Natrinema gari sp. nov., a halophilic archaeon isolated from fish sauce in Thailand

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Two Gram-negative, rod-shaped, halophilic archaea, designated strains HIS40-3^T and HDS3-1, were isolated from anchovy fish sauce (nam-pla) collected from two different locations in Thailand. The two strains were able to grow at 20–60 °C (optimum 37–40 °C), at 1.7–5.1 M NaCl (optimum 2.6–3.4 M NaCl) and at pH 5.5–8.5 (optimum pH 6.0–6.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolates were C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G + C contents were 64.0–65.4 mol%. In addition to phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequence similarities showed that strains HIS40-3^T and HDS3-1 were related most closely to species of the genus *Natrinema*. Levels of 16S rRNA gene sequence similarity between strains HIS40-3^T and HDS3-1 and the type strains of recognized *Natrinema* species were 99.1–96.6 %. The two novel strains could be distinguished from recognized *Natrinema* species on the basis of low levels of DNA–DNA relatedness and differences in whole-cell protein patterns and phenotypic properties. Levels of 16S rRNA gene sequence similarity and DNA–DNA relatedness between the two strains were 99.7 and 77.7 %, respectively, suggesting that they should be classified as representing a single species. Based on these taxonomic data, strains HIS40-3^T and HDS3-1 are considered to represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed. The type strain is HIS40-3^T (=BCC 24370^T =JCM 14663^T =PCU 303^T).

Fish sauce (nam-pla) is a traditional fermented fish product commonly used as a condiment in South-East Asia. Apart from its unique and pleasant flavour, it provides an important supplementary source of nitrogen

in the diet of people in this region. Fish sauce contains nitrogen at 20 g l⁻¹, of which 16 % is present as amino acids (Phithakpol *et al.*, 1995). Fish sauce contains a high concentration of NaCl, allowing various halophilic microorganisms to thrive (Lopetcharat *et al.*, 2001; Tanasupawat & Komagata, 2001). Nevertheless, just a few types of bacteria have been isolated from fish sauce samples and subjected to taxonomic study. In Thailand, *Halobacterium salinarum* and *Halococcus thailandensis* have been isolated from fish sauce samples: these are extremely halophilic archaea that grow optimally at 20–25 % NaCl (Thongthai *et al.*, 1992; Namwong *et al.*, 2007). Other bacteria isolated from fish sauce are moderately halophilic micro-organisms

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HIS40-3^T and HDS3-1 are AB289741 and AB289743, respectively.

Two-dimensional TLC of polar lipids extracted from strains HIS40-3^T and HDS3-1, maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences and patterns of whole-cell proteins are available as supplementary material with the online version of this paper.

that grow optimally at 3–15 % NaCl, such as *Tetragenococcus halophilus*, *Tetragenococcus muriaticus*, *Halobacillus thailandensis* and *Lentibacillus juripiscarius* (Thongsanit *et al.*, 2002; Chaiyanan *et al.*, 1999; Namwong *et al.*, 2005). At the time of writing, the genus *Natrinema* comprises five recognized species. *Natrinema pellirubrum* and *Natrinema pallidum* were created by McGenity *et al.* (1998) as a result of reclassification of strains of *Halobacterium salinarum* and *Halobacterium halobium*, respectively. On the basis of 16S rRNA gene sequence analysis, phenotypic properties and polar lipid composition, *Natrinema versiforme* (Xin *et al.*, 2000) and *Natrinema altunense* (Xu *et al.*, 2005) were also included within the genus. *Natrinema ejinorensis* has been described more recently (Castillo *et al.*, 2006). Here, we describe the taxonomic properties of two extremely halophilic archaea, strains HIS40-3^T and HDS3-1, isolated from fermented fish sauce, and these are suggested to represent a novel species of the genus *Natrinema*.

Strains HIS40-3^T and HDS3-1 were isolated from anchovy fish sauce fermented for 40 days collected from Samut Prakarn Province and an anchovy fish sauce sample fermented for 3 months collected from Samut Songkram Province, central Thailand, respectively. Samples were plated on agar plates of halophilic medium [per litre: 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 0.036 g FeCl₄·4H₂O, 0.36 mg MnCl₂·4H₂O, 20 g agar (pH 7.2)] and incubated at 37 °C for 1–2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T were used as reference strains in all tests of phenotypic characteristics. Unless otherwise stated, strains were grown in liquid medium (with shaking at 200 r.p.m.) or on agar plates of the halophilic medium and cultivated at 37 °C for 1–2 weeks.

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Colony and cell morphology were examined for cells grown on agar plates at 37 °C for 14 days. Catalase and oxidase activities and hydrolysis of gelatin, casein, starch and Tween 80 were determined according to the methods of Barrow & Feltham (1993). Casamino acids were omitted from the test medium for determination of the hydrolysis of gelatin and casein. Utilization of sugars, alcohols, amino acids and organic acids and acid production were determined in modified Leifson medium supplemented with 0.01 % (w/v) yeast extract and 4.3 M NaCl, but with casitone and Tris/HCl omitted (Leifson, 1963). Growth at various temperatures (20–60 °C) was examined. NaCl requirement was determined in medium containing various NaCl concentrations (0–5.1 M). Similarly, the requirement of the strains for Mg²⁺ was tested in halophilic medium without MgSO₄·7H₂O but supplemented with 0–1.0 M MgCl₂. Growth was determined by measuring culture turbidity at

600 nm. Anaerobic growth was tested on agar plates in the presence of nitrate (1 g l⁻¹), L-arginine (1 g l⁻¹) or DMSO (10 g l⁻¹). Production of indole and reduction of nitrate and nitrite were also tested. Determination of the antibiotic susceptibility of the strains was tested according to the methods described by Stan-Lotter *et al.* (2002). Menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984).

DNA was isolated and purified according to the method of Saito & Miura (1963). The G+C content was determined by the method of Tamaoka & Komagata (1984) by using reversed-phase HPLC. DNA–DNA hybridization was determined as reported by Ezaki *et al.* (1989) and levels of relatedness were determined by the colorimetric method as reported by Tanasupawat *et al.* (2000). The 16S rRNA gene sequences of strains HIS40-3^T and HDS3-1, comprising 1405 and 1353 bp, respectively, were amplified by PCR with primers D30F (5'-ATTCCGGTTGATCCTGC-3', positions 6–12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-3', positions 1492–1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (v. 3.0; Applied Biosystems) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with the following primers: D30F, D33R (5'-TCGCGCCTGCGCCCCGT-3', positions 344–360), D34R (5'-GGTCTCGCTCGTTGCCTG-3', positions 1096–1113) and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood and maximum-parsimony methods, by using programs in the CLUSTAL_X and MEGA 4 packages (Thompson *et al.*, 1997; Tamura *et al.*, 2007). Confidence in the branching pattern was assessed by analysis of 1000 bootstrap replicates (Felsenstein, 1985).

Cells of strains HIS40-3^T and HDS3-1 were motile, Gram-negative rods (0.5–0.8 × 2.0–3.0 µm) (Fig. 1). Colonies formed on agar plates were circular (1–2 mm in diameter), smooth, translucent and pale-orange pigmented. The two strains were able to grow over a wide range of NaCl concentrations, from 1.7 M (approximately 10 %) to 5.1 M (approximately 30 %). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. Strains HIS40-3^T and HDS3-1 grew optimally in the presence of 2.6–3.4 M (15–20 %) NaCl, similar to most halophilic archaea (Grant *et al.*, 2001). The strains grew over a wide range of MgCl₂ concentrations from 0 to 1.0 M and grew optimally at around 0.1–0.2 M. The two strains grew at 20–60 °C (optimum 37–40 °C) and at pH 5.5–8.5 (optimum pH 6.0–6.5). The two strains were positive for catalase and oxidase. Indole production from tryptophan was

Fig. 1. Scanning electron micrographs of cells of strain HIS40-3^T (a) and strain HDS3-1 (b) grown on halophilic medium at 37 °C. Bars, 1 µm.

negative. Casein, starch and Tween 80 were not hydrolysed. Gelatin was liquefied. The strains showed anaerobic growth in the presence of DMSO but not with nitrate or arginine. Nitrate was not reduced and gas formation was not observed. The strains were sensitive to rifampicin, bacitracin and novobiocin. Strains HIS40-3^T and HDS3-1 utilized several carbohydrates. Among them, strong acid formation was observed only from glycerol for both strains and from arabinose for strain HIS40-3^T. There were some differences in the utilization of carbon sources between strains HIS40-3^T and HDS3-1. Some characteristics that distinguish strains HIS40-3^T and HDS3-1 from other members of the genus *Natrinema* are summarized in Table 1.

Strains HIS40-3^T and HDS3-1 possessed two menaquinones, MK-8 (76.3 and 74.6 %, respectively) and MK-8(H₂) (23.7 and 25.5 %), which are commonly detected in species of the genus *Natrinema* (McGenity *et al.*, 1998; Xin *et al.*, 2000). Two-dimensional TLC revealed that the two novel strains possessed glycerol diether analogues of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP), phosphatidylglycerol sulfate (PGS), pigment, two glycolipids and one unidentified lipid (see Supplementary Fig. S1, available in IJSEM Online). The core lipids were C₂₀C₂₀ and C₂₀C₂₅ diether, as shown

from the two PG and PGP spots. The two strains showed two glycolipid spots, tentatively designated GL1 and 2, which might correspond to molecules containing C₂₀C₂₀ and C₂₀C₂₅ diether moieties. These two glycolipids were also found in varying amounts in the reference strains *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T (McGenity *et al.*, 1998; Xu *et al.*, 2005). The DNA G + C contents of strains HIS40-3^T and HDS3-1 were 65.4 and 64.0 mol%, respectively.

The neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequence data for the two new isolates and other representative *Natrinema* species is shown in Fig. 2. Strains HIS40-3^T and HDS3-1 formed a distinct cluster that fell within the genus *Natrinema*. Trees constructed according to the maximum-likelihood and maximum-parsimony methods are shown in Supplementary Fig. S2. 16S rRNA gene sequence similarity between the two novel strains was 99.7 %, suggesting that they should be classified as representing a single species or as members of very closely related species. This is in accordance with the morphological and chemotaxonomic similarities detailed above. The nearest neighbours of strains HIS40-3^T and HDS3-1 were *Nnm. pallidum* JCM 8980^T (99.1 and 99.1 % 16S rRNA gene sequence similarity, respectively), *Nnm. pellirubrum* JCM 10476^T (98.7 and 98.7 %), *Nnm. altunense* JCM 12890^T (98.5 and 98.5 %), *Nnm. versiforme* JCM 10478^T (98.4 and 98.4 %) and *Nnm. ejinorensis* JCM 13890^T (96.6 and 96.7 %).

The DNA–DNA hybridization study revealed that strains HIS40-3^T and HDS3-1 were closely related, exhibiting levels of relatedness of 73.5–77.7 % to each other; however, strains HIS40-3^T and HDS3-1 showed only low levels of DNA–DNA relatedness to *Nnm. pallidum* JCM 8980^T (40.5 and 42.5 %, respectively), *Nnm. pellirubrum* JCM 10476^T (18.7 and 22.0 %), *Nnm. altunense* JCM 12890^T (13.2 and 16.0 %) and *Nnm. versiforme* JCM 10478^T (19.5 and 18.2 %), indicating that these two novel strains are not members of any of these *Natrinema* species. Each of the values was obtained from two independent determinations. Moreover, the protein pattern of the novel strains was markedly different from those of the *Natrinema* species representatives analysed herein (Supplementary Fig. S3).

In conclusion, on the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G + C contents and 16S rRNA gene sequence analysis, strains HIS40-3^T and HDS3-1 are considered to represent a single species of the genus *Natrinema*. However, they could be differentiated from recognized *Natrinema* species based on levels of DNA–DNA relatedness and differences in whole-cell protein patterns. The results of the present study thus suggest that strains HIS40-3^T and HDS3-1 represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed.

Table 1. Differential characteristics between strains HIS40-3^T and HDS3-1 and recognized *Natrinema* species

Strains: 1, strain HIS40-3^T; 2, strain HDS3-1; 3, *Nnm. pallidum* JCM 8980^T; 4, *Nnm. pellirubrum* JCM 10476^T; 5, *Nnm. altunense* JCM 12890^T (data in columns 1–5 from this study unless indicated); 6, *Nnm. versiforme* JCM 10478^T (data from Xin *et al.*, 2000); 7, *Nnm. ejinorensis* JCM 13890^T (Castillo *et al.*, 2006). +, Positive; –, negative; +/–, doubtful; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Cell morphology	Rods	Rods	Rods	Rods	Rods	Pleomorphic	Pleomorphic
Motility	+	+	+	+	+	–	–
Pigmentation	Pale orange	Pale orange	Pale orange	Pale orange	Pale orange	Light red	Light red
NaCl concentration (M) required to prevent cell lysis	>1.7	>1.7	>1.7	>1.7	>2.1	>1.0	>1.5
NaCl requirement (M)	≥1.7	≥1.7	≥1.7	≥1.7	≥2.1	≥1.5	≥1.8
NaCl optimum (M)	2.6–3.4	2.6–3.4	3.4–4.3	2.6–3.4	2.6–3.4	3.4–4.3	3.4
MgCl ₂ optimum (M)	0.1–0.2	0.1–0.2	0.1–0.2	0.1–0.2	0.1–0.2	0.15	ND
Temperature for growth (°C)							
Range	20–60	20–60	25–60	20–60	20–60	20–53	25–50
Optimum	37–40	37–40	30–37	30–37	37–40	37–46	37
pH for growth							
Range	5.5–8.5	5.5–8.5	5.5–8.5	5.5–8.5	6.0–8.5	6.0–8.0	6.0–8.5
Optimum	6.0–6.5	6.0–6.5	7.0–7.5	7.0–7.5	6.5–7.0	6.5–7.0	7.0
Anaerobic growth in the presence of nitrate	–	–	+	–	+	+	–
Reduction of nitrate to nitrite	–	–	+	+	+	+	–
Gas formation from nitrate	–	–	–	–	–	+	+
Oxidase activity	+	+	+	–	–	+	+
Indole formation	–	–	–	–	–	+	–
Hydrolysis of:							
Gelatin	+	+	+	–	+	–	+
Starch	–	–	–	–	–	+/–	+
Tween 80	–	–	+	–	+	+	+
DNA G + C content (mol%)	65.4	64.0	63.9	62.9	65.6*	64.2	64.7

*Data from Xu *et al.* (2005).

Description of *Natrinema gari* sp. nov.

Natrinema gari (ga'ri. L. gen. n. *gari* of a fish sauce, pertaining to the isolation of strains from fermented fish sauce).

Cells are motile, Gram-negative rods, 0.5–0.8 × 2.0–3.0 µm in size. Colonies are pale orange, smooth, circular and elevated. Growth is chemo-organotrophic. Requires at least 1.7 M NaCl for growth (optimum 2.6–3.4 M NaCl).

Fig. 2. Phylogenetic tree showing the relationships between strains HIS40-3^T, HDS3-1 and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap values above 70 %, based on 1000 replications, are shown at nodes. Bar, 0.5 substitutions per 100 nucleotide positions.

Growth occurs at 0–1.0 M MgCl₂ (optimum 0.1–0.2 M MgCl₂). The pH range for growth is 5.5–8.5 (optimum pH 6.0–6.5). The temperature range for growth is 20–60 °C (optimum 37–40 °C). Grows anaerobically in the presence of DMSO but not nitrate. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. Negative for production of indole. Casein, starch and Tween 80 are not hydrolysed. Gelatin is hydrolysed. L-Arabinose, D-glucose and glycerol are utilized for growth. Does not utilize inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, D-ribose, sorbitol, sucrose, D-xylose or citrate. Acid is produced from L-arabinose and glycerol. Susceptible to bacitracin (10 U), novobiocin (5 µg) and rifampicin (30 µg), but resistant to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The predominant menaquinone is MK-8. Cells contain C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G+C content of the type strain is 65.4 mol%.

The type strain, HIS40-3^T (=BCC 24370^T =JCM 14663^T =PCU 303^T), was isolated from fermented fish sauce in Thailand.

Acknowledgements

We would like to thank the Thailand Research Fund for financial support under the TRF Senior Research Scholar programme to S. B., the Royal Golden Jubilee PhD programme (grant no. PHD/0119/2548) and Prince of Songkla University for an operating grant under the graduate study programme to W.T. Thanks are also due to the National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing laboratory equipment and experimental space.

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