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

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A novel, extremely halophilic archaean, 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 pauchihalophilus DX253\(^T\). The sequence from strain D43\(^T\) formed a clade with those from Hal. pauchihalophilus regardless of which tree-generating algorithm was used. DNA–DNA hybridization experiments showed 25.8 % relatedness between the isolate and Hal. pauchihalophilus 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 pauchihalophilus, which was isolated from a low-salt, sulfide- and sulfur-rich spring. It was reported that the colonies of Hal. pauchihalophilus 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.

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\(_2\)O, 2 g KCl, 12 g Tris base, 0.2 g CaCl\(_2\), 2H\(_2\)O, 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 80i; Nikon) and motility was examined on semi-solid agar plates and using electron microscopy. Gram staining was performed using the standard staining method for halarchaea 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\(_2\)O, 5 g K\(_2\)SO\(_4\), 0.1 g CaCl\(_2\), 2H\(_2\)O, 0.1 g yeast extract, 0.5 g NH\(_4\)Cl, 0.05 g KH\(_2\)PO\(_4\), 0.5 g Casamino acids as carbon source, 180 g NaCl; Savage et al.,...
at pH 3.0–11.0 (at intervals of 1 pH unit). The requirement for and minimal concentration of Mg²⁺ for growth were examined using medium 954 containing 0.01 % yeast extract without MgCl₂·6H₂O at different Mg²⁺ 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 Savage et al. (2007). Antibiotic sensitivity was performed using the diffusion agar method (Bauer et al., 1960) 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′-TTCCGTTGATCCTGCCGGA-3′) and 1492R (5′-GGYTACCTTGTGACAGTT-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 halooarchaeon strains RO1-28 (98.8 %) and RO1-22 (98.5 %), halooarchaeon 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 strains.

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain D43 T</th>
<th>Hap. paucihalophilus DX253 T</th>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Salt-rich, fermented seafood</td>
<td>Low-salt, sulfide-rich spring</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>NaCl range (optimum) (%)</td>
<td>10–30 (15)</td>
<td>5–30 (18)</td>
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<tr>
<td>Temperature range (optimum) (°C)</td>
<td>15–50 (37)</td>
<td>25–45 (30)</td>
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<td>Hydrolysis of:</td>
<td></td>
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<tr>
<td>Casein</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>−</td>
<td>+</td>
</tr>
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<td>Utilization of:</td>
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</tr>
<tr>
<td>Citrate</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Lactose</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Mannitol</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>56.5</td>
<td>60.5</td>
</tr>
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</table>
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 D43T in the genus *Haladaptatus* of the family Haloarchaeaceae.

To determine the genetic distance between strain D43T 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 D43T and *Hap. paucihalophilus* KCTC 4006T 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* PR5T and *Halorubrum tibetense* AS 1.3239T. The DNA G+C content of strain D43T was 56.5 mol%. The G+C content of genomic DNA of *Hap. paucihalophilus* strains DX253T and GY252 is 60.5 mol% (Savage et al., 2007). Thus, the G+C content of strain D43T 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 F254 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 D43T 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 D43T 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). Mg2+ is required for growth. The minimal Mg2+ concentration for growth and the Mg2+ concentration for optimal growth are 5 and 20 mM, respectively. Cell lysis does not occur in...
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, apheridicolin, 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 D43T (DSM 19505T = JCM 15962T), which was isolated from Korean salt-fermented seafood made from shellfish.

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References


