Haladaptatus cibarius sp. nov., an extremely halophilic archaeon from seafood, and emended description of the genus *Haladaptatus*

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A novel, extremely halophilic archaeon, D43^T, was isolated from traditional salt-fermented seafood in Korea. The cells were Gram-negative-staining and motile. The strain grew at 15–50 °C, 10–30 % (w/v) NaCl and pH 6.0–8.0. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain D43^T is affiliated with the family *Halobacteriaceae* in the domain *Archaea* and had 95.5 % 16S rRNA gene sequence similarity with *Haladaptatus paucihalophilus* DX253^T. The sequence from strain D43^T formed a clade with those from *Hap. paucihalophilus* regardless of which tree-generating algorithm was used. DNA–DNA hybridization experiments showed 25.8 % relatedness between the isolate and *Hap. paucihalophilus* KCTC 4006^T. Major lipids were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two unidentified glycolipids. The DNA G+C content of the isolate was 56.5 mol%. On the basis of this polyphasic taxonomic study, strain D43^T represents a novel species in the genus *Haladaptatus*, for which the name *Haladaptatus cibarius* sp. nov. is proposed. The type strain is D43^T (=DSM 19505^T =JCM 15962^T).

The genus *Haladaptatus* in the family *Halobacteriaceae* was first proposed by Savage *et al.* (2007) and currently comprises only one species, *Haladaptatus paucihalophilus*, which was isolated from a low-salt, sulfide- and sulfur-rich spring. It was reported that the colonies of *Hap. paucihalophilus* are pink and that the cells are Gram-negative and non-motile and have the phospholipids phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS) (Savage *et al.*, 2007). In a study of archaeal diversity in traditional salt-fermented seafood in Korea, the extremely halophilic archaea *Natronococcus jeotgali* (Roh *et al.*, 2007a), *Halalkalicoccus jeotgali* (Roh *et al.*, 2007b), *Halorubrum cibi* (Roh & Bae, 2009) and *Haloterrigena jeotgali* (Roh *et al.*, 2009) were proposed as novel species in the family *Halobacteriaceae*. Through further study of archaeal diversity in salt-fermented seafood that comprises fish or shellfish with lots of rock salt, we identified a novel strain, designated D43^T, that was obtained from a salt-rich fermented seafood made from shellfish.

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence from strain D43^T is EF660747.

A thin-layer chromatogram of the polar lipids in *Haladaptatus* species is available as a supplementary figure with the online version of this paper.

The salt-fermented seafood was purchased from a distributor of a commercially available brand in Korea. A sample (1 ml), obtained just after the pack was opened, was serially diluted and spread onto a complex medium (DSM medium 954) adjusted to pH 7.0 [containing (l^{-1}) 5 g Casamino acids (Difco), 5 g yeast extract (Difco), 20 g $MgCl_2 . 6H_2O$, 2 g KCl, 12 g Tris base, 0.2 g $CaCl_2 . 2H_2O$, 200 g NaCl, 20 g agar], with antimicrobial compounds as described previously (Roh *et al.*, 2007a). The plates were incubated at 37 °C for 1 month and a single colony was streaked at least three times on the halophile medium to obtain a pure culture. The characterization of strain D43^T was guided by the proposed minimal standards for describing extremely halophilic archaea (Oren *et al.*, 1997). All tests were performed in triplicate unless stated otherwise. Cell morphology was examined by light microscopy (Eclipse 80*i*; Nikon) and motility was examined on semi-solid agar plates and using electron microscopy. Gram staining was performed using the standard staining method for haloarchaea as described by Dussault (1955). Cell lysis in distilled water was detected by microscopic examination. Optimal conditions for growth were determined in medium 954 with 0–30 % (w/v) NaCl (at intervals of 5 %) and at 4, 10, 15, 20, 25, 30, 37, 40, 50 and 60 °C and in halophilic medium [HMD; containing (l^{-1}) 20 g $MgCl_2 . 6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2 . 2H_2O$, 0.1 g yeast extract, 0.5 g NH_4Cl, 0.05 g KH_2PO_4, 0.5 g Casamino acids as carbon source, 180 g NaCl; Savage *et al.*,

2007] at pH 3.0–11.0 (at intervals of 1 pH unit). The requirement for and minimal concentration of Mg^{2+} for growth were examined using medium 954 containing 0.01 % yeast extract without $MgCl_2 . 6H_2O$ at different Mg^{2+} concentrations (0, 5, 10, 20, 50, 100, 200 and 500 mM). Standard phenotypic tests for nitrate reduction under aerobic conditions, indole formation, activity of oxidase and catalase and hydrolysis of casein, starch and urea were conducted as described by Gerhardt *et al.* (1994). Hydrolysis of gelatin and Tween 80 were tested simultaneously through the procedure of Gutierrez & Gonzalez (1972). Utilization of sole carbon and energy sources as well as acid production was determined using HMD as described by Savage *et al.* (2007) with 20 mM carbon source. Tests for anaerobic growth in the presence of 30 mM nitrate, sulfate, thiosulfate or DMSO were performed in stopped tubes as described by Sehgal & Gibbons (1960). Antibiotic sensitivity was performed using the diffusion agar method (Bauer *et al.*, 1966) with the following antimicrobial compounds (μg unless otherwise stated): ampicillin (10), anisomycin (30), aphidicolin (30), chloramphenicol (30), erythromycin (15), kanamycin (30), rifampicin (30), streptomycin (10) and polymycin B (300 UI).

Colonies of strain D43^T were pink and cells were Gram-negative-staining and motile on semi-solid agar medium. Strain D43^T was catalase- and oxidase-positive and did not reduce nitrate to nitrite under aerobic conditions. Lysis of cells and changes of cell-wall morphology were not detected in distilled water after 2 weeks and cells remained alive under these conditions. Detailed characteristics of strain D43^T are presented in the species description and compared with those of *Hap. paucihalophilus* DX253^T in Table 1.

Chromosomal DNA of strain D43^T and *Hap. paucihalophilus* KCTC 4006^T was extracted and purified as described by Sambrook *et al.* (1989) and the 16S rRNA gene sequence of strain D43^T was amplified by PCR using archaea-specific primer set 21F (5′-TTCCGGTTGATCCTGCCGGA-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′). Sequencing of the amplified gene fragments and assembly of the sequences were performed as described previously (Roh *et al.*, 2008). Identification of phylogenetic neighbours and the calculation of pairwise sequence similarities were carried out by a BLAST search of GenBank (Altschul *et al.*, 1997). Phylogenetic relationships between the isolate and phylogenetic neighbours were determined using MEGA 4.0 (Tamura *et al.*, 2007) and PHYLIP software (Felsenstein, 2005). A distance matrix was determined using the two-parameter model of Kimura (1980). Phylogenetic trees were generated by three algorithms: neighbour joining (Saitou & Nei, 1987), maximum parsimony (Kluge & Farris, 1969) and maximum likelihood (Felsenstein, 1981). Bootstrap analysis to evaluate the stability of phylogenetic trees was achieved using a consensus tree from the neighbour-joining, maximum-parsimony and maximum-likelihood methods, based on 1000, 1000 and 100 replicates, respectively.

The nearly complete 16S rRNA gene sequence of strain D43^T (1405 bp) was obtained. *Hap. paucihalophilus* DX253^T, as well as other uncharacterized strains isolated by Purdy *et al.* (2004), has two distinct 16S rRNA gene sequences; however, no multiple heterogeneous sequences were detected in strain D43^T through the cloning approach. Comparison of 16S rRNA gene sequences indicated that the isolate is associated with the family *Halobacteriaceae*. Strain D43^T exhibited high 16S rRNA gene sequence similarity with uncharacterized haloarchaeon strains RO1-28 (98.8 %) and RO1-22 (98.5 %), haloarchaeon clone W1 (96.1 %), *Hap. paucihalophilus* DX253^T (95.5 and 93.0 %), *Hap. paucihalophilus* GY252 (95.3 and 94.3 %) and other uncharacterized or uncultured

Table 1. Differentiating characteristics of strain D43^T and *Hap. paucihalophilus* DX253^T

Data for *Hap. paucihalophilus* DX253^T were obtained in this study or taken from Savage *et al.* (2007).

Characteristic	Strain D43^T	*Hap. paucihalophilus* DX253^T
Isolation source	Salt-rich, fermented seafood	Low-salt, sulfide-rich spring
Motility	+	−
NaCl range (optimum) (%)	10–30 (15)	5–30 (18)
Temperature range (optimum) (°C)	15–50 (37)	25–45 (30)
Hydrolysis of:		
Casein	−	+
Starch	−	+
Utilization of:		
Citrate	−	+
Lactose	+	−
Mannitol	−	+
Acid production from:		
Fructose	−	+
Mannitol	−	+
DNA G+C content (mol%)	56.5	60.5

haloarchaeon strains (95.4 % or less). The isolate formed a clade with *Hap. paucihalophilus*, uncharacterized strains and uncultured haloarchaeon clones in phylogenetic trees based on 16S rRNA gene sequences, with high bootstrap values (Fig. 1), regardless of which tree-generating algorithm was used (data not shown). The molecular phylogenetic analyses supported the placement of strain D43^T in the genus *Haladaptatus* of the family *Halobacteriaceae*.

To determine the genetic distance between strain D43^T and *Hap. paucihalophilus*, a DNA–DNA hybridization experiment was performed with the modified method of Ezaki *et al.* (1989) as described previously (Roh *et al.*, 2008). The mean DNA–DNA relatedness between strain D43^T and *Hap. paucihalophilus* KCTC 4006^T was 25.8 %. DNA–DNA relatedness values below a threshold of 70 % indicated that the isolate represents a distinct genospecies (Wayne *et al.*, 1987). The G+C content was determined by a fluorimetric method using SYBR Green and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002) with the calibration references *Haloterrigena thermotolerans* PR5^T and *Halorubrum tibetense* AS 1.3239^T. The DNA G+C content of strain D43^T was 56.5 mol%. The G+C content of genomic DNA of *Hap. paucihalophilus* strains DX253^T and GY252 is 60.5 mol% (Savage *et al.*, 2007). Thus, the G+C content of strain D43^T is relatively lower than the value reported previously for the genus *Haladaptatus*.

Polar lipids were extracted and detected with specific reagents (Dittmer & Lester, 1964; Xin *et al.*, 2000) sprayed on a Merck silica gel 60 F_{254} aluminium-backed plate, as described by Oren *et al.* (1996). The designations of all lipid spots were given according to Savage *et al.* (2007). The major lipids of strain D43^T comprised PG, PGP-Me and two unidentified glycolipids (Supplementary Fig. S1, available in IJSEM Online), in agreement with those reported for all strains of haloarchaea and *Hap. paucihalophilus* described by Savage *et al.* (2007); however, PGS was not detected. It is concluded that the presence of PGS is variable within the genus *Haladaptatus*.

Our polyphasic taxonomic study, including data from molecular phylogenetic analysis, DNA–DNA relatedness, genomic DNA G+C content, polar lipid profile and physiological and biochemical tests, showed genotypic and phenotypic differences between the new isolate and *Hap. paucihalophilus*. On the basis of genetic, chemotaxonomic and phenotypic comparisons with previously described taxa, strain D43^T is affiliated with the genus *Haladaptatus* and represents a novel species in the genus *Haladaptatus*, for which the name *Haladaptatus cibarius* sp. nov. is proposed.

Emended description of the genus *Haladaptatus* Savage *et al.* 2007

The description is based on that given by Savage *et al.* (2007), with the following amendments. Cells contain PG, PGP-Me and two unidentified glycolipids. The presence of PGS is variable. The DNA G+C content is 56.5–60.5 mol%.

Description of *Haladaptatus cibarius* sp. nov.

Haladaptatus cibarius (ci.ba′ri.us. L. masc. adj. *cibarius* pertaining to or suitable for food).

Cells are aerobic, Gram-negative-staining cocci or coccobacilli with a diameter of 1.0 μm, motile with a single polar flagellum. The colonies are pink, circular with entire margins and 1.0 mm in diameter after 3 weeks of incubation on a complex agar medium (DSM medium 954) at 37 °C. Growth occurs at 15–50 °C (optimum 37 °C), in the presence of 10–30 % (w/v) NaCl (optimum 15 %) and at pH 6.0–8.0 (optimum pH 7.0). Mg^{2+} is required for growth. The minimal Mg^{2+} concentration for growth and the Mg^{2+} concentration for optimal growth are 5 and 20 mM, respectively. Cell lysis does not occur in

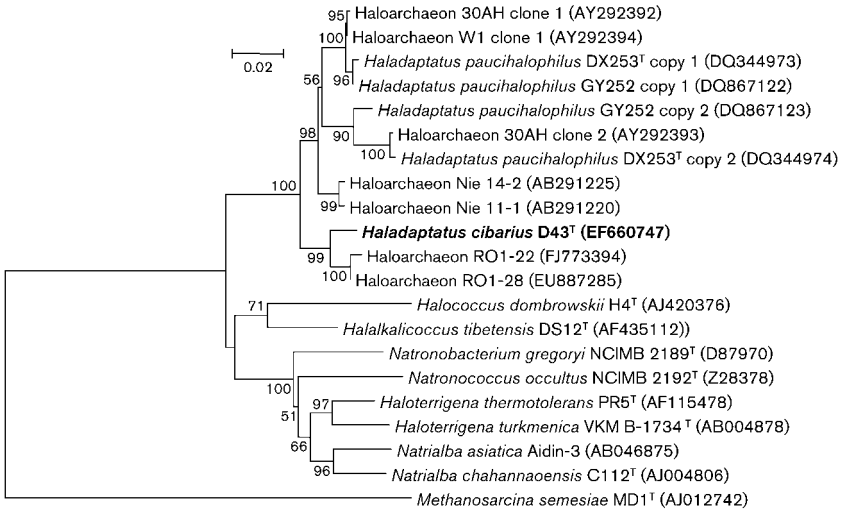

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences generated using the neighbour-joining method. Bootstrap values (>50 %) based on 1000 replicates are shown as percentages at branch nodes. *Methanosarcina semesiae* MD1^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

distilled water. Positive for catalase, oxidase and indole formation. Does not reduce nitrate to nitrite under aerobic conditions. Gelatin and Tween 80 are hydrolysed, but starch, casein and urea are not. Sucrose, D-fructose, D-glucose, lactose, formate and acetate are utilized as carbon and energy sources, but citrate and D-mannitol are not. Acid is produced from sucrose and D-glucose, but not from D-fructose, citrate, lactose, formate, acetate or D-mannitol. Anaerobic growth with nitrate, sulfate, thiosulfate or DMSO does not occur. Sensitive to anisomycin, aphidicolin, chloramphenicol and rifampicin, and resistant to ampicillin, erythromycin, kanamycin, streptomycin and polymycin B. The polar lipids are PG, PGP-Me and two unidentified glycolipids. The genomic DNA G+C content of the type strain is 56.5 mol%.

The type strain is D43^T (=DSM 19505^T =JCM 15962^T), which was isolated from Korean salt-fermented seafood made from shellfish.

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