Piscibacillus salipiscarius gen. nov., sp. nov., a moderately halophilic bacterium from fermented fish (pla-ra) in Thailand

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A Gram-positive, spore-forming and moderately halophilic bacterium was isolated from fermented fish (pla-ra) in Thailand. Cells of the isolate, RBU1-1^T, were strictly aerobic, motile rods and contained meso-diaminopimelic acid in the cell-wall peptidoglycan. Menaquinone with seven isoprene units (MK-7) was the predominant quinone. This isolate grew at 15–48 °C, pH 5–9 and in 2–30 % NaCl (optimally 10–20 %). The major cellular fatty acids were iso-C_{15:0} and anteiso-C_{15:0}. Polar lipid analysis revealed the presence of phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content was 36.7 mol%. 16S rRNA gene sequence analysis revealed that strain RBU1-1^T was a member of the family *Bacillaceae*, and belonged to a cluster with *Filobacillus* and *Tenuibacillus*; strain RBU1-1^T showed 16S rRNA gene sequence similarities of 96.0–96.9 % to members of these two genera. Strain RBU1-1^T could also be differentiated from members of the genera *Filobacillus* and *Tenuibacillus* based on certain phenotypic characteristics such as cell-wall composition, mode of flagellation and growth pH range. Therefore, strain RBU1-1^T is considered to represent a novel species in a new genus in the family *Bacillaceae*, for which the name *Piscibacillus salipiscarius* gen. nov., sp. nov. is proposed. The type strain of *Piscibacillus salipiscarius* is RBU1-1^T (=JCM 13188^T=PCU 270^T=TISTR 1571^T).

Moderately halophilic, aerobic, endospore-forming, Gram-positive, rod-shaped bacteria, isolated from various saline environments, such as marine environments, salt lakes and fermented fish-foods, are widely found in the family *Bacillaceae*, with representatives in the genera *Bacillus*, *Halobacillus*, *Virgibacillus*, *Filobacillus*, *Oceanobacillus*, *Lentibacillus*, *Paralibacillus*, *Pontibacillus*, *Tenuibacillus*, *Salinibacillus*, *Alkalibacillus* and *Thalassobacillus* (Ventosa *et al.*, 1989; Spring *et al.*, 1996; Heyndrickx *et al.*, 1999; Schlesner *et al.*, 2001; Lu *et al.*, 2001; Yoon *et al.*, 2002; Ishikawa *et al.*, 2002; Lim *et al.*, 2005; Ren & Zhou, 2005a, b; Jeon *et al.*, 2005; García *et al.*, 2005). Recently, we have described novel species of the genus *Lentibacillus*, *Lentibacillus juripiscarius* and *Lentibacillus halophilus* from fish

sauce (Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006) and *Lentibacillus kapialis* from fermented shrimp paste (Pakdeeto *et al.*, 2007), suggesting that such fermented foods may harbour many undescribed species of halophilic or halotolerant bacteria. Here we describe the isolation and phenotypic, chemotaxonomic and phylogenetic properties of a novel, moderately halophilic isolate, designated RBU1-1^T, from pla-ra (fermented fish), which is fermented mainly from freshwater fish and contains roasted rice and high levels of added salt (Phithakpol *et al.*, 1995).

Strain RBU1-1^T was isolated from fermented fish (pla-ra) collected from a market in Ratchaburi Province, Thailand. Isolation was by the spread-plate technique on agar plates of JCM medium no. 377 (designated *Lentibacillus* medium; Namwong *et al.*, 2005) incubated at 37 °C for 7 days. Strain RBU1-1^T and reference strain *Filobacillus milosensis* JCM 12288^T were routinely cultivated in JCM medium no. 377.

Cell form, cell size, cell arrangement and colonial appearance were examined for cells grown on *Lentibacillus* medium

Abbreviation: meso-DAP, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of RBU1-1^T is AB194046.

A chromatogram of the polar lipids of strain RBU1-1^T is available as a supplementary figure with the online version of this paper.

at 37 °C for 5 days. The Gram reaction was investigated according to the procedures of Hucker & Conn (1923). Critical-point-dried cells and spores were observed under a scanning electron microscope. Flagella were stained by the method described by Forbes (1981). Tests for catalase, oxidase, indole production, Simmons' citrate, nitrate reduction and hydrolysis of DNA, starch, tyrosine, Tween 80, phenylalanine, xanthine and hypoxanthine were performed according to the methods of Barrow & Feltham (1993). The test for arginine hydrolysis was performed by using the medium given by Thornley (1960). Casamino acids in JCM medium no. 377 were omitted from the test medium for determination of the hydrolysis of gelatin and casein. Urease was detected on Christensen's medium (Barrow & Feltham, 1993) supplemented with 10 % NaCl. Acid production from carbohydrate was determined in the medium described by Leifson (1963) supplemented with 6.5 % NaCl. Growth under anaerobic conditions on agar plates was performed by using a Gaspak (BBL). Growth at various temperatures (5–60 °C) was examined by using a temperature gradient incubator (model TN-3; Advantec). NaCl requirement for growth was determined in the medium containing various NaCl concentrations (0–30 %). At lower NaCl concentrations (0–2.0 %), MgSO₄·7H₂O was omitted from the test medium and KCl and trisodium citrate were used. Growth was monitored by measuring culture turbidity at 660 nm. Isomers of diaminopimelic acid (DAP) in the peptidoglycan and menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984). Quantitative analysis of cellular fatty acids was done with the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). DNA was isolated from cells grown in JCM no. 377 medium broth incubated on a rotary shaker (150 r.p.m.) at 37 °C for 2 days and was 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 experiments were conducted as described by Ezaki *et al.* (1989) and with the colorimetric method as reported by Tanasupawat *et al.* (2000). 16S rRNA gene fragments were amplified by PCR and the sequence was determined as described by Tanasupawat *et al.* (2004). The 16S rRNA gene sequence was multiply aligned with the program CLUSTAL_X (Thompson *et al.*, 1997); the alignment was manually verified and edited prior to construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the program MEGA, version 2.1 (Kumar *et al.*, 2001). Confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resampled datasets.

Cells of strain RBU1-1^T were Gram-positive rods, 0.4–0.5 × 1.5–4 µm in size, and motile with peritrichous flagella. Oval to spherical endospores were formed at cell-terminal positions in swollen sporangia (Fig. 1). Colonies were white to cream, 0.9–3.9 mm in diameter after 5 days cultivation on *Lentibacillus* agar medium. Results of physiological,

Fig. 1. Scanning electron micrograph of cells of strain RBU1-1^T grown on JCM no. 377 medium at 37 °C. Bar, 1 µm.

biochemical and chemotaxonomic tests are given in the species description below and in Table 1.

16S rRNA gene sequence comparisons with known aerobic, Gram-positive, spore-forming halophilic bacteria revealed that strain RBU1-1^T formed a cluster together with *F. milosensis* SH714^T and *Tenuibacillus multivorans* NBRC 100370^T, with high bootstrap support (88 %), and this cluster was also closely related to another cluster of *Alkalibacillus* species with 92 % bootstrap support, as shown in Fig. 2. 16S rRNA gene sequence similarity values between strain RBU1-1^T and *F. milosensis* SH714^T and *T. multivorans* NBRC 100370^T were 96.9 and 96 %, respectively, while values between strain RBU1-1^T and species of the genus *Alkalibacillus* were in the range 94–95.3 %. In addition, strain RBU1-1^T was related to members of the genera *Bacillus* (*sensu lato*), *Halobacillus*, *Virgibacillus*, *Oceanobacillus*, *Lentibacillus*, *Paraliobacillus*, *Pontibacillus*, *Salinibacillus* and *Thalassobacillus* with levels of 16S rRNA gene sequence similarity of 91.5–94.7 %. In DNA–DNA hybridization experiments (Wayne *et al.*, 1987), labelled DNA from *F. milosensis* JCM 12288^T showed 9.5 % DNA–DNA relatedness to strain RBU1-1^T, and reciprocally, that of RBU1-1^T showed 23.4 % DNA–DNA relatedness to *F. milosensis* JCM 12288^T.

Strain RBU1-1^T is a moderately halophilic, aerobic, spore-forming, Gram-positive, rod-shaped bacterium belonging to the family *Bacillaceae*. 16S rRNA gene sequence analysis revealed that strain RBU1-1^T is related closely to the monospecific genera *Filobacillus* and *Tenuibacillus*. In agreement with the phylogenetic relationships, strain RBU1-1^T shared similar DNA G+C content range, polar lipid pattern and fatty acid profile with these two genera. However, *F. milosensis* JCM 12288^T has L-ornithine as the diagnostic diamino acid in the cell wall, whereas strain RBU1-1^T and *T. multivorans* NBRC 100370^T have meso-DAP. Strain RBU1-1^T and *T. multivorans* can be differentiated phenotypically, for example based on mode of flagellation and acid tolerance. On the other hand, strain RBU1-1^T is related quite closely phylogenetically to members of the genus *Alkalibacillus*; however, all *Alkalibacillus* species are

Table 1. Differential characteristics between strain RBU1-1^T and related taxa

Taxa: 1, strain RBU1-1^T; 2, *Filobacillus* (data from this study and Schlesner *et al.*, 2001); 3, *Tenuibacillus* (Ren & Zhou, 2005a); 4, *Alkalibacillus* (Fritze, 1996; Jeon *et al.*, 2005). +, Positive; w, weakly positive; –, negative; NA, not available. All taxa produce spores in a terminal position.

Characteristic	1	2	3	4
Spore shape	Oval	Spherical	Spherical	Spherical
Gram reaction	+	–	+	+
Flagella	Peritrichous	Single lateral	Single polar	Peritrichous
NaCl range (% w/v)	2–30	2–23	1–20	0–25
Maximum growth temperature (°C)	48	42	42	40
Growth at pH 6.0	+	–	–	–
Growth at pH 7.0	+	+	+	–
Hydrolysis of:				
Gelatin	+	–	+	+
Casein	+	–	+	+
Starch	+	–	–	w
Murein type	meso-DAP	L-Orn	meso-DAP	meso-DAP
Major fatty acids	iso-C _{15:0} (35.3 %), anteiso-C _{15:0} (30.8 %)	anteiso-C _{15:0} (41.5 %), iso-C _{15:0} (23.2 %)	iso-C _{15:0} (64.7 %), anteiso-C _{15:0} (12.7 %)	iso-C _{15:0} (48 %), anteiso-C _{15:0} (11 %)
DNA G+C content (mol%)	36.7	35.7	36.5–37	38–41

alkalophilic (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005). Differential characteristics between strain RBU1-1^T and the genera *Filobacillus*, *Tenuibacillus* and *Alkalibacillus* are given in Table 1.

Strain RBU1-1^T can be separated from the genera *Filobacillus*, *Tenuibacillus* and *Alkalibacillus* and is considered to represent a novel species of a new genus. We propose the name *Piscibacillus salipiscarius* gen. nov., sp. nov. to accommodate this organism.

Description of *Piscibacillus* gen. nov.

Piscibacillus (Pis.ci.ba.cil'lus. L. n. *piscis* fish; L. masc. n. *bacillus* small rod; N.L. masc. n. *Piscibacillus* a rod from fish).

Gram-positive rods, measuring 0.4–0.5 × 1.5–4 µm. Cells are motile with peritrichous flagella. Oval terminal endospores are formed in swollen sporangia. Positive for catalase and oxidase, but negative for urease and reduction

Fig. 2. Phylogenetic tree showing the relationships between strain RBU1-1^T and related bacterial species based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages ≥ 78 %, based on 1000 replications, are shown at nodes. Bar, 1 substitution per 100 nucleotide positions.

Table 2. Cellular fatty acid composition of strains RBU1-1^T and *F. milosensis* JCM 12288^T

Values are percentage of total fatty acids. ND, Not detected.

Fatty acid	RBU1-1 ^T	<i>F. milosensis</i> JCM 12288 ^T
Saturated		
C _{14:0}	0.2	0.2
C _{15:0}	0.5	0.6
C _{16:0}	1.3	1.3
C _{16:0} N alcohol	0.8	0.2
Unsaturated		
C _{16:1} ω7c	2.0	1.1
C _{16:1} ω11c	1.2	0.2
Branched-chain		
iso-C _{14:0}	4.5	2.7
iso-C _{15:0}	35.3	23.2
anteiso-C _{15:0}	30.8	41.5
iso-C _{16:0}	10.1	9.5
iso-C _{17:0}	3.5	4.0
anteiso-C _{17:0}	6.1	11.2
iso-C _{19:0}	ND	0.4
anteiso-C _{19:0}	ND	0.2

of nitrate, production of indole and utilization of citrate. Casein, DNA and gelatin are hydrolysed, but arginine, starch, Tween 80, tyrosine, phenylalanine, xanthine and hypoxanthine are not. *meso*-DAP is present in the peptidoglycan. The predominant menaquinone is MK-7. The major fatty acids are iso-C_{15:0} and anteiso-C_{15:0} (Table 2). Phosphatidylglycerol and diphosphatidylglycerol are the predominant polar lipids (see Supplementary Fig. S1 in IJSEM Online). The type species is *Piscibacillus salipiscarius*.

Description of *Piscibacillus salipiscarius* sp. nov.

Piscibacillus salipiscarius (sa.li.pis.ca'ri.us. L. n. *sal*, *salis* salt; L. adj. *piscarius* of or belonging to fish; N.L. masc. adj. *salipiscarius* of or belonging to a salted fish).

Colonies are white to cream, 0.9–3.9 mm in diameter after 5 days growth on *Lentibacillus* agar medium. Grows aerobically but weakly under anaerobic conditions. Grows at 15–48 °C (optimally at 37 °C), pH 5–9 (optimally at pH 7.0) and in 2–30 % NaCl (optimally in 10–20 %). Acid is produced from D-fructose, D-galactose, D-glucose, glycerol, D-ribose and sucrose, but not from D-amygdalin, L-arabinose, cellobiose, inulin, lactose, maltose, mannitol, D-mannose, melibiose, melezitose, methyl α-D-glucoside, *myo*-inositol, raffinose, rhamnose, salicin, sorbitol, trehalose or D-xylose. The DNA G + C content of the type strain is 36.7 mol%.

The type strain, RBU1-1^T (=JCM 13188^T=PCU 270^T=TISTR 1571^T), was isolated from fermented fish (pla-ra) in Thailand.

Acknowledgements

This study was supported in part by a Rachadapiseksomphot Research Grant, Chulalongkorn University (2002).

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