Isolation of *Lentibacillus salicampi* strains and *Lentibacillus juripiscarius* sp. nov. from fish sauce in Thailand

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Eight strains of aerobic, spore-forming, Gram-positive, moderately halophilic bacteria were isolated from sauce (nam-pla and bu-du) produced in Thailand by the fermentation of fish. They grew optimally in the presence of 10 % NaCl, at 37 °C and pH 7.0. A diagnostic diamino acid, meso-diaminopimelic acid, was present in the cell-wall peptidoglycan. The predominant menaquinone was MK-7. The major cellular fatty acids were anteiso-C_{15:0} and iso-C_{16:0}. Phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid were found to be the major polar lipid components. The DNA G+C content was 42–43 mol%. These bacteria were further divided into two groups based on phenotypic characteristics and DNA–DNA similarities. Three strains of Group I were highly affiliated to the type strain of *Lentibacillus salicampi* in terms of phenotypic characterization and DNA–DNA similarities (96–102 %); accordingly, they were identified as strains of *L. salicampi*. A representative strain of Group II, strain IS40-3^T, was most closely related to *L. salicampi* in terms of 16S rRNA-based phylogenetic analysis, although five strains of Group II could be distinguished from *L. salicampi* by means of several phenotypic properties, low 16S rRNA gene sequence similarity (95.2 %) and low DNA–DNA similarities (12–32 %). Therefore, the Group II strains should be included in a novel species of the genus *Lentibacillus*, for which the name *Lentibacillus juripiscarius* sp. nov. is proposed. The type strain is IS40-3^T (=JCM 12147^T=PCU 229^T=TISTR 1535^T).

Nam-pla (fish sauce) is a clear brown liquid traditionally produced by fermentation of fish and widely used in Southeast Asia and, to some extent, in some other parts of the world (Saisithi *et al.*, 1966; Beddows, 1998). A related product, bu-du (Muslim sauce or fish sauce), is a viscous brown liquid with grey colloidal fish separated from the fish bones suspended in it, and it has a salty taste (Phithakpol *et al.*, 1995). These products are rich in various nutrients, particularly amino acids and peptides, and contain a high concentration of NaCl, which allow various halophilic micro-organisms to thrive (Lopetcharat *et al.*, 2001; Tanasupawat & Komagata, 2001). Nevertheless, only a

few types of bacteria have been isolated from the fish sauce samples and subjected to taxonomic study. In Thailand, *Halobacterium salinarum* and '*Halobacillus thailandensis*' have been isolated from the fish sauce samples: these are extremely halophilic bacteria/archaea that grow optimally at 20–25 % NaCl. Other bacteria isolated were moderately halophilic bacteria that grew optimally at 3–15 % NaCl, such as *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* (Chaiyanan *et al.*, 1999; Thongthai *et al.*, 1992; Thongsanit *et al.*, 2002). Recently, we isolated a number of halophilic bacteria/archaea from fish sauce samples to study the microbial diversity of this product. In this report, we consider eight isolates of aerobic, spore-forming, Gram-positive, moderately halophilic bacteria by characterizing their phenotypic, chemotaxonomic and phylogenetic properties to elucidate their taxonomic positions.

The halophilic bacteria were isolated from the fish sauce

Published online ahead of print on 27 August 2004 as DOI 10.1099/ij.s.0.63272-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IS40-3^T is AB127980.

samples (nam-pla and bu-du) that were collected from factories and markets in Thailand by the spread-plate technique on agar plates of JCM medium no. 168 [composed of (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 $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 20 g agar; pH 7.2] incubated at 37 °C for 1–2 weeks. The isolates were routinely cultivated in modified JCM medium no. 168, in which the NaCl concentration had been reduced to 100 g l^{-1} (designated *Lentibacillus* medium: JCM medium no. 377). *Lentibacillus salicampi* JCM 11462^T, which was used as a reference strain, was cultivated in the same way.

Unless otherwise stated, the tested strains were grown in liquid or on agar forms of JCM medium no. 377. Colony and cell morphologies were examined using cells grown on agar plates at 37 °C for 5 days. The Hucker–Conn modification was used for Gram staining (Hucker & Conn, 1923). Critical-point-dried cells were observed under a scanning electron microscope. Flagella were observed by transmission electron microscopy of cells shadowed with platinum/palladium. Methyl red and Voges–Proskauer tests were performed and catalase and oxidase activities, indole production, nitrate reduction and hydrolysis of arginine, starch, tyrosine, tributyrin, Tween 80, xanthine and hypoxanthine were tested for by using the methods of Barrow & Feltham (1993). The Casamino acids in JCM medium no. 377 were omitted from the medium used to test for the hydrolysis of gelatin and casein. Urease was detected on Christensen's medium (Barrow & Feltham, 1993) supplemented with 10 % (w/v) NaCl. Acid production from carbohydrate was determined in the medium described by Leifson (1963) supplemented with 6.5 % (w/v) NaCl. Growth under anaerobic conditions on agar plates with or without nitrate (1 %, w/v) was investigated using a Gaspak (BBL) anaerobic jar. Growth at various temperatures (5–60 °C) was examined using a temperature-gradient

incubator (model TN-3; Advantec). NaCl requirements were determined in medium containing various NaCl concentrations (0–30 %, w/v). At lower NaCl concentrations (0–2.0 %, w/v), $MgSO_4 \cdot 7H_2O$ was omitted from the test medium, while KCl and $C_6H_5Na_3O_7 \cdot 2H_2O$ (tri-sodium citrate dihydrate) were added. Growth was monitored by measuring culture turbidity at 660 nm.

meso-Diaminopimelic acid, in the peptidoglycan, and menaquinones were analysed as described previously (Komagata & Suzuki, 1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984). Quantitative analysis of cellular fatty acids was performed with the Microbial Identification System (MIDI). DNAs were isolated 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 as reported previously (Ezaki *et al.*, 1989) and detected by the colorimetric method (Tanasupawat *et al.*, 2000). The 16S rRNA gene fragments were PCR-amplified with primers EB-20F (5'-AGTTTGATCCTGGCTC-3', positions 10–25 according to the *Escherichia coli* numbering system) and EB-1530R (5'-AAGGAGGTGATCCAGCC-3', positions 1541–1525). The 16S rRNA gene fragment was separated by agarose gel electrophoresis and recovered by using the GenElute Minus EtBr spin column (Sigma). The sequence was determined using the BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.0; Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: EB-10F and EB-520R (5'-ACCGCGGCTGCTGGC-3', positions 531–516), EB-530F (5'-GTGCCAGCAGCCGCGG-3', positions 515–530), EB-1100R (5'-AGGGTTGCGCTCGTTG-3', positions 1115–1100), EB-1110F (5'-GCAACGAGCGCAACCC-3', positions 1099–1114) and EB-1530R. The sequence was multiply aligned with the CLUSTAL W program (version 1.81; Thompson *et al.*, 1994), then the alignment was manually

Table 1. Strain designations, sources, DNA G + C content and DNA–DNA relatedness among the isolates and *L. salicampi* JCM 11462^T

Strain	Source	G + C content (mol%)	DNA–DNA similarity with labelled strain (%):		
			JCM 11462 ^T	IS40-3 ^T	PS9-2
Group I					
BN2-2 (=PCU 226)	Bu-du	42.5	96	1	12
DB8-4 (=PCU 227)	Nam-pla	42.3	71	1	19
KS333-3B (=JCM 12151=PCU 228=TISTR 1536)	Nam-pla	42.5	102	14	8
Group II					
IS40-3 ^T (=JCM 12147 ^T =PCU 229 ^T =TISTR 1535 ^T)	Nam-pla	43.4	12	100	113
IS40-2 (=PCU 230)	Nam-pla	43.5	32	98	111
IS10-5 (=PCU 231)	Nam-pla	43.3	27	96	118
IO-1 (=PCU 232)	Nam-pla	43.2	13	82	85
PS9-2 (=JCM 12148=PCU 233)	Nam-pla	43.3	14	79	100
<i>L. salicampi</i> JCM 11462 ^T	Salt field	42.4	100	13	11

Table 2. Phenotypic characteristics of the Group I and Group II isolates

Taxa are identified as *L. salicampi* for Group I and *L. juripiscarius* sp. nov. for Group II. All strains grew at pH 5–9, produced terminal spores, showed catalase and oxidase activities, reduced nitrate, hydrolysed casein, gelatin and Tween 80, and produced acid from D-fructose, D-glucose, glycerol, D-ribose and D-xylose. They showed negative reactions for urease, in the Voges–Proskauer reaction, for H₂S formation, in the methyl red test, for indole formation and for hydrolysis of arginine, starch, tyrosine, tributyrin, phenylalanine, hypoxanthine and xanthine. Anaerobic growth was observed in medium containing nitrate.

Characteristic	JCM 11462 ^T	Group I (3 strains)	IS40-3 ^T	Group II (5 strains)
Spore shape	Spherical or oval	Spherical or oval	Oval	Oval
Chains of cells	—	—	+	+
Motility	+	+	—	—
Colony size (mm, diameter)	0.2–1.3	0.2–1.3	1.3–3.2	0.9–3.9
Temperature range (°C)	10–40	10–40	10–45	10–45
NaCl range (%)	3–25	3–25	3–30	3–30
Acid production from:				
Cellobiose	w	+	—	—
D-Galactose	w	+	—	—
D-Mannose	w	+	—	—
Salicin	w	w (2)*	—	—
Sucrose	—	—	w	w (4)*

*The numbers of weakly positive strains are shown in parentheses.

verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) in MEGA program 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.

Eight aerobic, spore-forming, Gram-positive, moderately halophilic rods were isolated from the fish sauce samples, as shown in Table 1. These isolates were divided into two groups, Group I and Group II, according to their morphological, cultural, physiological and biochemical characteristics and their DNA–DNA similarities, as shown in Tables 1 and 2.

The cells of the isolates were rod-shaped, measuring approximately 0.4–0.5 × 2–6 µm (for Group I) or 0.4–0.5 × 1.5–6 µm (for Group II). The Group I isolates and *L. salicampi* JCM 11462^T occurred singly, in pairs or in short chains, whereas the Group II isolates tended to form crooked chains comprising several cells. Oval spores lay terminally in swollen sporangia (Fig. 1). They grew optimally in the presence of 10–15 % NaCl, at 30–39 °C and approximately pH 7. Other morphological, cultural, physiological and biochemical properties are shown in Table 2. The properties of *L. salicampi* JCM 11462^T determined by us were basically in agreement with those reported by Yoon *et al.* (2002), except for acid production from carbohydrates. In our analysis, the type strain of *L. salicampi* (JCM 11462^T) produced acid from cellobiose (weak), D-fructose, D-galactose (weak), D-glucose, D-mannose (weak), D-ribose and xylose (sometimes weak);

according to Yoon *et al.* (2002), strain SF-20^T (=JCM 11462^T) does not produce acid from any of these sugars. The reason for the discrepancy is not clear, and our results were reproducible.

All strains, including *L. salicampi* JCM 11462^T, contained *meso*-diaminopimelic acid as a diagnostic diamino acid, as well as glutamic acid and alanine in the cell-wall peptidoglycan. The predominant isoprenoid quinone found was MK-7. Polar lipid analysis revealed the presence of phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The cellular fatty acid profiles for all strains

Fig. 1. Scanning electron micrograph of strain IS40-3^T grown in *Lentibacillus* medium at 37 °C. Bar, 1 µm.

tested contained the following components (%): anteiso- $C_{15:0}$ (38–54), iso- $C_{16:0}$ (13–30), anteiso- $C_{17:0}$ (13–18), iso- $C_{15:0}$ (3–18) and iso- $C_{14:0}$ (5–13). The G + C contents of the DNAs of Group I and Group II were 42.2–42.5 and 43.2–43.5 mol%, respectively, whereas that for *L. salicampi* JCM 11462^T was 42.4 mol%. As shown in Table 1, *L. salicampi* JCM 11462^T showed high levels of DNA–DNA similarity to the Group I isolates (96–102 %). Also, high DNA–DNA similarity values were detected between the Group II strains (85–118 %); however, the values between the Group II and Group I strains (or *L. salicampi* JCM 11462^T) were significantly low (<32 %).

Almost-complete 16S rRNA gene sequences of strain IS40-3^T, used to represent Group II, determined in this study contained 1524 nucleotide positions, and the phylogenetic analysis was conducted with sequences of representative strains of the family *Bacillaceae* and related taxa. Ambiguous and unalignable bases were eliminated, leaving 1233 positions for comparison in the phylogenetic analysis. The analysis revealed that strain IS40-3^T was part of a coherent cluster that included the genera *Lentibacillus* and *Virgibacillus*, as shown in Fig. 2. In this cluster, the close relationship between strain IS40-3^T and *L. salicampi* was supported by high 16S rRNA gene sequence similarity

Fig. 2. Phylogenetic tree showing the relationships between *L. juripiscarius* IS40-3^T and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages above 60 %, based on 1000 replications, are shown at the nodes. Bar, 2 substitutions per 100 nucleotide positions.

(95.2%) and a high bootstrap value (96%). Strain IS40-3^T showed 93.3–95.6% 16S rRNA gene sequence similarity to members of the genus *Virgibacillus*, and less than 94.2% to other members of the family *Bacillaceae*.

Our novel isolates were aerobic, spore-forming, Gram-positive, rod-shaped, moderately halophilic bacteria. Bacteria with such characteristics are commonly observed in salty environments such as salterns, salted soils, soda lakes and salted foods (including fish sauce); however, they constitute a phylogenetically heterogeneous group of micro-organisms, represented by *Halobacillus* (Spring *et al.*, 1996), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* (Wainø *et al.*, 1999), *Filobacillus* (Schlesner *et al.*, 2001), *Jeotgalibacillus* (Yoon *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002), *Paraliobacillus* (Ishikawa *et al.*, 2002) and certain *Bacillus* species. These genera could be differentiated not only by means of phylogenetic distances but also by their chemotaxonomic profiles, including the diamino acid composition of cell walls, cellular fatty acid patterns and DNA G+C content. Our isolates can be divided into two groups, Group I and Group II, according to their cultural, physiological and biochemical properties and by means of the DNA–DNA hybridization study as described above. Although the 16S rRNA gene sequences of the Group I strains have not been determined, they shared almost identical phenotypic properties with the type strain of *L. salicampi*, the sole member of the genus *Lentibacillus*. Furthermore, the high DNA–DNA similarities with *L. salicampi* JCM 11462^T warrant the inclusion of Group I strains in *L. salicampi*. On the other hand, the representative strain of Group II, IS40-3^T, is most closely affiliated to *L. salicampi* on the basis of 16S rRNA gene sequence comparisons, indicating that the Group II strains are also members of the genus *Lentibacillus*. The generic assignment is supported by the fact that their chemotaxonomic profiles (i.e. the presence of *meso*-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan, MK-7 as the major menaquinone, anteiso-C_{15:0} and iso-C_{16:0} as the major cellular fatty acids, and phosphatidylglycerol and diphosphatidylglycerol as the major polar lipids, and the DNA G+C content of 42–43 mol%) correspond with that of the genus *Lentibacillus*. The genus *Virgibacillus* now incorporates the former *Salibacillus* species (Heyrman *et al.*, 2003); however, the genera *Lentibacillus* and *Virgibacillus* retain separate phylogenetic status, as shown in Fig. 2. In addition, these two genera can be differentiated on the basis of iso-C_{16:0} content (13–30% for *Lentibacillus*, 3–8% for *Virgibacillus*) and, possibly, on the basis of DNA G+C content (42–43% for *Lentibacillus*, 36–41% for *Virgibacillus*, except for *Virgibacillus marismortui*).

As shown in Table 2, Group II strains can be differentiated from *L. salicampi* strains by cell shape, motility, maximum growth temperature and NaCl concentration requirements, and by the production of acid from sucrose but not from D-mannose, cellobiose or salicin. The DNA–DNA hybridization study supports the view that Group II strains are

separate from *L. salicampi*. In addition, 16S rRNA gene sequence comparison reveals that the representative strain, IS40-3^T, is closely related to *L. salicampi*; however, the sequence similarity is still too low (<96%) to allow inclusion in the same species. Therefore, Group II isolates should be classified as a novel species of the genus *Lentibacillus*, for which we propose the name *Lentibacillus juripiscarius* sp. nov.

Description of *Lentibacillus juripiscarius* sp. nov.

Lentibacillus juripiscarius (ju.ris.pis'ca.ri.us. L. n. *jus juris* sauce; L. adj. *piscarius* -a -um of, or belonging to, fish; N.L. masc. adj. *juripiscarius* of a fish sauce).

Cells are Gram-positive, rod-shaped, 0.4–0.5 × 1.5–6 µm, non-motile, lack flagella, and form crooked chains made up of several cells. Oval endospores form at cell terminal position in swollen sporangia. Colonies are white to cream and are 0.9–3.9 mm in diameter after 5 days on *Lentibacillus* agar medium. Grows aerobically, and anaerobically in the presence of nitrate. Grows between 10 and 45 °C (optimally at 37 °C) and between pH 5 and 9 (optimally at pH 7.0). Requires 3–30% NaCl (optimally, 10% NaCl) for growth. Catalase- and oxidase-positive but urease-negative. Reduces nitrate. Hydrolyses casein, Tween 80 and gelatin, but not arginine, starch, tyrosine, tributyrin, phenylalanine, xanthine or hypoxanthine. Produces acid from D-fructose, D-glucose, glycerol, D-ribose, sucrose and D-xylose, but not from amygdalin, L-arabinose, cellobiose, D-galactose, inulin, lactose, maltose, mannitol, D-mannose, melibiose, melezitose, methyl α-D-glucoside, *myo*-inositol, raffinose, rhamnose, salicin, sorbitol or trehalose. Peptidoglycan contains *meso*-diaminopimelic acid. Predominant menaquinone is MK-7. Fatty acid profile is largely composed of anteiso-C_{15:0} and iso-C_{16:0}. Predominant polar lipids are phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The DNA G+C content is 43 mol%.

The type strain, IS40-3^T (=JCM 12147^T=PCU 229^T=TISTR 1535^T), was isolated from sauce produced in Thailand by the fermentation of fish.

Acknowledgements

This study was supported by the Thailand Research Fund for a 2001 Royal Golden Jubilee Scholarship as a research grant to S.N., and, in part, by Rachadapiseksompoj Research Grant, Chulalongkorn University (2002).

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