Haloterrigena* based on the presence of S_2-DGD, the fact that it clusters with members of the genus *Haloterrigena* in phylogenetic trees, and the fact that the highest 16S rRNA gene sequence similarities are shared with members of the genus *Haloterrigena*.

Results from 16S rRNA gene sequence analysis and chemotaxonomic, physiological and biochemical tests have identified genotypic and phenotypic differences between strain A29^T and other species in the genus *Haloterrigena*. Taken together, these data clearly differentiate the new isolate from other *Haloterrigena* species. Thus, on the basis of genetic, chemotaxonomic and phenotypic comparisons to previously described taxa, strain A29^T constitutes a novel species of the genus *Haloterrigena*, for which the name *Haloterrigena jeotgali* sp. nov. is proposed.

Description of *Haloterrigena jeotgali* sp. nov.

Haloterrigena jeotgali (je.ot.ga′li. N.L. gen. n. *jeotgali* of jeotgal, a traditional Korean fermented seafood).

Cells are Gram-negative, non-motile, rod-shaped (0.4 μm wide, 1.0 μm long) and occur in irregular clusters. The colonies on complex agar medium are light red, circular and measure 0.5 mm in diameter after 7 days at 37 °C. Growth occurs in the presence of 10–30 % (w/v) NaCl at

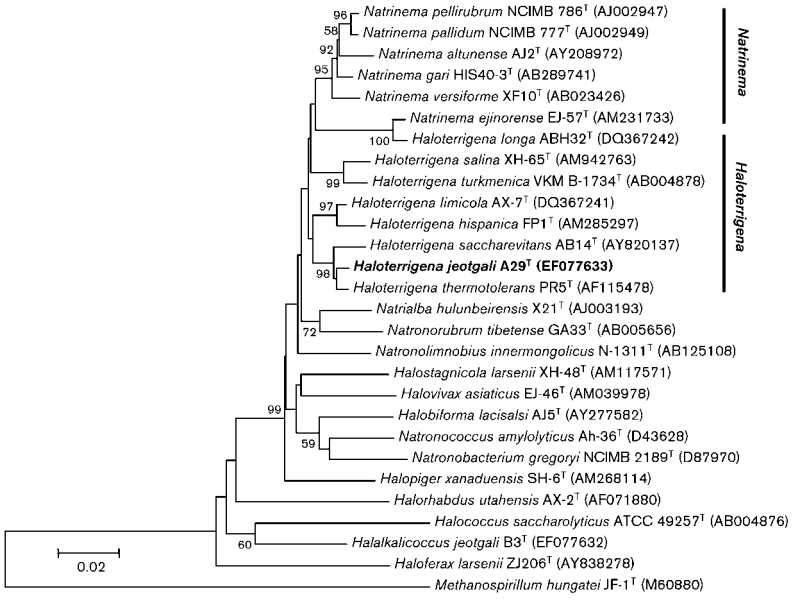

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain A29^T with respect to other species in the genus *Haloterrigena*, *Natrinema* and other haloarchaea. The tree was generated by the neighbour-joining method. The numbers at the nodes indicate bootstrap values (1000 replications). Bar, 0.02 accumulated changes per nucleotide.

temperatures ranging from 17 to 50 °C and in the pH range 6.5–8.5. Optimal conditions are an NaCl concentration of 15–20 %, temperatures ranging from 37 to 45 °C and a pH of 7.0–7.5. The isolate is catalase-positive, oxidase-negative and does not reduce nitrate to nitrite. Mg^{2+} is not required for growth. Cell lysis occurs in distilled water. Casein and Tween 80 are hydrolysed, but starch, gelatin, urea and DNA are not. Positive for indole formation. Anaerobic growth with nitrate occurs, but not with sulfate, thiosulfate, DMSO and TMAO. Fructose, lactose and acetate are utilized as carbon and energy sources, but sucrose, glucose, citrate and formate are not. Acid is not produced from fructose, lactose, acetate, sucrose, glucose, citrate or formate. The polar lipid fraction consists of PG, PGP-Me and S_2-DGD. Resistant to the following antimicrobial compounds (μg ml^{-1}): bacitracin (50), penicillin (50), ampicillin (50), chloramphenicol (50) and erythromycin (50). Sensitive to the following antimicrobial compounds (μg ml^{-1}): novobiocin (25), anisomycin (25) and aphidicolin (25). G+C content of genomic DNA of the type strain is 62.3 mol%.

The type strain is A29^T (=KCTC 4020^T=DSM 18794^T=JCM 14585^T=CECT 7218^T) and it was isolated from shrimp jeotgal, a traditional Korean fermented food.

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