Salinicoccus siamensis sp. nov., isolated from fermented shrimp paste in Thailand

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Fifteen strains of moderately halophilic, Gram-positive cocci were isolated from a traditional fermented shrimp paste ('ka-pi') produced in Thailand. These bacteria were strictly aerobic, non-motile, non-sporulating and catalase- and oxidase-positive. They produced orange pigment and grew in the presence of 1.5–25% (w/v) NaCl. They grew optimally in 10% (w/v) NaCl, at pH 8.5 and at 37 °C. The cell-wall peptidoglycan was of L-Lys type. Menaquinone with six isoprene units (MK-6) was a major component. The dominant cellular fatty acids were anteiso-C_{15:0} and iso-C_{15:0}. DNA G + C contents were in the range 44.5–47.5 mol%. Comparative 16S rRNA gene sequence analyses indicated that representative strain PN1-2^T was related most closely to *Salinicoccus roseus* JCM 14630^T, with 97.3% similarity. The other novel strains were included in the same species based on their levels of DNA–DNA relatedness to strain PN1-2^T (≥76.6%) but showed low DNA–DNA relatedness to *S. roseus* JCM 14630^T (21.7%). On the basis of the phenotypic and molecular data presented, the 15 novel strains are suggested to represent a single novel species of the genus *Salinicoccus*, for which the name *Salinicoccus siamensis* sp. nov. is proposed. The type strain is PN1-2^T (=JCM 12822^T =PCU 242^T =TISTR 1562^T).

Moderately halophilic Gram-positive cocci have been recognized within several bacterial genera, and include *Marinococcus albus*, *M. halophilus* and *M. halotolerans* (Hao *et al.*, 1984; Li *et al.*, 2005), *Salinicoccus roseus*, *S. hispanicus* and *S. alkaliphilus* (Ventosa *et al.*, 1990, 1992; Zhang *et al.*, 2002), *Nesterenkonia halobia* (Stackebrandt *et al.*, 1995), *Halobacillus halophilus* (Spring *et al.*, 1996), *Tetragenococcus muriaticus* (Satomi *et al.*, 1997), and *Jeotgalicoccus halotolerans* and *J. psychrophilus* (Yoon *et al.*, 2003). In this study, a novel orange-pigmented species isolated from

'ka-pi' was identified as belonging to the genus *Salinicoccus* on the basis of phenotypic and chemotaxonomic characterization and 16S rRNA gene sequence analysis.

The halophilic bacteria investigated herein were isolated from samples of fermented shrimp paste, 'ka-pi', collected from a market in Nakhonsrithammarat Province in southern Thailand. The cultures were isolated by using the spread-plate technique on agar plates of JCM medium no. 377 (per litre distilled water: 100 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g trisodium citrate, 20 g MgSO₄ · 7H₂O, 36 mg FeCl₂ · 4H₂O, 0.36 mg MnCl₂ · 4H₂O, 20 g agar; pH 7.2) after incubation at 37 °C for 7 days. Liquid cultures were cultivated in Erlenmeyer flasks containing the same medium and incubated on a rotary shaker. All media contained 10% (w/v) NaCl, except for tests of NaCl tolerance. Cell shape, size and arrangement and colony size were examined by using cells grown on JCM medium no. 377 agar at 37 °C for 5 days. The Hucker–Conn modification was used for Gram staining (Hucker & Conn, 1923). Spore formation was examined with Gram-stained specimens.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PN1-2^T and PN7-1 are AB258358 and EF397606, respectively.

A scanning electron micrograph of cells of strain PN1-2^T, an image showing the colonial appearance of strain PN1-2^T, a 16S rRNA gene sequence phylogenetic tree showing the relationships between strains PN1-2^T and PN7-1 and related species and a table giving DNA G + C contents and levels of DNA–DNA relatedness among the novel strains and *S. roseus* JCM 14630^T are available as supplementary material in IJSEM Online.

Critical-point-dried cells were observed under a scanning electron microscope. The presence of flagella was examined as described by Forbes (1981) and observed by transmission electron microscopy. Physiological and biochemical characteristics were determined as described by Thornley (1960), Leifson (1963) and Barrow & Feltham (1993), by using medium supplemented with 10% (w/v) NaCl. Growth under anaerobic conditions on agar plates with or without nitrate (1%, w/v) was investigated with a GasPak (BBL) anaerobic jar. Growth at various temperatures (10–50 °C), pH values (5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0) and NaCl concentrations (0–30%, w/v) was assessed. At the lower NaCl concentrations (0.0–2.0%, w/v), MgSO₄·7H₂O was omitted from the test medium. Growth was monitored by measuring culture turbidity at 660 nm. The presence of diaminopimelic acid in the cell-wall peptidoglycan and menaquinone profiles were analysed as described by Komagata & Suzuki (1987). A TLC plate (Merck no. 5577) developed with solvent systems of methanol/pyridine/12 M HCl/water (32:4:1:7, v/v) was used to determine lysine in the cell wall (Tanasupawat *et al.*, 1993). Quantitative analysis of the cellular fatty acids was

performed as described by Sasser (1990) and Kämpfer & Kroppenstedt (1996). DNA was isolated from cells grown in JCM medium no. 377 broth and purified according to the method of Saito & Miura (1963). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984), by using reversed-phase HPLC. DNA–DNA hybridization was conducted in microdilution-well plates, as described by Ezaki *et al.* (1989), and was detected by using the colorimetric method reported by Tanasupawat *et al.* (2000). 16S rRNA genes were amplified, purified and sequenced as described by Seearunruangchai *et al.* (2004). The sequences determined (1489–1503 bases) were aligned with selected sequences obtained from the GenBank database by using CLUSTAL W, version 1.81 (Thompson *et al.*, 1994). The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of the phylogenetic tree. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in MEGA, version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Table 1. Differential characteristics of strain PN1-2^T and related taxa

Strains: 1, PN1-2^T and 14 related strains; 2, *S. roseus* DSM 5351^T (data from Ventosa *et al.*, 1990); 3, *S. hispanicus* DSM 5352^T (Márquez *et al.*, 1990; Ventosa *et al.*, 1992); 4, *S. alkaliphilus* JCM 11311^T (Zhang *et al.*, 2002); 5, *J. halotolerans* JCM 11198^T (Yoon *et al.*, 2003). +, Positive; –, negative; v, variable; NA, no data available.

Characteristic	1	2	3	4	5
Cell size (µm)	0.6–0.8	1–2.5	1–2.5	0.5–0.8	0.6–1.1
Colony pigmentation	Orange	Pink–red	Reddish orange	Pinkish	Light yellow
pH range for growth	6–9	5–9	6–9	6.5–11.5	7–8
Optimum pH for growth	8.5	7.5	8.0	9.5	NA
NaCl range for growth (%)	1.5–25	0.5–25	0.9–25	0–25	0–20
Temperature range for growth (°C)	15–45	15–37	15–40	10–49	4–42
Optimum temperature for growth (°C)	37	37	37	32	30–35
Methyl red test	–	–	+	–	NA
Urease test	–	–	+	+	–
Nitrate reduction	–	–	+	+	+
Hydrolysis of:					
Casein	–	+	–	–	–
Aesculin	–	–	+	+	–
Gelatin	–	+	+	–	NA
Tween 80	–	+	–	–	–
Starch	–	+	–	–	–
Acid production from:					
D-Fructose	+	–	+	–	–
D-Galactose	–	–	+	–	–
D-Glucose	+	–	+	+	–
Maltose	–	–	+	–	–
D-Mannitol	–	–	+	+	+
Sucrose	v*	–	NA	–	–
DNA G+C content (mol%)	44.5–47.5†	51.2	45.6–49.3‡	49.6	42

*Two strains tested positive. Thirteen strains (including the type strain) tested negative.

†The G+C content of the type strain is 46 mol%.

‡Range of values for five strains given by Marquez *et al.* (1990). The value for the type strain is 45.7 mol%.

Table 2. Cellular fatty acid compositions of strain PN1-2^T and related taxa

Strains: 1, PN1-2^T; 2, PN1-8, PN2-2 and PN7-1; 3, *S. roseus* DSM 5351^T (data from Ventosa *et al.*, 1990); 4, *S. hispanicus* DSM 5352^T (Márquez *et al.*, 1990; Ventosa *et al.*, 1992); 5, *S. alkaliphilus* JCM 11311^T (Zhang *et al.*, 2002); 6, *J. halotolerans* JCM 11198^T (Yoon *et al.*, 2003). NA, No data available; ND, not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{14:0}	0.5	0.2–0.5	NA	0.5	1.6	0.4
C _{15:0}	ND	0.1–0.2	0.7	0.5	NA	1.0
C _{16:0}	2.4	0.9–2.4	1.0	2.7	1.5	3.4
Unsaturated						
C _{16:1} ω7c alcohol	ND	0.2–1.6	0.6	0.8	5.8	0.8
Branched						
iso-C _{14:0}	2.4	1.2–3.2	0.6	0.5	4.4	2.6
iso-C _{15:0}	14.2	10.4–24.5	12.5	20.8	22.3	16.7
anteiso-C _{15:0}	38.7	49.2–49.8	32.9	35.9	27.6	33.5
iso-C _{16:0}	12.1	8.3–17.0	4.3	2.5	10.1	12.6
iso-C _{17:0}	4.9	3.3–6.1	6.2	8.8	3.3	5.7
iso-C _{17:1} ω10c	4.5	3.4–10.8	7.3	3.5	4.0	4.7
anteiso-C _{17:0}	12.1	12.1–24.2	13.5	11.9	8.9	8.9
iso-C _{19:0}	0.4	0.2–0.4	2.3	2.5	2.8	1.8
Unknown fatty acids	3.4	1.6–3.4	1.4	1.2	0.9	5.0

We isolated 15 strains of moderately halophilic, strictly aerobic, Gram-positive cocci, approximately 0.6–0.8 µm in diameter, occurring singly and in pairs or in tetrads (see Supplementary Fig. S1 in IJSEM Online). Endospores and flagella were not observed. Colonies were smooth, circular, of low convexity and orange in colour (Supplementary Fig. S2). Anaerobic growth was not observed. They grew in JCM medium no. 377 with 1.5–25 % NaCl, at pH 6–9 and at 15–45 °C, but no growth was observed at 10 or 50 °C. The phenotypic and chemotaxonomic characteristics of the 15 novel strains are detailed in the species description below and in Tables 1 and 2. Representative strain PN1-2^T contained L-Lys in the cell-wall peptidoglycan. This strain had isoprenoid quinone with six isoprene units (MK-6) as a predominant component, and had a polar lipid profile of

phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The dominant cellular fatty acids of strains PN1-2^T, PN1-8, PN2-2 and PN7-1 were anteiso-C_{15:0} (38.7–49.8 %) and iso-C_{15:0} (10.4–24.5 %) (Table 2). The DNA G + C contents of the novel strains ranged from 44.5 to 47.5 mol%. On the basis of 16S rRNA gene sequence analyses with the neighbour-joining algorithm, strains PN1-2^T and PN7-1 were included in a monophyletic cluster consisting of species of the genus *Salinicoccus*, as shown in Fig. 1 (the maximum-parsimony tree is shown in Supplementary Fig. S3) (Ventosa *et al.*, 1990, 1992; Zhang *et al.*, 2002). Levels of 16S rRNA gene sequence similarity between strains PN1-2^T and PN7-1 and *S. roseus* DSM 5351^T, *S. hispanicus* DSM 5352^T, *S. alkaliphilus* T8^T and *J. halotolerans* YKJ-101^T were 97.3–97.7, 96.5, 95.9 and 93.5 %, respectively. The novel strains were considered to represent the same species based on levels of DNA–DNA relatedness of over 76.6 % with strain PN1-2^T (Wayne *et al.*, 1987) (Supplementary Table S1). Strain PN1-2^T showed low DNA–DNA relatedness to *S. roseus* JCM 14630^T (21.7 %) and, reciprocally, *S. roseus* JCM 14630^T showed low DNA–DNA relatedness to strain PN1-2^T and the other 14 related strains (2.0–29.0 %). In addition, all 15 novel strains could be differentiated from *S. roseus* JCM 14630^T and related species based on pigmentation, maximum growth temperature, NaCl tolerance, hydrolysis of casein, gelatin, Tween 80 and starch, acid production from fructose and D-glucose and DNA G + C content, as detailed in Table 1. Furthermore, they could be differentiated from *Salinicoccus salsiraiiae* LMG 22840^T based on pigmentation, maximum growth temperature, NaCl tolerance, hydrolysis of casein and gelatin and acid production from maltose and sucrose (França *et al.*, 2006). Thus, the 15 novel strains isolated herein are considered to represent a single novel species of the genus *Salinicoccus*, for which we propose the name *Salinicoccus siamensis* sp. nov.

Description of *Salinicoccus siamensis* sp. nov.

Salinicoccus siamensis (si.am.en'sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, from where the first strains were isolated).

Fig. 1. Phylogenetic tree showing the relationships between strains PN1-2^T and PN7-1 and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages above 50 %, based on 1000 replications, are shown at nodes. Bar, 2 substitutions per 100 nucleotide positions.

Cells are Gram-positive, strictly aerobic cocci, approximately 0.6–0.8 µm in diameter, occurring singly and in pairs or in tetrads. Non-motile. Endospores are not formed. Colonies are smooth, circular, of low convexity and orange in colour. Growth occurs at pH 6 and 9 (optimally at pH 8.5), at temperatures of 15–45 °C (optimally at 37 °C) and in media with 1.5–25 % (w/v) NaCl (optimally at 10 % NaCl). Catalase- and oxidase-positive, but urease-negative. Unable to reduce nitrate. Negative for the indole, methyl red and Voges–Proskauer tests and for utilization of citrate. Aesculin, casein, arginine, gelatin, starch and Tween 80 are not hydrolysed. Acid is produced from D-fructose, D-glucose, glycerol, D-ribose and trehalose, but not from amygdalin, L-arabinose, cellobiose, aesculin, D-galactose, gluconate, *myo*-inositol, inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, melezitose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin, sorbitol, sucrose or D-xylose. The cell-wall peptidoglycan is L-Lys type. The predominant isoprenoid quinone is MK-6. The major cellular fatty acids are anteiso-C_{15:0} and iso-C_{15:0}. Phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid are predominant in the polar lipid profile. The DNA G+C content ranges from 44.5 to 47.5 mol%.

The type strain, PN1-2^T (=JCM 12822^T =PCU 242^T =TISTR 1562^T), was isolated from fermented shrimp paste ('ka-pi') in Thailand.

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