Haloarcula salaria sp. nov. and *Haloarcula tradensis* sp. nov., isolated from salt in Thai fish sauce

Sirilak Namwong,¹ Somboon Tanasupawat,² Takuji Kudo³ and Takashi Itoh³

Correspondence

Somboon Tanasupawat
Somboon.T@chula.ac.th

¹Department of Biotechnology, Faculty of Science and Technology, Suan Sunandha Rajabhat University, Bangkok 10300, Thailand

²Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

³Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

Two red-pigmented, strictly aerobic, pleomorphic rod-shaped and extremely halophilic archaea, designated strains HST01-2R^T and HST03^T, were isolated from salt in a fish sauce sample from Thailand. The novel strains grew optimally at 37 °C, pH 7.0, and in the presence of 20–25 % (w/v) NaCl. The DNA G + C contents of the isolates were 61.6–62.2 mol%. Phylogenetic analysis based on a comparison of 16S rRNA gene sequences revealed that strains HST01-2R^T and HST03^T were placed in the radiation of species of the genus *Haloarcula*. The chemotaxonomic properties of the two strains, i.e. the presence of MK-8 and MK-8(H₂) as the major menaquinone components and C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and a triglycosyl diether as major polar lipids, supported the assignment of the two strains to the genus *Haloarcula*. Nevertheless, several phenotypic features and the low DNA–DNA relatedness between the two strains and related species of the genus *Haloarcula* (13.4–46.9 %) enabled the strains to be distinguished from each other and from recognized species. Therefore, strains HST01-2R^T and HST03^T represent two novel species in the genus *Haloarcula*, for which the names *Haloarcula salaria* sp. nov. and *Haloarcula tradensis* sp. nov. are proposed, respectively. The type strains are HST01-2R^T (=BCC 40029^T=JCM 15759^T=PCU 313^T) and HST03^T (=BCC 40030^T=JCM 15760^T=PCU 314^T).

The genus *Haloarcula* was firstly described by Torreblanca *et al.* (1986) with the type species *Haloarcula vallismortis*. To date, seven additional species have been described, *Har. amylolytica*, *Har. argentinensis*, *Har. hispanica*, *Har. japonica*, *Har. marismortui*, *Har. mukohataei* and *Har. quadrata* (Juez *et al.*, 1986; Oren *et al.*, 1990, 1999; Takashina *et al.*, 1990, 1991; Ihara *et al.*, 1997; Yang *et al.*, 2007). *Har. mukohataei* has since been transferred to the genus *Halomicrobium* as *Hmc. mukohataei* (Oren *et al.*, 2002). These extremely halophilic archaea are red-pigmented

and short pleomorphic rods ranging from almost regular to irregular shapes. Fish sauce, which is a common fermented food in Thailand, is rich in various nutrients, particularly amino acids and peptides. It also contains a high concentration of NaCl, thus allowing halophilic bacteria and archaea to thrive (Lopetcharat *et al.*, 2001). Previous studies of this product have found strains of halophilic archaea, *Halobacterium salinarum*, *Halococcus thailandensis*, *Hcc. saccharolyticus* and *Natrinema gari* (Thongthai *et al.*, 1992; Namwong *et al.*, 2007; Tapingkae *et al.*, 2008; Tanasupawat *et al.*, 2008–2009), and moderately halophilic bacteria, ‘*Halobacillus thailandensis*’, *Lentibacillus salicampi*, *L. juripiscarius*, *L. halophilus* and *Chromohalobacter salexigens* (Chaiyanan *et al.*, 1999; Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006, 2008–2009). This paper deals with the characterization of two novel extremely halophilic archaea from the salt in a sample of fish sauce based on their phenotypic and chemotaxonomic characteristics, DNA–DNA relatedness and 16S rRNA gene sequences.

Abbreviations: DGD, diglycosyl diether; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; TGD-2, triglycosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain HST01-2R^T are FJ429317 (*rrnA*) and FJ429318 (*rrnB*) and FJ429313 (*rrnA*), FJ429316 (*rrnB*) and FJ429314 (*rrnC*) for strain HST03^T.

Supplementary figures are available with the online version of this paper.

The halophilic archaeal strains were isolated from salt from a sample of fish sauce collected in Trad province, Thailand. The collected salt was suspended in JCM medium No. 168 broth and the suspension was spread on agar plates of the same medium [composed of (l^{-1}): 200 g NaCl, 5 g Casamino acids (Difco), 5 g yeast extract (Difco), 2 g KCl, 3 g $Na_3C_6H_5O_7$, 1 g glutamic acid, 20 g $MgSO_4 \cdot 7H_2O$, 0.36 g $FeCl_2 \cdot 4H_2O$, 0.0036 g $MnCl_2 \cdot 4H_2O$, 20 g agar, pH 7.2] and incubated at 37 °C for 1–2 weeks. Unless otherwise stated, the strains were grown in liquid or on agar media based on JCM medium No. 168.

Phenotypic characteristics were determined in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell morphology and motility were examined by phase-contrast microscopy (BX50; Olympus). Gram staining was performed as described by Dussault (1955). Colonies were observed on JCM medium No. 168 agar plates after incubation at 37 °C for 7 days. Tests for catalase and oxidase activities, nitrate reduction, methyl red/Voges–Proskauer reactions, the formation of indole and the hydrolysis of gelatin, skimmed milk, Tween 80, tyrosine and xanthine were performed as described by Barrow & Feltham (1993). Carbon utilization from carbohydrates was determined in Leifson (1963) medium. Casitone was omitted for the carbon utilization test whereas Tris/HCl was omitted for the acid production test. Anaerobic growth was observed in the standard growth medium with 0.5 % nitrate using a Gaspak (BBL) anaerobic jar. Growth at various temperatures (5–60 °C) was examined using a temperature gradient incubator (model TN-3; Advantec). The effects of initial pH (5–9) for growth were determined in JCM medium No. 168. The effects of NaCl concentration on growth were tested in a medium containing 0–30 % (w/v) NaCl. At lower NaCl concentrations [0–2 % (w/v) NaCl], $MgSO_4 \cdot 7H_2O$, KCl and $Na_3C_6H_5O_7 \cdot 2H_2O$ were omitted from the test medium. Likewise, the requirement for Mg^{2+} was tested in JCM medium No. 168 by omitting $MgSO_4 \cdot 7H_2O$ but supplementing with 2 % (w/v) Na_2SO_4 and 0–10 % (w/v) $MgCl_2$. Growth was determined by measuring the culture turbidity at 660 nm. The antibiotic susceptibility of the strains was tested as described by Stan-Lotter *et al.* (2002).

Menaquinones were analysed by the reversed-phase HPLC method of Komagata & Suzuki (1987) and confirmed by MS running in EI mode and using a direct insertion probe (QPMS-QP5050A; Shimadzu). Polar lipids were extracted serially by using four different solvent solutions [first extraction with methanol:0.3 % NaCl (100:1, v/v), followed by petroleum ether, chloroform:methanol:water (90:100:30, v/v) and finally chloroform:methanol:water (50:100:40, v/v)] and their extracts were separated by single dimensional silica gel TLC (Hezayen *et al.*, 2002). Methanolysis of the polar lipids was conducted by mixing the dried cells with methanol (3 ml), toluene (3 ml) and concentrated H_2SO_4 (0.1 ml), followed by heating at 55 °C for 9 h. Core diether moieties were extracted with hexane

and the hexane extracts were developed on TLC using the procedure described by Ross *et al.* (1981). Two-dimensional TLC of the total polar lipids was conducted according to the method of Minnikin *et al.* (1984).

Genomic DNA was isolated and purified according to the method of Saito & Miura (1963). The DNA G + C content was determined with the method of Tamaoka & Komagata (1984) using reversed-phase HPLC. DNA–DNA hybridization was conducted as reported previously (Ezaki *et al.*, 1989) and detected by the colorimetric method reported by Tanasupawat *et al.* (2000). The almost complete 16S rRNA gene sequences of strains HST01-2R^T and HST03^T were PCR-amplified with primers 20F (5'-TCCGGTTGATCC-TGCCG, position 8–24 according to the *Escherichia coli* numbering system) and 1540R (5'-GGAGGTGATCCA-GCCG, position 1540–1525) (Enache *et al.*, 2007). The amplified DNA fragments were separated by agarose gel electrophoresis and recovered by using a GenElute Minus EtBr Spin Column (Sigma). PCR products were ligated into the pT7blueT-vector (Novagen) using a Takara ligation kit version 2.1 according to the manufacturer's instructions and transformed into *E. coli* JM 109 by the heat-shock technique (Shao *et al.*, 2004). Plasmids were extracted with a QIAprep Spin Miniprep kit (Qiagen) using the protocol supplied by the manufacturer. Inserts were amplified using the primers T7 (5'-TAATACGACTCACT ATAGGG-3') and U19 (5'-GTTTTCCTCCAGTCACGACGT-3') (Enache *et al.*, 2007). PCR products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.0; Applied Biosystems) in the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The phylogenetic tree was constructed as described by Thompson *et al.* (1997), Saitou & Nei (1987), Kumar *et al.* (2001) and Felsenstein (1985).

Two strains of non-motile, red-pigmented and extremely halophilic archaea were isolated from the salt in the samples of fish sauce. Colonies on JCM medium No. 168 agar plates were small (0.8–2.3 mm in diameter), entire, translucent and convex. Cells from late-exponential to stationary phase cultures were pleomorphic, rod-shaped and approximately $0.6\text{--}1.2 \times 1.0\text{--}2.5 \mu\text{m}$ (Fig. 1a and b) and lysed when suspended in distilled water. The strains grew at salinities of 15–30 % (w/v) NaCl, showing optimal growth at 20–25 % (w/v) NaCl. They required at least 2 % (w/v) Mg^{2+} and grew

Fig. 1. Phase-contrast micrographs of cells of strains HST01-2R^T (a) and HST03^T (b) grown on JCM medium No. 168 agar plates. Bars, 10 μm .

optimally when 4–6 % (w/v) Mg^{+} was added to the medium with 20 % (w/v) NaCl. The growth temperature range was 15–45 °C (optimum, 37 °C) and the pH range was 6–8 (optimum, pH 7). The physiological and biochemical properties of the novel strains are summarized in the species description and Table 1.

The chemotaxonomic features of the two strains, i.e. the presence of $C_{20}C_{20}$ glycerol diether derivatives of phosphatidylglycerol sulfate (PGS) and triglycosyl diether (TGD-2), and MK-8 and MK-8(H_2) as the major menaquinone components, conformed to the previously published descriptions of the genus *Haloarcula* (Torreblanca *et al.*, 1986; Juez *et al.*, 1986; Grant & Larsen, 1990; Oren *et al.*, 1990, 1999, 2009; Takashina *et al.*, 1990; Ihara *et al.*, 1997; Yang *et al.*, 2007). Thin layer chromatograms of the polar lipid fractions of strains HST01-2R^T and HST03^T revealed $C_{20}C_{20}$ glycerol diether derivatives of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), PGS, TGD-2, diglycosyl diether (DGD) and two unknown phospholipids (see Supplementary Fig. S1 in IJSEM Online). The TLC of the methanolysis samples from strains HST01-2R^T and HST03^T, as well as those from *Har. amylolytica* JCM 13557^T, *Har. argentinensis* JCM 9737^T, *Har. hispanica* JCM 8911^T and *Hcc. thailandensis* JCM 13552^T revealed that the two novel strains contained only $C_{20}C_{20}$ core lipids (see Supplementary Fig. S2). The two-dimensional TLC of the total polar lipids of strains HST01-2R^T and

HST03^T is presented in Supplementary Fig. S3. The novel strains contained MK-8 (91.8–91.9 %) and MK-8(H_2) (8.2–8.8 %) as the menaquinone components. The genomic DNA G + C contents of the strains were 61.6–62.2 mol% (Table 2).

It has been demonstrated that the recognized members of the genus *Haloarcula* examined so far harbour at least two different 16S rRNA gene copies, which have been categorized into two 16S rRNA gene types (type I and II) showing 4.8–5.6 % divergence from each other (Gemmell *et al.*, 1998; Arahal *et al.*, 1996; Ihara *et al.*, 1997; Oren *et al.*, 1999; Yang *et al.*, 2007; Baliga *et al.*, 2004; Cui *et al.*, 2009). The two novel isolates also revealed this 16S rRNA gene polymorphism: strain HST01-2R^T contained at least two 16S rRNA gene copies, *rrnA* (1479 bp) and *rrnB* (1484 bp), and strain HST03^T contained three 16S rRNA gene copies, *rrnA* (1489 bp), *rrnB* (1487 bp) and *rrnC* (1487 bp), as shown in Fig. 2. All of the 16S rRNA gene copies belonged to type II. On the phylogenetic tree (Fig. 2), the three gene copies of strain HST03^T seemed to be separated from the hitherto reported type II genes, and only one sequence from *Haloarcula hispanica* ATCC 33960^T (GenBank accession no. U68541) was closely related to the *rrnC* gene (97.91 % similarity). On the other hand, the *rrnA* and *rrnB* gene sequences of strain HST01-2R^T were included in the radiation of the type II genes. The DNA–DNA hybridization study revealed that strains HST01-2R^T and HST03^T had low DNA–DNA relatedness (<46.9 %) to each other

Table 1. Differential characteristics of strains HST01-2R^T, HST03^T and species of the genus *Haloarcula*

Taxa: 1, HST01-2R^T; 2, HST03^T; 3, *H. argentinensis* JCM 9737^T; 4, *H. amylolytica* JCM 13557^T; 5, *H. hispanica* JCM 8911^T; 6, *H. quadrata* JCM 11048^T; 7, *H. vallismortis* JCM 8877^T. +, Positive; –, negative; w, weak.

Characteristic	1	2	3	4	5	6	7
Cell shape	Pleomorphic	Pleomorphic	Triangular	Short rods	Short rods	Square	Rods
Range for growth:							
NaCl (% w/v)	15–30	15–30	15–30	15–30	15–30	15–30	15–30
MgCl ₂ (% w/v)	2–10	2–10	2–10	0–10	0–10	1–10	1–10
Hydrolysis of							
Starch	+	w	w	+	+	–	–
Tween 80	+	+	+	+	+	+	–
Tyrosine	–	–	–	+	+	–	–
Nitrate reduction	–	–	–	–	–	+	+
Acid from							
D-Glucose	–	–	+	–	–	–	–
D-Galactose	–	–	+	+	–	–	–
Raffinose	+	–	–	–	–	–	–
Utilization of							
Aspartic acid	+	+	–	–	–	+	+
Cellobiose	+	+	–	+	+	+	–
D-Galactose	–	+	+	+	+	–	–
D-Glucose	+	+	–	+	+	+	–
Glycerol	+	+	–	–	+	–	–
Lysine	–	+	+	+	+	+	–
Raffinose	+	+	+	–	–	–	–
D-Sorbitol	–	+	+	+	+	–	+
Trehalose	–	–	+	+	–	+	+

Table 2. DNA G+C contents and DNA–DNA relatedness of strains HST01-2R^T, HST03^T and other species of the genus *Haloarcula*

Strain	DNA G+C content (mol%)	DNA–DNA relatedness (%) with labelled DNA from:					
		1	2	3	4	5	6
1. HST01-2R ^T	61.6	100	34.0	46.9	18.8	25.5	14.0
2. HST03 ^T	62.2	32.8	100	37.9	20.7	28.0	13.4
3. <i>H. argentinensis</i> JCM 9737 ^T	62.8	36.3	41.1	100	14.3	18.5	12.7
4. <i>H. amylolytica</i> JCM 13557 ^T	61.4	34.0	29.0	54.1	100	57.2	14.9
5. <i>H. hispanica</i> JCM 8911 ^T	62.8	27.0	36.1	52.8	51.2	100	11.3
6. <i>H. vallismortis</i> JCM 8877 ^T	64.7*	20.1	30.7	25.6	25.9	24.4	100
<i>H. marismortui</i> JCM 8966 ^T	61.8*	27.8	37.3	52.0	27.9	28.3	14.5
<i>H. japonica</i> JCM 7785 ^T	63.3*	39.6	42.5	32.3	23.9	25.6	15.5
<i>H. quadrata</i> JCM 11048 ^T	60.1*	24.8	38.7	39.9	21.0	28.1	28.5

*Data from Oren *et al.* (1999).

and to the type strains of recognized species of the genus *Haloarcula*, i.e. *Har. argentinensis* JCM 9737^T, *Har. amylolytica* JCM 13557^T, *Har. hispanica* JCM 8911^T, *Har. vallismortis* JCM 8877^T, *Har. marismortui* JCM 8966^T, *Har. japonica* JCM 7785^T and *Har. quadrata* JCM 11048^T (Wayne *et al.*, 1987), as shown Table 2. In addition, several phenotypic characteristics of strains HST01-2R^T and HST03^T, i.e. acid production from raffinose, utilization of sugars and amino acids as energy sources, the NaCl and Mg²⁺ ranges for growth and the hydrolysis of tyrosine,

clearly distinguished these strains from each other and from recognized species of the genus *Haloarcula*. Therefore, strains HST01-2R^T and HST03^T represent two novel species in the genus *Haloarcula*, for which the names *Haloarcula salaria* sp. nov. and *Haloarcula tradensis* sp. nov. are proposed, respectively.

Description of *Haloarcula salaria* sp. nov.

Haloarcula salaria (sa.la'ri.a. L. fem. adj. *salaria* of or belonging to salt).

Fig. 2. Phylogenetic tree reconstruction showing the relationships between strains HST01-2R^T, HST03^T and related species of the genus *Haloarcula* based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages >56%, based on 1000 replications, are shown at the nodes. Bar, 1 substitution per 100 nt positions.

Cells are Gram-stain negative, non-motile, strictly aerobic pleomorphic rods, 0.6–0.8 µm in width and 1.0–2.0 µm in length. When grown on a complex medium of neutral pH, colonies are small, entire, translucent and convex (0.8–2.1 mm in diameter) after 1 week of incubation at 37 °C and show red pigmentation. Cells lyse in distilled water. Grows at between 15 and 45 °C (optimally at 37 °C) and at between pH 6.0 and pH 8.0 (optimally pH 7.0). Grows aerobically but does not grow anaerobically, even in the presence of nitrate. Tests for nitrate reduction, the methyl red/Voges–Proskauer reaction and the formation of indole are negative. Extremely halophilic, requires at least 15 % (w/v) NaCl for growth and the optimal concentration for growth is 20–25 % (w/v) NaCl. Growth occurs at Mg²⁺ concentrations of 2–10 % (w/v). Catalase and oxidase reactions are positive. Tween 80 and starch are hydrolysed, but skimmed milk, gelatin, tyrosine and xanthine are not hydrolysed. Produces acids from raffinose, but not from cellobiose, glycerol, D-glucose, D-sorbitol, or trehalose. Utilizes cellobiose, glycerol, D-glucose, raffinose, glutamic acid and aspartic acid, but not D-galactose, D-sorbitol, trehalose or lysine as the sole energy source. The type strain is susceptible to bacitracin (10 µg), but resistant to chloramphenicol (30 µg), tetracycline (30 µg), ampicillin (30 µg), gentamicin (30 µg), rifampicin (30 µg), streptomycin (30 µg) and novobiocin (5 µg). Possesses C₂₀C₂₀ diether core lipids. MK-8 (91.9 %) and MK-8(H₂) (8.8 %) are present. Possesses PG, PGP-Me, PGS, TGD-2, DGD and two unknown phospholipids.

The type strain, HST01-2R^T (=BCC 40029^T=JCM 15759^T=PCU 313^T) was isolated from salt from a sample of fish sauce from Thailand. The DNA G+C content of the type strain is 61.6 mol%.

Description of *Haloarcula tradensis* sp. nov.

Haloarcula tradensis (tra.den'sis. N.L. fem. adj. *tradensis* of or belonging to Trad, the province in Thailand from where the strain was isolated).

Cells are Gram-stain negative, non-motile, strictly aerobic pleomorphic rods, 0.8–1.2 µm in width and 1.0–2.5 µm in length. When grown on a complex medium of neutral pH, colonies are small, entire, translucent and convex (0.9–2.3 mm in diameter) after 1 week of incubation at 37 °C and show red pigmentation. Cells lyse in distilled water. Grows at between 15 and 45 °C (optimally at 37 °C) and at between pH 6.0 and pH 8.0 (optimally at pH 7.0). Grows aerobically, but does not grow anaerobically, even in the presence of nitrate. Tests for nitrate reduction, the methyl red/Voges–Proskauer reaction and the formation of indole are negative. Extremely halophilic, requires at least 15 % (w/v) NaCl for growth and optimal growth occurs at 20–25 % (w/v) NaCl. Growth occurs at Mg²⁺ concentrations of 2–10 % (w/v). Catalase and oxidase reactions are positive. Tween 80 and starch (weak reaction) are hydrolysed, but skimmed milk, gelatin, tyrosine and xanthine are not hydrolysed. No acid production from cellobiose, glycerol,

D-glucose, raffinose, D-sorbitol or trehalose. Utilizes cellobiose, glycerol, D-glucose, D-galactose, raffinose, D-sorbitol, glutamic acid, aspartic acid and lysine, but not trehalose as sole energy sources. The type strain is susceptible to bacitracin (10 µg) and novobiocin (5 µg), but resistant to chloramphenicol (30 µg), tetracycline (30 µg), ampicillin (30 µg), gentamicin (30 µg), rifampicin (30 µg), streptomycin (30 µg) and novobiocin (5 µg). Possesses C₂₀C₂₀ diether core lipids. MK-8 (91.8 %) and MK-8(H₂) (8.2 %) are present. Possesses PG, PGP-Me, PGS, TGD-2, DGD and two unknown phospholipids.

The type strain, HST03^T (=BCC 40030^T=JCM 15760^T=PCU 314^T) was isolated from salt from a sample of fish sauce in Thailand. The DNA G+C content of the type strain is 62.2 mol%.

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