Salinivibrio siamensis sp. nov., from fermented fish (*pla-ra*) in Thailand

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A Gram-negative, facultatively anaerobic, moderately halophilic bacterium, strain ND1-1^T, was isolated from fermented fish (*pla-ra*) in Thailand. The cells were curved rods, motile and non-endospore-forming. The novel strain grew optimally at 37 °C, at pH 8 and in the presence of 9–10 % (w/v) NaCl. The predominant respiratory lipoquinone was Q-8. The major cellular fatty acids were C_{16:0} and C_{12:0}. Polar lipid analysis revealed the presence of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content was 49.0 mol%. Comparative 16S rRNA gene sequence analyses indicated that strain ND1-1^T was closely related to *Salinivibrio costicola*, which comprises three subspecies, and *Salinivibrio proteolyticus* with gene sequence similarities of 98.3–98.6 %. Strain ND1-1^T showed low levels of DNA–DNA relatedness with *S. costicola* subsp. *costicola* JCM 15095^T (33.2 %), *S. costicola* subsp. *alcaliphilus* DSM 16359^T (38.4 %), *S. costicola* subsp. *vallismortis* JCM 15096^T (59.7 %), and *S. proteolyticus* AF-2004^T (42.1 %). On the basis of the physiological and biochemical characteristics and the molecular data presented, strain ND1-1^T should be classified as a novel species of the genus *Salinivibrio* for which the name *Salinivibrio siamensis* sp. nov. is proposed. The type strain is ND1-1^T (=JCM 14472^T=PCU 301^T=TISTR 1810^T).

The genus *Salinivibrio* is included in the family *Vibrionaceae*, belonging to the class *Gammaproteobacteria* of the phylum *Proteobacteria*. This genus was created for *Vibrio costicola* based on the significant phenotypic and genotypic differences between this species and other species of the genus *Vibrio* (Mellado *et al.*, 1996). At present, the genus comprises two species, *Salinivibrio costicola* subsp. *costicola* (Smith, 1938; Garcia *et al.*, 1987), *S. costicola* subsp. *vallismortis* (Huang *et al.*, 2000), *S. costicola* subsp. *alcaliphilus* (Romano *et al.*, 2005) and *Salinivibrio proteolyticus* (Amoozgar *et al.*, 2008). Members of the genus *Salinivibrio* are moderately halophilic bacteria which are

distributed in salted meats, brines and hypersaline environments and grow in the presence of 0–20 % NaCl. During our investigation into halophilic bacteria in fermented fish in Thailand, strains of *Lentibacillus juripiscarius* and *Lentibacillus halophilus* from fish sauce (Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006), *Lentibacillus kapialis* from fermented shrimp paste (Pakdeeto *et al.*, 2007) and *Piscibacillus salipiscarius* from fermented fish (Tanasupawat *et al.*, 2007) have been reported. In this paper, a *Salinivibrio*-like, moderately halophilic bacterium, strain ND1-1^T, isolated from fermented fish (*pla-ra*), is described based on phenotypic, chemotaxonomic and genotypic characteristics, including DNA–DNA relatedness and 16S rRNA gene sequence analyses.

Strain ND1-1^T was isolated from a fermented fish product, *pla-ra*, which was collected from a market in Nakornnayok province, Thailand. *Pla-ra* contains 7.8–17.9 % NaCl (w/v) and has a pH of 4.3–6.2 (Phithakpol *et al.*, 1995). The

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ND1-1^T is AB285018.

Additional phylogenetic trees constructed by the maximum-parsimony and maximum-likelihood methods are available with the online version of this paper.

novel strain was isolated from the sample by the spread-plate technique on agar plates of JCM medium no. 377 (containing 1^{-1} : 100 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g $C_6H_5Na_3O_7 \cdot 2 H_2O$, 20 g $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 20 g agar; pH 7.2) and incubated at 37 °C for 3–7 days. Cell shape, size and arrangement were examined on JCM medium no. 377 agar at 37 °C for 3 days. The Hucker–Conn modification was used for Gram stain (Hucker & Conn, 1923). Spore formation was examined on Gram-stained specimens. Critical-point-dried cells were observed by scanning electron microscopy. Flagella were examined as described by Forbes (1981) and observed by transmission electron microscopy.

The effects of various NaCl concentrations (0, 0.5 and 1–25 %, w/v with a 1% interval), initial pH values (5–10, with intervals of 1 pH unit) and temperatures (5–55 °C with intervals of 1, 1.5, 2, 2.5 and 3 °C) on the growth of the novel strain were investigated with JCM medium no. 377 broth (Namwong *et al.*, 2005). Catalase and oxidase activity, aesculin hydrolysis, citrate utilization and nitrate reduction were determined as described by Barrow & Feltham (1993). The hydrolysis of casein, gelatin, starch and Tween 80 and the activities of DNase and urease were determined as described by Namwong *et al.* (2005). Arginine decarboxylase activity was determined by using the medium reported by Thornley (1960). Acid production from carbohydrate was determined with the medium described by Leifson (1963) and the ability to utilize various compounds was examined in a mineral liquid medium as reported by Romanenko *et al.* (2003) supplemented with 10 % NaCl. Growth under anaerobic conditions on agar plates with or without 1 % (w/v) KNO_3 was performed in Gaspak (BBL) anaerobic jars. Additional enzyme activities were recorded after 24 h incubation with API ZYM strips (bioMérieux).

Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). Plates were incubated at 37 °C for 48 h and the inhibition zone was interpreted according to the manufacturer's manual. For chemotaxonomic characterization, cell biomass was produced in JCM medium no. 377 at 37 °C for 3 days. Quinones were analysed using reversed-phase HPLC as described by Komagata & Suzuki (1987). Polar lipids were extracted as described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). For quantitative analysis of cellular fatty acid content, cell mass was harvested and fatty acid methyl esters (FAMES) were prepared and identified according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

Chromosomal DNA was isolated and purified from cells grown in JCM medium no. 377 according to the method of Saito & Miura (1963). The DNA G+C content was determined by reversed-phase HPLC (Tamaoka &

Komagata, 1984). DNA–DNA hybridization tests were conducted in microdilution well plates as reported by Ezaki *et al.* (1989). The 16S rRNA gene of the novel isolate was amplified, purified and sequenced as described by Namwong *et al.* (2005). The sequence determined was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ database by using CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). The alignment was manually edited and positions with gaps and ambiguous bases were eliminated prior to the construction of the phylogenetic tree. Phylogenetic trees based on the neighbour-joining method (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood methods were constructed with the NJPlot program (Perrière & Gouy, 1996) with MEGA4 v4 software (Tamura *et al.*, 2007). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain ND1-1^T were Gram-negative, non-endo-spore-forming, curved rods, approximately $0.4\text{--}0.5 \times 2\text{--}8 \mu\text{m}$ after 3 days incubation at 37 °C on JCM medium no. 377 agar (Fig. 1a). Cells were motile by means of a single polar flagellum (Fig. 1b). Colonies were round with entire edges, smooth, convex, opaque and cream-pigmented. The diameter of the colonies was 0.9–2.5 mm. The results of the biochemical tests are listed in the species description and in Table 1. The novel isolate grew over a pH range of 5–9 (optimally at pH 8), at 10–47 °C (optimally at 37 °C) and in 1–22 % (w/v) NaCl (optimally in 9–10 %), but not in 0.5 % NaCl or below. No growth was observed at or above 50 °C. The novel strain grew under anaerobic conditions even if KNO_3 was omitted from the medium. The novel strain contained Q-8 as the major respiratory lipoquinone. The major cellular fatty acids were $C_{16:0}$ (16.4 %) and $C_{12:0}$ (14.0 %). When analysed in this study using the same conditions, strain ND1-1^T, *S. costicola* subsp. *costicola* JCM 15095^T, *S. costicola* subsp. *vallismortis* DSM 8285^T, *S. costicola* subsp. *alcaliphilus* JCM 15096^T and *S. proteolyticus* AF-2004^T contained roughly the same fatty acid pattern, with a large amount of $C_{16:1\omega7c}$ and/or iso- $C_{15:0}$ 2-OH

Fig. 1. (a) Scanning electron micrograph of cells of strain ND1-1^T grown on JCM medium no. 377 at 37 °C. Bar, 1 μm . (b) Transmission electron micrograph of a cell of strain ND1-1^T showing the single polar flagellum. Bar, 2 μm .

Table 1. Differential characteristics of strain ND1-1^T and related species of the genus *Salinivibrio*

Strains: 1, ND1-1^T; 2, *S. costicola* subsp. *costicola* JCM 15095^T; 3, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 4, *S. costicola* subsp. *vallismortis* JCM 15096^T; 5, *S. proteolyticus* AF-2004^T. +, Positive; w, weakly positive; –, negative; A, aerobic; FA, facultative anaerobe; ND, no data.

Characteristic	1	2	3	4	5
Colony colour	Cream	Cream	Cream–pink	Cream–white	Cream–white
Oxygen requirement	FA	FA	A	FA	FA
Flagellation	One polar flagellum	One polar flagellum*	ND	One or two polar flagella†	One polar flagellum‡
NaCl range (% w/v)	1–22	2–18	2–22	0–12.5	1–17
Optimum NaCl concentration (%)	9–10	10	9	2.5	5
Temperature range (°C)	10–47	15–45	10–45	20–50	10–45
Optimum temperature (°C)	37	34	37	37	32–35
pH range	5–9	6–9	6–10	5.5–8.2	5–9.5
Optimum pH	8	8	8	7.3	8–8.5
Voges–Proskauer	+	+	–	+	+
Nitrate reduction	–	–	+	–	–
Esterase (C4)	–	+	–	+	w
Naphthol-AS-BI phosphohydrolase	+	+	–	w	–
<i>N</i> -acetyl- β -glucosaminidase	+	+	–	+	w
Hydrolysis of:					
Aesculin	–	–	+	–	–
Starch	+	–	–	+	+
L-Tyrosine	+	+	w	–	–
Acid from:					
Cellobiose	–	–	+	+	–
D-Xylose	+	–	–	–	+
Utilization of:					
Cellobiose	–	+	–	+	–
Crotonic acid	+	–	+	–	ND
D-Fructose	–	–	–	+	+
Glycine	+	–	+	–	+
Malonic acid	–	–	+	–	+
Succinate	+	–	+	–	+
D-Xylose	+	–	–	–	–
DNA G + C content (mol%)	49	49.9 or 50.0*	49.3†	50.0†	49.5‡
Source of isolation	Fermented fish	Cured meat and salterns*	Saltish spring†	Death valley†	Hypersaline lake‡

*Data from Mellado *et al.* (1996) and Huang *et al.* (2000).

†Data from Romano *et al.* (2005).

‡Data from Amoozegar *et al.* (2008).

(25.3–35.7 %, summed feature 3) (Table 2). Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) were detected. The DNA G+C content of the novel strain was 49.0 mol%.

A comparison of the 16S rRNA gene sequence of strain ND1-1^T (1487 nucleotides) with those of other members of the family *Vibrionaceae* indicated that the novel strain was closely related to members of the genus *Salinivibrio*, showing 98.3 % gene sequence similarity to *S. costicola* subsp. *costicola* NCIMB 701^T, 98.3 % to *S. costicola* subsp. *alcaliphilus* DSM 16359^T, 98.2 % to *S. costicola* subsp. *vallismortis* DSM 8285^T and 98.6 % to *S. proteolyticus* AF-2004^T (Fig. 2).

In agreement with the phylogenetic assignment, the phenotypic properties of strain ND1-1^T, such as cell morphology, NaCl range for growth, major lipoquinone composition, fatty acid profile and DNA G+C content, it is concluded that strain ND1-1^T can be placed within the genus *Salinivibrio*. Additionally, the 16S rRNA gene sequence of strain ND1-1^T contained the signature nucleotides found in the genus *Salinivibrio* (Mellado *et al.*, 1996) and its secondary structure at positions 178–197 was more closely related to that of *S. costicola* subsp. *vallismortis* DSM 8285^T and *S. proteolyticus* AF-2004^T than to those of *S. costicola* subsp. *costicola* NCIMB 701^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T as reported by Huang *et al.* (2000). However, strain ND1-1^T did not show

Table 2. Cellular fatty acid content (%) of strain ND1-1^T and related species of the genus *Salinivibrio*

Strains: 1, ND1-1^T; 2, *S. costicola* subsp. *costicola* JCM 15095^T; 3, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 4, *S. costicola* subsp. *vallismortis* JCM 15096^T; 5, *S. proteolyticus* AF-2004^T. –, Not detected. Values are % of total cellular fatty acids.

Fatty acid	1	2	3	4	5
Saturated straight-chain					
C _{12:0}	14.0	5.1	7.2	6.6	4.0
C _{14:0}	8.1	3.7	2.8	6.8	3.6
C _{16:0}	16.4	24.4	15.0	16.3	20.2
C _{18:0}	1.5	1.2	1.6	1.4	3.0
Unsaturated straight-chain					
C _{16:1} ω9 <i>c</i>	2.1	4.3	3.1	3.7	3.7
C _{18:1} ω5 <i>c</i>	–	–	–	–	1.2
C _{18:1} ω7 <i>c</i>	10.3	14.3	11.8	21.7	27.3
C _{18:1} ω9 <i>c</i>	1.2	1.6	1.8	2.8	4.2
Saturated branched-chain					
iso-C _{13:0}	–	–	4.6	1.4	–
iso-C _{14:0}	1.7	–	–	–	–
iso-C _{15:0}	–	–	1.3	1.9	–
iso-C _{16:0}	2.8	–	1.9	1.7	1.4
iso-C _{17:0}	–	–	2.0	1.2	–
Hydroxylated fatty acids					
C _{12:0} 3-OH	8.7	3.7	4.4	3.4	2.7
Summed features*					
2	5.4	4.6	6.3	5.8	3.4
3	27.7	37.1	35.7	25.4	25.3

*Summed features are groups of two or three fatty acids that can not be separated using the MIDI system. Summed feature 2 comprises iso-C_{16:1} and/or C_{14:0} 3-OH. Summed feature 3 comprises C_{16:1}ω7*c* and/or iso-C_{15:0} 2-OH.

clear affiliation with these two type strains in the 16S rRNA gene sequence-based trees based on the different algorithms (Fig. 2 and see Supplementary Figs S1 and S2 in IJSEM Online). The 16S rRNA gene sequence similarities between strain ND1-1^T and the four type strains (98.2–98.6%) of the genus *Salinivibrio* did not suggest that strain ND1-1^T should be considered as a separate species (Stackebrandt & Goebel, 1994). However, the DNA–DNA hybridization studies, conducted with three replicates, showed that strain ND1-1^T had low levels of DNA–DNA relatedness to *S. costicola* subsp. *costicola* JCM 15095^T (33.2%), *S. costicola* subsp. *alcaliphilus* DSM 16359^T (38.4%), *S. costicola* subsp. *vallismortis* JCM 15096^T (59.7%) and *S. proteolyticus* AF-2004^T (42.1%). Strain ND1-1^T could be distinguished from the type strains of the genus *Salinivibrio* on the basis of the phenotypic properties shown in Table 1. Therefore, on the basis of phenotypic and genotypic properties, strain ND1-1^T represents a new member of the genus *Salinivibrio*, for which the name *Salinivibrio siamensis* sp. nov. is proposed.

Description of *Salinivibrio siamensis* sp. nov.

Salinivibrio siamensis (si.am.en'sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, where the strain was isolated).

Cells are Gram-negative, non-endospore-forming, curved rods, 0.4–0.5 × 2–8 µm. Forms circular, convex, opaque, cream-coloured colonies (0.9–2.5 mm). Motile by means of a single polar flagellum. Facultatively anaerobic. Growth at pH values of 5–9 (optimally at pH 8), at 10–47 °C (optimally at 37 °C) and in 1–22% (w/v) NaCl (optimally in 9–10% NaCl), but no growth in the absence of NaCl. Positive in tests for catalase, oxidase, hydrolysis of casein, gelatin, starch and L-tyrosine, for nitrate reduction, for the Voges–Proskauer reaction and for activities of arginine decarboxylase, acid phosphatase, alkaline phosphatase, cystine arylamidase, DNase, esterase lipase (C8), *N*-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase. Negative result in tests for the hydrolysis of aesculin and Tween 80, for citrate utilization, for activities of α-chymotrypsin, esterase (C4), α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase and urease, for H₂S production, for the methyl-red test and for indole formation. Acid is produced from D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, ribose, sucrose, trehalose and D-xylose, but not from L-arabinose, cellobiose, *myo*-inositol, lactose, melibiose, melezitose, raffinose, rhamnose, salicin or sorbitol. Utilizes L-alanine, crotonic acid, fumaric acid, glucoric acid, glycine, propionate, pyruvate, sodium glycolate, succinate and D-xylose. Slight growth in sodium hippurate, but does not utilize L-arginine, L-arabinose, betaine, cellobiose, citric acid, D-fructose, galactose, glucose, *p*-hydroxybenzoic acid, lactose, sucrose, malonic acid, L-ornithine, sodium benzoate, sodium tartrate or sorbitol. The type strain is susceptible to ampicillin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), novobiocin (5 µg), rifampicin (30 µg) and tetracycline (30 µg), but resistant to bacitracin (10 U), gentamicin (10 µg), kanamycin (30 µg) and neomycin (30 µg). Q-8 is the major respiratory lipoquinone. Major cellular fatty acids are C_{16:0} and C_{12:0}. PE, PG and DPG are the predominant polar lipids.

The type strain, ND1-1^T (=JCM 14472^T=PCU 301^T=TISTR 1810^T), was isolated from fermented fish (*pla-ra*) in Thailand. The DNA G + C content of the type strain is 49.0 mol%.

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Fig. 2. Phylogenetic tree showing the relationships between strain ND1-1^T, species of the genus *Salinivibrio* and related taxa based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages $\geq 55\%$, based on 1000 replications are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

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