***Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand**

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Eleven strains of homofermentative, rod-shaped lactic acid bacteria and five strains of heterofermentative, sphere-shaped lactic acid bacteria were isolated from fermented fish (pla-ra and pla-chom) in Thailand. They were identified as new species and named *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., respectively, on the basis of phylogenetic analysis of the 16S rRNA gene sequences, DNA relatedness and phenotypic characteristics. The type strain of *L. acidipiscis* is FS60-1^T (= PCU 207^T = NRIC 0300^T = HSCC 1411^T = JCM 10692^T = TISTR 1386^T) and the type strain of *Weissella thailandensis* is FS61-1^T (= PCU 210^T = NRIC 0298^T = HSCC 1412^T = JCM 10695^T = TISTR 1384^T).

Keywords: *Lactobacillus acidipiscis*, *Weissella thailandensis*, fermented fish, lactic acid bacteria

INTRODUCTION

Lactic acid bacteria are widely distributed in Thailand in foods such as fermented fish (pla-ra, pla-paeng-daeng, pla-chao, pla-chom, pla-som, etc.) and shrimp (kung-chom), fermented pork (nham), fermented vegetables (phak-gard-dong, phak-koom-dong, thua-ngok-dong, hom-dong, etc.), tea leaves (miang), sweetened rice (khaomak), etc. (Tanasupawat & Komagata, 1995). These fermented foods are used for main- and side dishes, condiments, desserts and so on. The lactic acid bacteria are responsible for the souring and ripening of these foods. Some of these foods contain a high concentration of salt (pla-ra and pla-chom). *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Staphylococcus* strains have been isolated from the above-mentioned foods so far (Tanasupawat & Komagata, 1995; Tanasupawat *et al.*, 1998).

In a previous paper, Tanasupawat *et al.* (1998) reported the isolation of L-lactic-acid-producing rod-shaped and D-lactic-acid-producing sphere-shaped lactic acid bacteria from fermented fish (pla-ra and

pla-chom) in Thailand. These bacteria were tentatively identified as *Lactobacillus* sp. (group III) and *Leuconostoc* species (group IV), respectively. On the basis of phylogenetic analysis of 16S rRNA gene sequences and DNA relatedness, these strains were included in the genera *Lactobacillus* and *Weissella*, respectively, and identified as new species. *Lactobacillus acidipiscis* sp. nov. is proposed for group III and *Weissella thailandensis* sp. nov. is proposed for group IV. This paper deals with the descriptions of the above species. The type strain of *L. acidipiscis* is FS60-1^T (= PCU 207^T = NRIC 0300^T = HSCC 1411^T = JCM 10692^T = TISTR 1386^T) and the type strain of *W. thailandensis* is FS61-1^T (= PCU 210^T = NRIC 0298^T = HSCC 1412^T = JCM 10695^T = TISTR 1384^T).

METHODS

Bacterial strains and growth media. Eleven strains of group III (rod-shaped) and five strains of group IV (sphere-shaped) were subjected to this study (Tanasupawat *et al.*, 1998). The strain designations and isolation sources are shown in Tables 1 and 3. MRSH medium (a half-strength version of MRS medium; De Man *et al.*, 1960) and GYPB medium (Tanasupawat *et al.*, 1998) were used throughout the study.

Phenotypic and chemotaxonomic characterization. The phenotypic characteristics, the isomers of lactic acid produced, the cellular fatty acids and the DNA base com-

The DDBJ accession numbers for the 16S rRNA gene sequences reported in this paper are AB023836 (*Lactobacillus acidipiscis* FS60-1^T), AB023837 (*Lactobacillus acidipiscis* FS111), AB023838 (*Weissella thailandensis* FS61-1^T) and AB023839 (*Weissella thailandensis* FS45-1).

Fig. 1. Phylogenetic relationships between *Lactobacillus acidipiscis* FS60-1^T, FS111 and *Lactobacillus* species on the basis of 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. The numbers indicate bootstrap values >700. Bar, 0.01 nucleotide substitutions per site.

position were all from a previous paper (Tanasupawat *et al.*, 1998) and additional tests were carried out in this study. Cell morphologies were examined by using scanning electron microscopy.

Cell wall peptidoglycan type. The composition of cell walls was determined using the method of Komagata & Suzuki (1987).

DNA relatedness. DNA relatedness was determined by using the fluorometric method, as described by Tanasupawat *et al.* (1998), and by using the colorimetric method, as described by Verlander (1992). The substrate, 3,3',5,5'-tetramethylbenzidine/H₂O₂ solution and streptavidin-peroxidase conjugate (Boehringer Mannheim) were used for the colorimetric method. The enzyme reaction was stopped with 2 M H₂SO₄ and the absorbance was measured at 450 nm with a Microplate Reader (model 3550; Bio-Rad). DNA relatedness was determined for *Lactobacillus* species by using the fluorometric method and for *Weissella* species by using the colorimetric method.

Sequencing of 16S rRNA gene. Cloning and sequencing of 16S rRNA genes were carried out as described by Shida *et al.* (1997). Oligonucleotides 5'-AGTTTGATCCTGGCTCAG-3' (primer 27FC; 5' end of the 16S rRNA gene) and 5'-GTTACCTTGTTACGACTTC-3' (primer 1490RC; 3' end of the 16S rRNA gene) were used as primers for the amplification of 16S rRNA gene by the PCR. Amplified 16S rRNA gene fragments were purified with a QIA quick Spin PCR purification kit (Qiagen) and used for sequencing templates. Sequencing was carried out by the method described by Sanger *et al.* (1977). A Dye Terminator Cycle-sequencing FS Ready Reaction kit (Perkin-Elmer) and a model ABI 373A automatic DNA sequencer (Perkin-Elmer) were used. The seven oligonucleotides 5'-CTGCTGCCT-CCCGTAG-3' (primer 341R), 5'-GTATTACCGCGGCTGCTG-3' (primer 518R), 5'-TACGGGAGGCAGCAG-3' (primer 356F), 5'-GTGCCAGCAGCCGCGG-3' (primer 530F), 5'-GATTAGATACCCTGGTAG-3' (primer 803F), 5'-ACTCAAAGGAATTGACGG-3' (primer 926F) and 5'-GCAACGAGCGCAACCC-3' (primer 1111F) were used as primers for sequencing of 16S rRNA gene fragments.

Comparison of 16S rRNA gene sequences. Previously published 16S rRNA gene sequences were obtained from the EMBL/GenBank/DBJ database. Multiple alignment of sequences, calculation of the nucleotide substitution rate (K_{nuc} ; Kimura, 1980), construction of a neighbour-joining

phylogenetic tree (Saito & Nei, 1987) and 1000-replicate bootstrap analysis for evaluation of phylogenetic tree topology (Felsenstein, 1985) were carried out with the CLUSTAL W version 1.6 program (Thompson *et al.*, 1994). Alignment gaps and unidentified base positions were not taken into account. Accession numbers for the nucleotide sequences used for the phylogenetic analysis are shown in Figs 1 and 2.

RESULTS AND DISCUSSION

16S rRNA sequences

Nucleotide sequences (1417 nucleotides) of 16S rRNA genes were determined for strain FS60-1^T, FS61-1^T and FS45-1, isolated in Thailand from the popular fermented fish pla-ra, and for strain FS111, isolated from pla-chom. These strains were studied in detail. Phylogenetic trees are shown in Figs 1 and 2.

Phenotypic and chemotaxonomic characterization

The phenotypic characteristics, the isomers of lactic acid produced, the cellular fatty acids and the DNA base composition were cited from a previous paper (Tanasupawat *et al.*, 1998) and are mentioned in the descriptions. The cell morphologies of FS60-1^T and FS61-1^T are shown in Fig. 3.

Characteristics of *Lactobacillus acidipiscis* sp. nov.

L. acidipiscis strains FS60-1^T and FS111 were included in a monophyletic cluster containing *Lactobacillus salivarius* subsp. *salicinius*, *Lactobacillus aviarius*, *Lactobacillus ruminis*, *Lactobacillus agilis*, *Lactobacillus murinus*, *Lactobacillus animalis* and *Lactobacillus mali* on the basis of 16S rRNA gene sequence analysis as shown in Fig. 1. FS60-1^T and FS111 were placed in a robust (1000 counts of bootstrap values) monophyletic cluster consisting of the *Lactobacillus* species. The similarity values of the sequence were 99.8% between FS60-1^T and FS111, and the values were higher than 93.0% between each of these strains and each of the above seven *Lactobacillus* species. The

Fig. 2. Phylogenetic relationships between *Weissella thailandensis* FS61-1^T, FS45-1, *Weissella* species and related bacteria on the basis of 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. The numbers indicate bootstrap values >700. Bar, 0.01 nucleotide substitutions per site.

Fig. 3. Scanning electron micrographs of (top) *Lactobacillus acidipiscis* FS60-1^T and (bottom) *Weissella thailandensis* FS61-1^T. Cells were grown in MRSH broth. Bar, 1 μm.

similarity values were lower than 91.3% between each of FS60-1^T and FS111 and the other *Lactobacillus* species (data not shown). FS111 contained L-Lys-D-

Asp in the cell wall. Values for the DNA–DNA relatedness of FS60-1^T and FS111 to other strains and related species are shown in Table 1. The level of DNA–DNA relatedness clearly supported the separation of the isolates from other L-lactic-acid-producing *Lactobacillus* species. *L. acidipiscis* was differentiated from other L-lactic-acid-producing *Lactobacillus* species on the basis of the descriptions reported so far (Kandler & Weiss, 1986; Hammes *et al.*, 1992). *L. acidipiscis* strains grew in 10% NaCl and seemed to be close to *Lactobacillus farciminis* but they differed in arginine hydrolysis, growth at 15 °C, acid production from D-maltose, D-mannitol and D-ribose, DNA base composition and cellular fatty acid profile (Tanasupawat *et al.*, 1998). Characteristics differentiating *L. acidipiscis* from other *Lactobacillus* species in the same phylogenetic cluster are shown in Table 2. Recently, the starch-hydrolyser *Lactobacillus manihotivorans* was described, but this species clearly differs from *L. acidipiscis* by the hydrolysis of starch and a high (48.4 mol%) G+C content (Morlon-Guyot *et al.*, 1998).

Description of *Lactobacillus acidipiscis* sp. nov.

Lactobacillus acidipiscis (a.ci.di'pis.cis. L. adj. *acidus* sour; L. n. *piscis* fish; L. adj. *acidipiscis* pertaining to sour fish, an isolation source of strains of this species). Cells are Gram-positive rods (0.4–0.6 × 1.2–5.0 μm), non-motile, non-spore-forming and occur singly, in pairs and in chains. Colonies on MRSH agar plate are circular, slightly convex with an entire margin and unpigmented. Microaerophilic. Utilizes D-glucose homo-fermentatively and does not produce gas from glucose. Produces L-lactic acid from glucose (>91%). Negative for catalase activity, nitrate reduction, hydrolysis of arginine, aesculin, gelatin and starch and for the formation of slime from sucrose. Most strains show no reaction in litmus milk. Grows at 25–37 °C but not at 15 or 42 °C. Does not grow at pH 4.0 or pH 8.5. Grows in 10% NaCl. Some strains grow in 12% NaCl. Produces acid from D-fructose, D-galactose, D-glucose and D-mannose but does not produce acid from

Table 1. Strain designations, isolation sources, DNA base composition and DNA relatedness of *Lactobacillus* species

Abbreviations: NRIC, Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan; PCU, Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; HSCC, Culture Collection of the Research and Development Department, Higeta Shoyu, Co., Ltd, Choshi, Japan; JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan; TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. T, Type strain.

Strain	Culture collection					Source (fermented fish)	G + G content (mol %)	DNA–DNA relatedness with labelled strains (%)		
	NRIC	PCU	HSCC	JCM	TISTR			FS60-1 ^T	FS111 [*]	FS111
FS60-1 ^T	0300 ^T	207 ^T	1411 ^T	10692 ^T	1386 ^T	Pla-ra	38.7*	100.0	94.6	—
FS109	0301	214				Pla-chom	41.5*	96.5	101.0	—
FS111	0302	208	1413	10693	1387	Pla-chom	40.3*	107.6	100.0	100.0
FS4-1	0303	215				Pla-chom	40.3*	87.8	92.8	—
FS49-1	0304	216				Pla-ra	40.6*	106.9	99.1	—
FS53-2	0305	217				Pla-ra	—	99.5	—	—
FS53-4	0306	218				Pla-ra	39.5*	93.6	93.0	—
FS55-2	0307	219				Pla-ra	—	83.2	—	—
FS56-1	0308	206			1388	Pla-ra	39.3*	85.2	92.1	—
FS58-1	0309	220				Pla-ra	38.6*	77.5	92.9	—
FS62-1	0310	221				Pla-ra	38.7*	78.9	95.7	—
<i>L. agilis</i>	1642 ^T						43.4†	4.9	—	6.7
<i>L. animalis</i>				5670 ^T	1115 ^T		42.4‡	22.5	26.7	—
<i>L. aviaris</i> subsp. <i>araffinosus</i>	1774 ^T			5667 ^T			41.3‡	3.6	—	3.8
<i>L. farciminis</i>				1097 ^T	1113 ^T		36.0‡	8.3	15.7	—
<i>L. mali</i>	1692 ^T			1116 ^T			37.6†	3.6	—	4.3
<i>L. murinus</i>				1717 ^T	1111 ^T		—	3.6	—	3.8
<i>L. ruminis</i>	1689 ^T			1152 ^T			45.5†	3.6	—	4.3
<i>L. salivarius</i> subsp. <i>salicinicus</i>	1072 ^T			1150 ^T			28.5§, 36.6‡	—	—	—
<i>L. salivarius</i> subsp. <i>salivarius</i>				1231 ^T	1112 ^T		28.3§	11.3	27.3	—

* Data from Tanasupawat *et al.* (1998).

† Data from Weiss *et al.* (1981).

‡ Data from Gasser & Mandel (1968).

§ Data from Entani *et al.* (1986).

Table 2. Characteristics that differentiate *Lactobacillus acidipiscis* from other L-lactic-acid-producing species

T, Type strain; ND, not determined.

Characteristic	<i>L. acidipiscis</i> *		<i>L. agilis</i> NRIC 1642 ^T	<i>L. animalis</i> TISTR 1115 ^T	<i>L. aviaris</i> subsp. <i>araffinosus</i> NRIC 1774 ^{T†}	<i>L. farciminis</i> TISTR 1113 ^T	<i>L. mali</i> NRIC 1692 ^T	<i>L. murinus</i> TISTR 1111 ^T	<i>L. ruminis</i> NRIC 1689 ^T	<i>L. salivarius</i> subsp. <i>salicinicus</i> NRIC 1072 ^T
	FS60-1 ^T	11 strains								
Diaminopimelic acid in cell wall	—	0	+‡	—	—	—	+‡	—	+§	—
Arginine hydrolysis	—	0	—	—	ND	+	—	—	—	—
Growth at 15 °C	—	0	—‡	—	—	+	+‡	—	—§	—
Growth in 10 % NaCl	+	11¶	—	—	ND	+	—	—	—	—
Acid from:										
D-Maltose	+	10	+	+	+	—	—	+	+	+
D-Mannitol	+	2	+	—	—	—	+	+	—	+
Raffinose	—	0	+	+	—	—	—	+	+	+
D-Ribose	+	11	+	—	—	—	—	—	+	+
Sorbitol	—	0	—	—	—	—	+	+	—	+

* Data from Tanasupawat *et al.* (1998).

† Data from Fujisawa *et al.* (1984).

‡ Data from Weiss *et al.* (1981).

§ Data from Sharpe *et al.* (1973).

|| Data from Entani *et al.* (1986).

¶ Number of positive strains.

cellobiose, gluconate, D-melibiose, D-melezitose, methyl α -D-glucoside, raffinose, D-sorbitol and D-xylose. Some strains produce acid weakly from lactose

and salicin. Variable reactions for D-amygdalin, L-arabinose, glycerol, maltose, D-mannitol, L-rhamnose, sucrose and D-trehalose. Requires niacin and

Table 3. Strain designations, isolation sources, DNA base composition and DNA relatedness of *Weisselia* species

T, Type strain.

Species	Strain	Culture collection					Source (fermented fish)	G + C content (mol %)	DNA–DNA relatedness with labelled strains (%)	
		NRIC	JCM	PCU	HSCC	TISTR			FS45	FS61-1 ^T
<i>W. thailandensis</i>	FS61-1 ^T	0298 ^T	10695 ^T	210 ^T	1412 ^T	1384 ^T	Pla-ra	41.2*	96.9	100.0
<i>W. thailandensis</i>	FS45	0295		211			Pla-ra	40.0*	100.0	97.9
<i>W. thailandensis</i>	FS45-1	0296	10694	209	1410	1385	Pla-ra	38.0*	92.1	95.5
<i>W. thailandensis</i>	FS46-2	0297		212			Pla-ra	39.8*	104.6	102.5
<i>W. thailandensis</i>	FS61-2	0299		213			Pla-ra		84.6	106.1
<i>W. paramesenteroides</i>		1542 ^T	9890 ^T					40.4†	36.0	21.5
<i>W. hellenica</i>		0203 ^T	10103 ^T					39.4‡	28.2	19.3
<i>W. confusa</i>		0207 ^T	1093 ^T					47.0†	15.7	16.7

* Data from Tanasupawat *et al.* (1998).† Data from Schillinger *et al.* (1989).‡ Data from Collins *et al.* (1993).**Table 4.** Characteristics that differentiate *Weisselia thailandensis* from related species

T, Type strain.

Characteristic	<i>W. thailandensis</i> *		<i>W. confusa</i> NRIC 0207 ^T	<i>W. hellenica</i> NRIC 0203 ^T	<i>W. paramesenteroides</i> NRIC 1542 ^T	<i>Leuconostoc mesenteroides</i> NRIC 1541 ^{T†}
	FS61-1 ^T	5 strains				
NH ₃ from arginine	—	0	+	—	—	—
Slime from sucrose	—	0	+	—	—	+
Growth in 10% NaCl	+	5‡	—	—	—	—§
Acid from:						
L-Arabinose	+	5	—	+	+	+
Cellobiose	—	0	+	+	—	+
D-Galactose	+	5	—	+	+	+
Lactose	—	3	—	—	—	+
D-Mannitol	—	0	—	—	—	+
D-Melibiose	+	5	—	—	+	+
Raffinose	+	5	—	—	—	+
D-Ribose	+	5	—	+	+	—
Salicin	—	0	—	+	—	+
Sucrose	+	4	+	—	+	+
D-Trehalose	+	4	+	—	+	+
D-Xylose	—	0	—	+	—	+

* Data from Tanasupawat *et al.* (1998).† Data from Takahashi *et al.* (1992).

§ Data from this study.

‡ Number of positive strains.

calcium pantothenate for growth. Major cellular fatty acids are straight-chained C_{16:0} and C_{18:1}. L-Lys-D-Asp in the cell wall. The G + C content of the DNA ranges from 38.6 to 41.5 mol%. Isolated from fer-

mented fish (pla-ra and pla-chom) in Thailand. The type strain is FS60-1^T (= PCU 207^T = NRIC 0300^T = HSCC 1411^T = JCM 10692^T = TISTR 1386^T). The type strain has the characteristics given in the descrip-

tion of species and in Tables 1 and 2. The G+C content of the type strain is 38.7 mol%.

Characteristics of *Weissella thailandensis* sp. nov.

Weissella thailandensis strains FS61-1^T and FS45-1 were included in a monophyletic cluster containing *Weissella* species on the basis of 16S rRNA gene sequence analysis, as shown in Fig. 2. FS61-1^T and FS45-1 were placed in a robust (1000 counts of bootstrap values) monophyletic cluster consisting of the *Weissella* species. The similarity values of the sequence were 99.9% between FS61-1^T and FS45-1 and higher than 94.0% between FS61-1^T or FS45-1 and each of seven *Weissella* species. The similarity values were lower than 90.2% between FS61-1^T or FS45-1 and each of the *Leuconostoc* and *Lactococcus* strains (data not shown). FS61-1^T contained L-Lys-L-Ala₂ in the cell walls. Values of DNA–DNA relatedness of FS61-1^T and FS45-1 to other strains and related species are shown in Table 3. The level of DNA–DNA relatedness clearly supported the separation of the isolates from other *Weissella* species. Garvie (1986) mentioned the growth of some (not all) *Weissella* (*Leuconostoc*) *paramesenteroides* strains in 3 and 6.5% NaCl. In contrast, all *W. thailandensis* strains tested grew in 10% NaCl. This is regarded as a characteristic of the species. Characteristics differentiating *W. thailandensis* from other *Weissella* species in the same phylogenetic cluster are shown in Table 4. The *W. thailandensis* strains produced D-lactic acid along with other *Weissella* species (Tsakalidou *et al.*, 1997).

Description of *Weissella thailandensis* sp. nov.

Weissella thailandensis (thai.lan'den.sis. M.L. fem. adj. *thailandensis* pertaining to Thailand, where the strains were first isolated).

Cells are Gram-positive cocci (0.5–0.7 µm), non-motile, non-sporing and occur in pairs or in chains. Colonies on MRSH agar plates are usually opaque, circular, slightly convex with an entire margin and unpigmented. Microaerophilic. Utilizes D-glucose heterofermentatively and produces gas from glucose. Produces D-lactic acid from glucose (>92%). Negative for catalase activity, nitrate reduction, hydrolysis of arginine, aesculin, gelatin and starch, for the formation of slime from sucrose and for the reaction in litmus milk. Final pH in MRSH medium is pH 4.3–4.6. Grows at 25–37 °C but not at 42 °C. Grows at pH 8.0 but not at pH 4.5 or pH 8.5. Grows in 10% NaCl. Produces acid from D-ribose, L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, D-melibiose, raffinose and L-rhamnose but does not produce acid from D-amygdalin, D-cellobiose, glycerol, D-mannitol, D-melezitose, salicin, D-sorbitol or D-xylose. Requires niacin and calcium pantothenate for growth. In addition, some strains require thiamine or riboflavin. Major cellular fatty acids are straight-chained C_{16:0} and C_{20:1}. L-Lys-L-Ala₂ in the cell wall. The G+C content of the DNA ranges from 38.0 to

41.2 mol%. Isolated from fermented fish (pla-ra) in Thailand. The type strain is FS61-1^T (PCU 210^T = NRIC 0298^T = HSCC 1412^T = JCM 10695^T = TISTR 1384^T). The type strain has the characteristics given in the description of species and in Tables 3 and 4. The G+C content of the type strain is 41.2 mol%.

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