

Lentibacillus halophilus sp. nov., from fish sauce in Thailand

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Fifteen strains of extremely halophilic bacteria were isolated from fish sauce (nam-pla) collected in Thailand at various stages of the fish-fermentation process. The isolates were strictly aerobic, spore-forming, Gram-positive rods. They grew optimally in the presence of 20–26% NaCl. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The predominant menaquinone was MK-7. The major cellular fatty acids were anteiso-C_{15:0} and anteiso-C_{17:0}. Polar lipid analysis revealed the presence of phosphatidylglycerol, diphosphatidylglycerol and two unidentified glycolipids. The DNA G + C content was 42.1–43.1 mol%. On the basis of the 16S rRNA gene sequence, a representative strain, PS11-2^T, was found to be closely related to *Lentibacillus juripiscarius* JCM 12147^T (97.3% similarity). The 15 strains were included in the same species on the basis that the levels of DNA–DNA relatedness with strain PS11-2^T were greater than 70%. They could be distinguished from *L. juripiscarius* and other *Lentibacillus* species on the basis of several phenotypic characteristics and low levels of DNA–DNA relatedness (≤19.4%). Therefore, the strains represent a novel species of the genus *Lentibacillus*, for which the name *Lentibacillus halophilus* sp. nov. is proposed. The type strain is PS11-2^T (=JCM 12149^T = TISTR 1549^T = PCU 240^T).

Moderately halophilic, endospore-forming, rod-shaped bacteria are widely distributed in environments containing high NaCl concentrations, such as saline lakes and fish sauce. They are a diverse group of bacteria belonging to the genera *Bacillus*, *Halobacillus*, *Virgibacillus*, *Filobacillus*, *Oceanobacillus*, *Lentibacillus* and *Pontibacillus* (Ventosa *et al.*, 1989; Spring *et al.*, 1996; Heydrickx *et al.*, 1998; Arahal *et al.*, 2000; Schlesner *et al.*, 2001; Lu *et al.*, 2001; Heyrman *et al.*, 2003; Yoon *et al.*, 2002, 2004; Lim *et al.*, 2005a). The genus *Lentibacillus*, which forms a phylogenetically coherent group, currently comprises four species: *Lentibacillus salicampi*, *L. juripiscarius*, *L. salarii* and *L. lacisalsi* (Yoon *et al.*, 2002; Namwong *et al.*, 2005; Jeon *et al.*, 2005; Lim *et al.*, 2005b). In this paper, we report the isolation and identification of some novel extremely halophilic strains representing a novel *Lentibacillus* species.

Fish-sauce samples were collected from fish-sauce factories in Thailand during the early, middle and late stages of the fermentation process. Halophilic bacteria were isolated from the samples by using the spread plate technique on agar plates of JCM medium no. 168 (containing, l⁻¹, 200 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 and 20 g agar; pH 7.2] with incubation at 37 °C for 7 days. Liquid cultures were cultivated in Erlenmeyer flasks containing the same medium without agar and were incubated on a rotary shaker. All media contained 20% (w/v) NaCl, except those used to investigate NaCl tolerance. Cell shape, cell size and cell arrangement were examined on JCM medium no. 168 agar at 37 °C for 5 days. The Hucker–Conn modification was used for Gram staining (Hucker & Conn, 1923). Spore formation was examined on Gram-stained specimens. Critical-point-dried cells were observed under a scanning electron microscope. Flagella were examined as described by Forbes (1981) and observed by transmission electron microscopy. Catalase activity, oxidase activity and the hydrolysis of aesculin were investigated as described by Barrow & Feltham (1993), while urease activity and the hydrolysis of gelatin, casein, starch, Tween 80, tyrosine,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PS11-2^T is AB191345.

A scanning electron micrograph of sporulating cells of strain PS11-2^T, a thin-layer chromatogram of polar lipids and details of the cellular fatty acids and levels of DNA–DNA relatedness of the novel strains and related taxa are available as supplementary material in IJSEM Online.

phenylalanine, xanthine and hypoxanthine were tested as described by Namwong *et al.* (2005). Arginine hydrolysis was investigated by using the medium reported by Thornley (1960). Acid production from carbohydrate was determined in the medium described by Leifson (1963), supplemented with 20% NaCl. Growth under anaerobic conditions on agar plates with or without KNO₃ (1%, w/v) was performed in a Gaspak (BBL) anaerobic jar. Growth at various temperatures (10–50 °C), pH values (5, 6, 7, 7.5, 8 and 9) and NaCl concentrations (0–30%, w/v) was tested by using JCM medium no. 168 as a basal medium (Namwong *et al.*, 2005).

The diaminopimelic acid in the peptidoglycan and the menaquinone composition were determined as described previously (Komagata & Suzuki, 1987). Polar lipids were investigated according to the methods of Minnikin *et al.* (1984) and Albert *et al.* (2005). A loop of cell mass was used for the extraction and quantitative analysis of the cellular fatty acids by means of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

DNA was isolated from cells grown in JCM medium no. 168 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), using reversed-phase HPLC. DNA–DNA hybridization was conducted in microdilution-well plates, as reported by Ezaki *et al.* (1989), and was detected by using the colorimetric method described by Tanasupawat *et al.* (2000). The 16S rRNA gene of the isolate was amplified, purified and sequenced as described previously (Seearunruangchai *et al.*, 2004). The sequence determined (1410 bases) was aligned with selected sequences (obtained from the GenBank/EMBL/DBJ database) by using CLUSTAL W, version 1.81 (Thompson *et al.*, 1994). The alignment was manually edited 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 using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Fifteen aerobic, extremely halophilic bacteria were isolated from the fish-sauce samples, and were characterized according to their morphological, cultural, physiological and

Table 1. Differential characteristics of *Lentibacillus* species

Strains: 1, PS11-2^T; 2, *L. juripiscarius* JCM 12147^T (data from Namwong *et al.*, 2005); 3, *L. salarius* KCTC 3911^T (Jeon *et al.*, 2005); 4, *L. salicampi* JCM 11462^T (Yoon *et al.*, 2002; Namwong *et al.*, 2005); 5, *L. lacisalsi* KCTC 3915^T (Lim *et al.*, 2005b). +, Positive; –, negative; w, weak; NA, no data available.

Characteristic	1	2	3	4	5
Spore shape	Spherical	Oval	Spherical/oval	Spherical/oval	Spherical
Motility	+	–	+	+	+
Colony diameter (mm)	0.2–0.6	1.3–3.2	NA	0.2–1.3	NA
Maximum temperature for growth (°C)	42	45	50	40	40
NaCl range for growth (%)	12–30	3–30	1–20	3–25	5–25
Oxidase	+	+	–	+	+
Nitrate reduction	–	+	+	+	+
Hydrolysis of:					
Aesculin	–	–	+	–	–
Casein	–	+	–	+	–
Tween 80	–	+	–	+	–
Acid production from:					
Cellobiose	–	–	NA	W	NA
D-Glucose	–	+	+	+	–
D-Galactose	–	–	NA	W	NA
D-Fructose	–	+	+	–	+
Maltose	–	–	+	W	–
D-Mannitol	–	–	W	–	–
D-Mannose	–	–	+	W	–
D-Ribose	–	+	+	–	+
Salicin	–	–	–	W	–
Sucrose	–	W	NA	–	NA
D-Trehalose	–	–	W	–	–
D-Xylose	–	+	+	–	W
DNA G+C content (mol%)	42	43	43	42	44

biochemical properties. The results are listed in the species description and Table 1. All 15 strains contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The menaquinones, cellular fatty acids and polar lipids of four of these strains, namely PS11-2^T, CB0-1, DS26-3 and DB9-1, were analysed. The four strains contained the following: MK-7 as a major menaquinone; anteiso-C_{15:0} (58.0–62.7%), anteiso-C_{17:0} (24.6–33.8%), C_{16:0} (1.7–3.0%), iso-C_{15:0} (2.7–7.8%) and iso-C_{16:0} (1.8–2.3%) as the cellular fatty acids (see Supplementary Table S1 available in IJSEM Online); and phosphatidylglycerol, diphosphatidylglycerol and two unidentified glycolipids as major polar lipids (see Supplementary Fig. S1 available in IJSEM Online).

The DNA G + C content of the isolates (13 strains) ranged from 42.1 to 43.1 mol%. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain PS11-2^T was positioned in a monophyletic cluster consisting of the members of the genus *Lentibacillus* (Fig. 1). The same topology was obtained by applying the maximum-parsimony method (result not shown). The 16S rRNA gene sequence similarity values between PS11-2^T and *L. juripiscarius* JCM 12147^T, *L. salarius* KCTC 3911^T, *L. lacisalsi* KCTC 3915^T and *L. salicampi* JCM 11462^T were 97.3, 95.5, 95.4 and 95.3%, respectively. Furthermore, strain PS11-2^T showed 93.3–94.2% 16S rRNA gene sequence similarity to members of the genus *Virgibacillus*. Hybridization studies revealed high levels of DNA–DNA relatedness between PS11-2^T and PB7-3 and to the other isolates (>70%), but only low levels with respect to *L. salicampi* JCM 11462^T (4.9–19.4%) and *L. juripiscarius*

JCM 12147^T (17.0–17.1%), as shown in Supplementary Table S2 (available in IJSEM Online).

The 16S rRNA gene sequence-based phylogenetic analysis clearly indicated that representative strain PS11-2^T belongs to the genus *Lentibacillus*. The chemotaxonomic properties (i.e. diamino acid content in the peptidoglycan, menaquinone content, the polar lipids and the cellular fatty acid profiles) of the isolates were in accordance with those of the genus *Lentibacillus* (Yoon *et al.*, 2002; Namwong *et al.*, 2005; Jeon *et al.*, 2005; Lim *et al.*, 2005b). The fatty acid profiles of the four strains examined were qualitatively similar to those of other *Lentibacillus* species, although the levels of iso-C_{14:0} and iso-C_{16:0} were significantly lower than those of the other *Lentibacillus* species reported ($\leq 0.7\%$ iso-C_{14:0} and 1.8–2.3% iso-C_{16:0} for the isolates; 5.7–13.9% iso-C_{14:0} and 16.3–26.5% iso-C_{16:0} for *Lentibacillus* species). When the cellular fatty acid profile of *L. juripiscarius* JCM 12147^T grown on JCM medium no. 168 (containing 20% NaCl) was compared with the profile from cells grown on the *Lentibacillus* medium (JCM medium no. 377, containing 10% NaCl), the levels of iso-C_{14:0} and iso-C_{16:0} were significantly lower (2.1 and 6.3%, respectively; see Supplementary Table S1 in IJSEM Online). Thus, the levels of iso-C_{14:0} and iso-C_{16:0} were affected by NaCl concentration during growth. The morphological, cultural, physiological and biochemical characteristics that differentiate the isolates from *Lentibacillus* species are shown in Table 1. It is noteworthy that all of the isolates are extreme halophiles requiring at least 12% NaCl for growth. In addition, the isolates do not reduce nitrate and do not produce acid from sugars, unlike *Lentibacillus* species. The 16S rRNA gene sequence of PS11-2^T shows a relatively

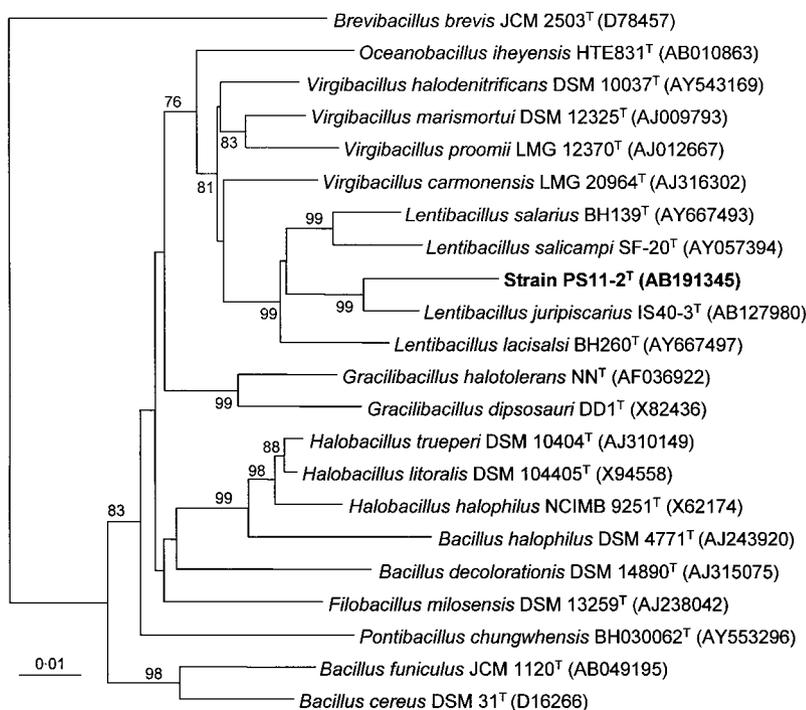


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain PS11-2^T and related bacterial species. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages above 75%, based on 1000 replications, are shown at the nodes. Bar, 1 substitution per 100 nucleotide positions.

high level of similarity to that of the type strain of *L. juripiscarius* (97.3%). However, the low levels of DNA–DNA relatedness ($\leq 19.4\%$) demonstrate that the novel strains do not belong to the species *L. juripiscarius*. On the other hand, inclusion of the 15 strains in the same species is supported by the fact that they share the same phenotypic properties and demonstrate high levels of DNA–DNA relatedness to each other ($> 70\%$) (Wayne *et al.*, 1987). Therefore, these 15 isolates represent a novel species in the genus *Lentibacillus*, for which we propose the name *Lentibacillus halophilus* sp. nov. The type strain is PS11-2^T (=JCM 12149^T=TISTR 1549^T=PCU 240^T).

Description of *Lentibacillus halophilus* sp. nov.

Lentibacillus halophilus (ha.lo'phi.us. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are Gram-positive, aerobic, motile rods and are mostly 0.4–0.6 μm wide and 1.0–3.0 μm long. Longer cells (up to 6 μm) or short filaments are observed. Spherical endospores are formed terminally in swollen sporangia (see Supplementary Fig. S2 available in IJSEM Online). Colonies are white to cream, low-convex or raised, smooth and circular (0.1–0.8 mm in diameter). Catalase- and oxidase-positive. Urease-negative. Growth occurs between 15 °C (weakly) and 42 °C (optimum, 30–37 °C) but not at 10, 45 or 50 °C. Growth is observed between pH 6 and 8 (optimum, pH 7.0–7.5) but not at pH 5 or 9. Extremely halophilic, growing in the presence of 12–30% (w/v) NaCl but not at or below 10% (w/v) NaCl (optimum, 20–26% NaCl, w/v). Anaerobic growth is not observed in the presence of 1% KNO₃ (w/v) or in other media. Aesculin, arginine, casein, gelatin, Tween 80, tyrosine, starch, phenylalanine, xanthine and hypoxanthine are not hydrolysed. Acid is not produced from D-glucose, glycerol, D-ribose, D-xylose, L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, *myo*-inositol, raffinose, L-rhamnose, salicin, sorbitol, sucrose or D-trehalose. Contains *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. MK-7 is the major menaquinone. The fatty acid profile consists of anteiso-C_{15:0} (58.0–62.7%), anteiso-C_{17:0} (24.6–33.8%), iso-C_{15:0} (2.4–7.8%), C_{16:0} (1.7–3.0%), iso-C_{16:0} (1.8–2.3%), iso-C_{17:0} (0.5–1.4%), C_{14:0} (0–0.9%), iso-C_{14:0} (0–0.7%), C_{15:0} (0–0.6%) and C_{12:0} (0–0.5%). Phosphatidylglycerol, diphosphatidylglycerol and two unidentified glycolipids are predominant in the polar lipid profile. The DNA G+C content is 42.1–43.1 mol% (type strain, 42.4 mol%).

The type strain, PS11-2^T (=JCM 12149^T=TISTR 1549^T=PCU 240^T), was isolated from a fish-sauce fermentation in Thailand.

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