

16S rRNA gene sequence analysis of lactic acid bacteria isolated from fermented foods in Thailand

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In our investigation of a variety of lactic acid bacteria in fermented foods in Thailand, we isolated 945 strains from 114 varieties of fermented foods from the northeastern, central, and southern parts of the northern Thailand. We determined their 16S ribosomal RNA (rRNA) gene sequences and selected 410 strains for further analyses based on differences of sequences and/or isolation source. The result of the 16S rRNA gene sequence analysis showed that the isolates were divided into 50 groups of six genera: *Aerococcus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Tetragenococcus*, and *Weissella*. Only one strain belonging to the genus *Aerococcus* showed 100% sequence similarity to *A. viridans*. Forty-six strains were assigned to the genus *Enterococcus*. They were divided into six groups to which they were closely related, namely, *E. avium*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. thailandicus*, or *E. gilvus/E. raffinosus*. We sorted 309 strains belonging to the genus *Lactobacillus* into 34 sequence groups. Most of them showed a sequence that is 100% identical to those of known species. However, 12 strains of eight groups showed slightly lower similarity rates of lower than 99.0%, suggesting that they may be classified into novel species. Thirty-eight strains of three groups were classified in the genus *Pediococcus*. One group was closely related to *P. acidilactici*, and the other groups were closely related to *P. pentosaceus*. Four isolates were classified in *Tetragenococcus halophilus*. Twelve strains belonging to the genus *Weissella* were divided into five species, namely, *W. cibaria*, *W. confusa*, *W. paramesenteroides*, *W. thailandensis*, and *W. viridescens*. The strains isolated in this study will be available for public use in the BIOTEC Culture Collection and the NITE Biological Resource Center, with the numbers NBRC 107174 to 107352 and NBRC 108277 to 108549.

Key words: lactic acid bacteria, Thailand, fermented foods, 16S rRNA gene sequence

INTRODUCTION

The National Institute of Technology and Evaluation (NITE) of Japan and the National Center for Genetic Engineering and Biotechnology (BIOTEC) of Thailand concluded their memorandum of understanding in accordance with the Convention on Biological Diversity, which promotes cooperative researches on the conservation and the sustainable use of biological resources in Japan and Thailand for academic, industrial, and other purposes. It is

expected that the utilization and commercialization of biological resources will provide some benefits to both countries. The NITE Biological Resource Center (NBRC) and the BIOTEC Culture Collection (BCC) started joint research projects on bacteria, yeasts, and fungi to enrich the microbiological resources and to develop the knowledge and skills of the scientists in both collections. We began a collaborative research focused on acetic acid bacteria in 2005 and published its results (Muramatsu *et al.*, 2009). In this paper, we report our study on lactic acid bacteria that we started in 2008.

Lactic acid bacteria have been used to ferment foods before the existence of microorganisms was

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even recognized. It is well known that they are beneficial for human health. Various fermented foods are eaten in Thailand, especially in the northeastern region of Thailand. These fermented foods are served as main dishes and also used as condiments. They give a unique taste and flavor to Thai foods. In our investigation of a variety of lactic acid bacteria in fermented Thai foods, we isolated more than 900 strains and performed phylogenetic analyses based on their 16S rRNA gene sequences.

MATERIALS AND METHODS

Sampling and isolation

The samples used in this study were collected from the local markets located in three regions; the northeastern, central, and southern parts of the northern Thailand. Sixty samples of fermented foods (mainly fermented freshwater fish) were collected from eight local markets located in eight provinces in the northeastern Thailand. Twenty-six samples of fermented freshwater fishes and crustaceans, one sample of fermented meat, and 27 samples of fermented plant materials such as vegetables, fruits, tea leaves, and soy beans, were collected from 31 different shops in 12 local markets located in eight provinces in the central and southern parts of the northern Thailand. Because the sodium chloride (NaCl) concentration of the samples varied from 0 to 50.0%, we used the MRS medium supplemented with 0, 5, 10, or 15% of NaCl for the isolation of lactic acid bacteria. In addition, we used pH levels between 4.0 and 10.0 for the culture media because the pH levels of the samples ranged from 4.0 to 8.0. The samples were diluted and inoculated on MRS agar plates and then incubated at 30°C in anaerobic conditions created by the AnaeroPack-Anaero system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). The colonies that emerged were repeatedly transferred on new MRS agar plates until purification was achieved.

Polymerase chain reaction amplification, sequencing, and phylogenetic analysis of 16S rRNA genes

The almost complete 16S rRNA gene sequences, ranging from positions 28 to 1494 on the *Escherichia coli* numbering system (Brosius *et al.*, 1978), were determined according to previously published procedures (Nakagawa *et al.*, 2002). The sequences obtained were aligned with those of the representative members of the lactic acid bacteria using

Clustal_X (Thompson *et al.*, 1997) and then modified manually by referring to the 16S rRNA secondary structure of *E. coli* (Gutell *et al.*, 1994) and using the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). A phylogenetic tree was reconstructed using the neighbor-joining method (Saitou & Nei, 1987) and the K_{nuc} values derived from the sequences (Kimura, 1980). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1,000 replicates.

RESULTS AND DISCUSSION

Phylogenetic positions of isolates

Initially, 945 strains of lactic acid bacteria were isolated from 114 varieties of fermented foods collected in Thailand. We determined their 16S rRNA gene sequences and selected 410 strains for further studies. Five hundred thirty-five strains whose isolation source and 16S rRNA gene sequences were identical were excluded from the study because we considered them as overlapping strains. Four hundred ten isolates were classified in the genera *Aerococcus* (number of isolates, 1), *Enterococcus* (46), *Lactobacillus* (309), *Pediococcus* (38), *Tetragenococcus* (4), and *Weissella* (12). They were divided into 50 groups according to the differences in their 16S rRNA gene sequences, and the species closely related to these groups are listed in Table 1.

Only one strain, NB527, belonging to the genus *Aerococcus* had a 16S rRNA gene sequence that coincided with the sequence of the type strain of *Aerococcus viridans*. Forty-six isolates belonged to the genus *Enterococcus* and were divided into six groups to which they were closely related, namely, *E. avium*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. thailandicus*, or *E. gilvus*/*E. raffinosus*. They showed greater than 99.8% sequence similarity to each type strain.

In the genus *Lactobacillus*, we grouped 309 strains and divided them into 34 sequence groups (Table 1). Twenty-five groups comprising 297 strains showed high rates of 16S rRNA gene sequence similarity (higher than 99.1%) to the following known species: *L. acidipiscis* (group L1), *L. brevis* (L3), *L. collinoides* (L4), *L. curvatus* (L6), *L. farciminis* (L7–L15), *L. fermentum* (L16), *L. futsaii* (L18, L19), *L. namurensis* (L21), *L. nantensis* (L23), *L. pantheris* (L24), *L. para-*

Table 1 List of the sequence groups of the Thai isolates

Genus	Phylogenetic group	Strains	Closest species based on 16S rRNA gene sequence similarity rates (%)
<i>Aerococcus</i>	A1	NB527	<i>A. viridans</i> (100)
<i>Enterococcus</i>	E1	NB1011	<i>E. avium</i> (99.8)
	E2	NB248 and 13 strains	<i>E. faecalis</i> (100)
	E3	NB211 and 16 strains	<i>E. faecium</i> (99.8)
	E4	NB860	<i>E. gilvus</i> / <i>E. raffinosus</i> (99.9)
	E5	NB152 and 9 strains	<i>E. hirae</i> (100)
	E6	NB320 and 2 strains	<i>E. thailandicus</i> (99.8)
<i>Lactobacillus</i>	L1	NB80 and 23 strains	<i>L. acidipiscis</i> (99.9)
	L2	NB278	<i>L. alimentarius</i> (98.5)
	L3	NB465 and 13 strains	<i>L. brevis</i> (100)
	L4	NB916	<i>L. collinoides</i> (100)
	L5	NB606	<i>L. crustorum</i> (98.7)
	L6	NB146	<i>L. curvatus</i> (99.8)
	L7	NB145 and 12 strains	<i>L. farciminis</i> (99.5)
	L8	NB85 and 4 strains	<i>L. farciminis</i> (99.6)
	L9	NB676 and 2 strains	<i>L. farciminis</i> (99.7)
	L10	NB730	<i>L. farciminis</i> (99.9)
	L11	NB08 and 3 strains	<i>L. farciminis</i> (99.4)
	L12	NB742 and 1 strain	<i>L. farciminis</i> (99.5)
	L13	NB1006	<i>L. farciminis</i> (99.6)
	L14	NB348	<i>L. farciminis</i> (99.9)
	L15	NB40 and 60 strains	<i>L. farciminis</i> (100)
	L16	NB523 and 16 strains	<i>L. fermentum</i> (100)
	L17	NB844 and NB1184	<i>L. futsaii</i> (98.7)
	L18	NB659	<i>L. futsaii</i> (99.2)
	L19	NB46 and 4 strains	<i>L. futsaii</i> (99.3)
	L20	NB61 and 33 strains	<i>L. futsaii</i> (100)
	L21	NB490 and 4 strains	<i>L. namurensis</i> (99.9)
	L22	NB483	<i>L. nantensis</i> (98.9)
	L23	NB522 and NB609	<i>L. nantensis</i> (99.4)
	L24	NB655	<i>L. pantheris</i> (99.7)
	L25	NB642 and 1 strain	<i>L. paracasei</i> (100)
	L26	NB1110	<i>L. paralimentarius</i> (99.8)
	L27	NB53 and NB834	<i>L. plantarum group</i> (98.9)
	L28	NB446 and NB702	<i>L. plantarum group</i> (98.7)
	L29	NB1140 and NB1183	<i>L. plantarum group</i> (99.9)
	L30	NB07 and 93 strains	<i>L. plantarum group</i> (100)
	L31	NB625	<i>L. pobuzihii</i> (100)
	L32	NB1023	<i>L. saerimneri</i> (100)
	L33	NB521	<i>L. senioris</i> (97.7)
	L34	NB196 and NB468	<i>L. versmoldensis</i> (99.0)
<i>Pediococcus</i>	P1	NB38 and 11 strains	<i>P. acidilactici</i> (99.7)
	P2	NB04 and 4 strains	<i>P. pentosaceus</i> (99.7)
	P3	NB20 and 20 strains	<i>P. pentosaceus</i> (99.8)
<i>Tetragenococcus</i>	T1	NB636 and 3 strains	<i>T. halophilus</i> (99.8)
<i>Weissella</i>	W1	NB27 and 2 strains	<i>W. cibaria</i> (100)
	W2	NB546	<i>W. confusa</i> (100)
	W3	NB442 and 1 strain	<i>W. paramesenteroides</i> (100)
	W4	NB173 and 4 strains	<i>W. thailandensis</i> (99.8)
	W5	NB25	<i>W. viridescens</i> (99.9)

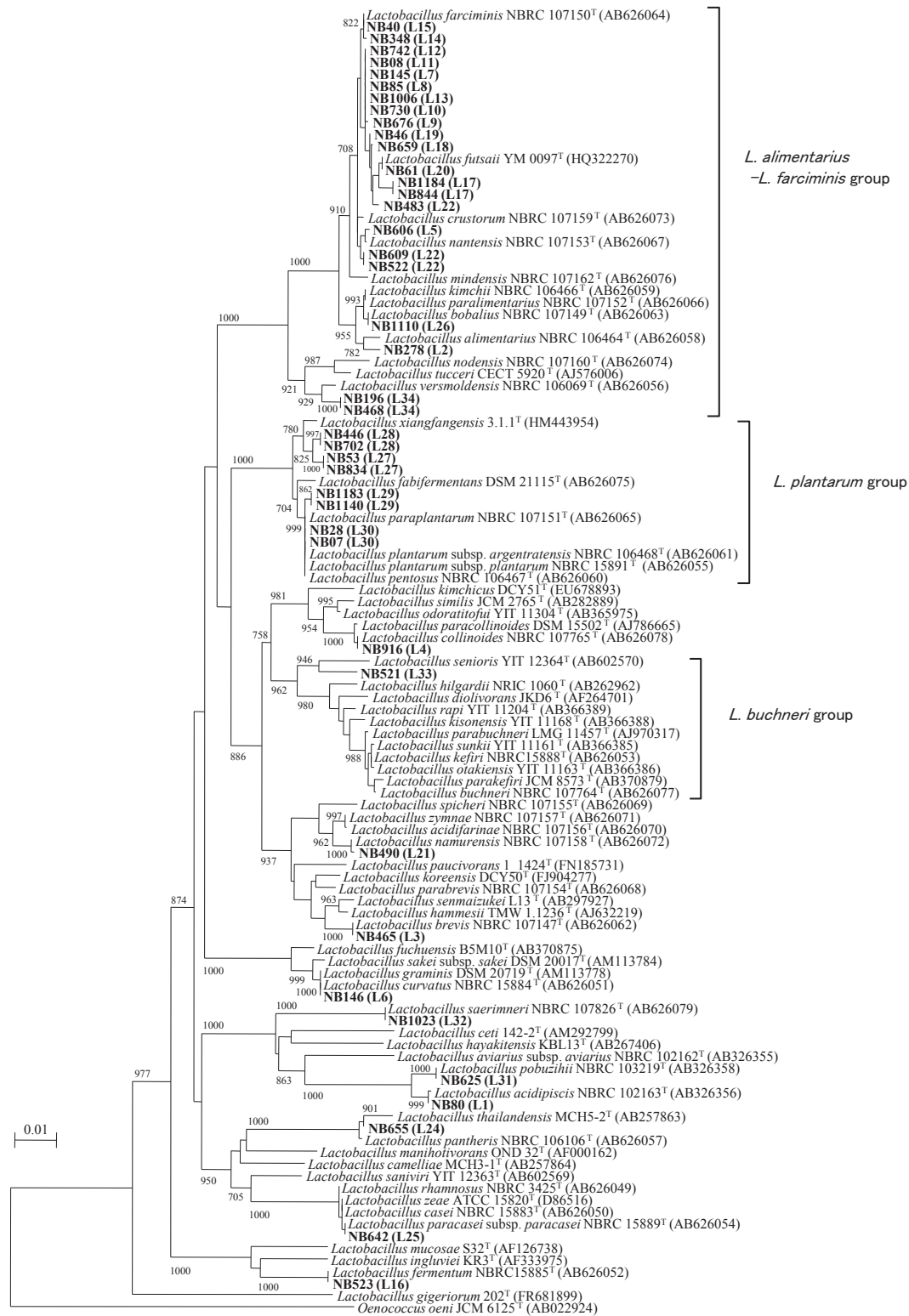


Fig. 1 The neighbor-joining tree showing the phylogenetic positions of the isolates in the genus *Lactobacillus* based on the 16S rRNA gene sequences. Bar, 0.01 K_{nuc}. Bootstrap values higher than 700 are shown in 1,000 replicates.

casei (L25), *L. paralimentarius* (L26), *L. plantarum* group (L29, L30), *L. pobuzihii* (L31), and *L. saerimneri* (L32). According to Stackebrandt and Ebers (2006), if the similarity rate between the 16S rRNA gene sequences of two organisms is lower than 98.7–99.0%, they belong to separate species. We found that eight groups that included 12 strains showed rates of similarity to known species lower than 99.0%, as will be discussed in the rest of the paragraph. Based on the system of classification of Felis and Dellaglio (2007), five of the eight groups (groups L2, L5, L17, L22, and L34) belonged to *L. alimentarius*–*L. farciminis* group, two (L28 and L37) to *L. plantarum* group, and one (L33) to *L. buchneri* group (Fig. 1). Isolates NB278 (group L2), NB606 (group L5), NB844 and NB1184 (group L17), NB483 (group L22), and NB196 and NB468 (group L34) were closely related to the following species based on the similarity of their 16S rRNA gene sequences: *L. alimentarius* (98.5%), *L. crustorum* (98.7%), *L. futsaii* (98.8%), *L. nantensis* (98.9%), and *L. versmoldensis* (99.0%), respectively. The 16S rRNA gene sequences of the strains NB446 and NB702 (group L28) and NB53 and NB834 (group L37) were similar to the sequences of the type strains of the *L. plantarum* group by 98.7% and 98.9%, respectively. The isolate NB521 (group L33) showed a 97.7% 16S rRNA gene sequence similarity to that of *L. senioris*. Because the similarity rates between the 16S rRNA gene sequences of the isolates and those of the strains considered closely related to them were lower than 99.0% (Table 1), the isolates may be classified in new species.

Thirty-eight strains belonging to the genus *Pediococcus* were divided into two species. Twelve strains (group P1) were closely related to *P. acidilactici* and 26 strains (P2 and P3) to *P. pentosaceus*. They showed higher than 99.7% 16S rRNA gene sequence similarity to each type strain.

Four isolates were classified in *Tetragenococcus halophilus*. The similarity rate between the 16S rRNA gene sequences of these isolates and that of the type strain was 99.9%.

Twelve strains belonging to the genus *Weissella* were divided into five species, namely, *W. cibaria*, *W. confusa*, *W. paramesenteroides*, *W. thailandensis*, and *W. viridescens*. The similarity rates between the 16S rRNA gene sequences of the isolates and those of the type strains were higher than 99.8%.

Distribution of lactic acid bacteria in fermented foods in Thailand

We used 26 kinds of 114 fermented foods as sources for isolation. These sources and the number of isolates from each are recorded in Table 2. Most *Enterococcus* strains were isolated from fermented fishes and crustaceans, and only eight of the 46 isolates were from fermented plant materials. *Lactobacillus* strains were found in all the fermented Thai food samples, except in pu-dong (fermented crab) and kapi (fermented shrimp paste). Most strains belonging to *L. acidiphiscis*, *L. farciminis*, and *L. futsaii* were isolated from fermented fish. On the other hand, strains of the *L. fermentum* were mainly isolated from fermented plant materials. The strains of the *L. plantarum* group were widely distributed in fermented Thai foods, having been found in 21 of the 26 kinds of samples. *Pediococcus pentosaceus* strains were isolated from various fermented fishes and crab. *Tetragenococcus halophilus* strains were isolated from pla-ra, pla-jaw (fermented fish), and tuaw jeaw (fermented soybean). Among the 26 kinds of fermented Thai foods, pla-ra, pla-som, pla-jom (fermented fish), and tuaw jeaw contained diverse species. However, most fermented plant materials, except tuaw jeaw, do not contain *Weissella* and *Tetragenococcus* species.

The NaCl concentration of the fermented foods collected from the central and southern parts of the northern Thailand ranged from 0 to 50%. Most fermented plant materials contained low amount of NaCl (lower than 6%). On the other hand, NaCl concentration of all fermented fishes, crustaceans, meat, and soybeans are high (10 to 50%) (Table 2). Relationships between isolated species and NaCl concentration of the 54 samples collected from central and southern parts of the northern region are summarized in Table 3. A lot of fermented fishes (16 samples) and fermented shrimp (1), crab (1), and soy beans (5) contained more than 22% NaCl. We found various lactic acid bacteria species in these high NaCl concentration sources (Table 3). Most of them were isolated from the medium without NaCl. These results suggested that they can survive in more than 22% NaCl concentration, even if they cannot grow in such high NaCl concentration. Most of *L. fermentum* were mainly isolated from the fermented plant materials of which NaCl concentrations were less than 6%. *L. plantarum* group were widely distributed in fermented foods, however more than half

Table 3 Relationships between isolated species and NaCl concentration range of the 54 samples collected from the central and southern parts of the northern region

Closest relative	Source					
	0 to 6 ^a		10 to 19 ^a		22 to 50 ^a	
	F ^b n=0	P ^c n=20	F n=9	P n=2	F n=18	P n=5
<i>Aerococcus viridans</i>					1	
<i>Enterococcus avium</i>		1				
<i>Enterococcus faecalis</i>			1		3	
<i>Enterococcus faecium</i>			3	2	4	3
<i>Enterococcus gilvus/raffinosis</i>				1		
<i>Enterococcus hirae</i>		1	2			
<i>Enterococcus thailandicus</i>			1			
<i>Lactobacillus acidipiscis</i>		1	4		10	1
<i>Lactobacillus brevis</i>		9	2		1	
<i>Lactobacillus collinoides</i>		1				
<i>Lactobacillus farciminis</i>		7	17	1	16	2
<i>Lactobacillus fermentum</i>		11	1	2	2	1
<i>Lactobacillus futsaii</i>		2	2		10	
<i>Lactobacillus namurensis</i>			3			1
<i>Lactobacillus nantensis</i>		2				
<i>Lactobacillus pantheris</i>		1				
<i>Lactobacillus paracasei</i>		1			1	
<i>Lactobacillus paralimentarius</i>		1				
<i>Lactobacillus plantarum group</i>		38	10	6	5	
<i>Lactobacillus pobuzihii</i>					1	
<i>Lactobacillus saerimneri</i>		1				
<i>Pediococcus acidilactici</i>		2		2	2	1
<i>Pediococcus pentosaceus</i>		3	3		3	2
<i>Tetragenococcus halophilus</i>					3	1
<i>Weissella confusa</i>						1
<i>Weissella paramesenteroides</i>				1		
<i>Weissella thailandensis</i>			3		1	
<i>Novel species candidates</i>						
<i>Lactobacillus sp.</i> NB606 (L5)		1				
<i>Lactobacillus sp.</i> NB844, NB1184 (L17)			1	1		
<i>Lactobacillus sp.</i> NB702 (L28)			1			
<i>Lactobacillus sp.</i> NB521 (L33)		1				
<i>Lactobacillus sp.</i> NB834 (L37)				1		
Total numbers of isolates	0	84	54	17	63	13

^aNaCl concentration (%) of samples, ^bSamples of fermented fishes, crustaceans, and meat, ^cSamples of fermented plant materials

of them were isolated from the fermented plant materials with low NaCl concentration. It is suggested that these two species have a high association to low NaCl concentration of fermented plant materials. *E. faecalis*, *E. faecium*, *L. acidipiscis*, *L. farciminis*, *L. futsaii*, *P. acidilactici*, *P. pentosaceus*, *T. halophi-*

lus, and *W. thailandensis* were frequently isolated from samples containing more than 10% NaCl (Table 3). It was reported that these species except for the *Enterococcus* species can grow in the presence of around 10% NaCl (Chao *et al.*, 2012; Dicks *et al.*, 2009; Hammes & Hertel, 2009; Holzapfel *et al.*, 2009;

Tanasupawat *et al.*, 2000). Of these species, *E. faecalis*, *E. faecium*, *L. acidipiscis*, *L. farciminis*, *L. futsaii*, and *P. pentosaceus* were mainly isolated from fermented fishes, as described above, but *E. faecium*, *L. farciminis*, *P. acidilactici*, and *P. pentosaceus* were also found in fermented vegetables and soybean to some extent. Cooked rice or roasted ground rice called kao-kouwa were added into most of pla-ra, and some of pla-jaw, pla-jom, and pla-som. Therefore, we cannot conclude that the species mainly found in fermented fishes are associated to fishes. However, because these species tolerate to high NaCl concentration, they were frequently isolated from fermented fishes.

L. plantarum and *L. farciminis* were popular in Thai fermented foods examined, and most of them appeared on the medium without NaCl. Frequency of appearance of these species could be suppressed by using the isolation medium containing high NaCl concentration. For example, four strains of *L. farciminis*, five strains of *L. plantarum* group, and two strains of *Enterococcus* species were isolated from one fermented fish (Pla-jom containing 13% NaCl, pH 4.5) by using the medium with 0 or 5 % NaCl. In contrast, when we apply the medium with 10 or 15% NaCl for the same source, the number of isolates of *L. farciminis* and *L. plantarum* groups is only one, respectively and we could obtain *L. acidipiscis* (1 strain), *P. pentosaceus* (1), *W. thailandensis* (2), and a novel species candidate (1). *T. halophilus* were isolated from sources containing high NaCl concentration (24–44%) with low pH (pH 5–5.5). However, they were isolated only from medium adjusted to pH 10 with various NaCl concentrations (0, 5, or 15%). For isolation of *T. halophilus* known as halophilic and alkaliphilic bacteria, use of alkaline condition seems effective, even if pH of isolation source was acidic. These results suggested that various NaCl concentration and pH should be used to isolate diverse lactic acid bacteria from fermented foods.

As described previously, we isolated 945 strains of lactic acid bacteria from 114 varieties of Thai fermented foods and selected 410 strains for further studies. The isolates were classified into 50 groups of six genera. We found several candidate groups that will be classified in new species. We are investigating their taxonomic characteristics to confirm whether they constitute new taxa. The results of the investigation will be published elsewhere by the

BCC and the NBRC. Some species of lactic acid bacteria, for example, *E. thailandicus*, *L. acidipiscis*, and *Weissella thailandensis*, were first found in fermented foods in Thailand (Tanasupawat *et al.*, 2000, 2008). Because we isolated 32 strains belonging to the three species, distributed in 27 samples of fermented foods (data not shown), these species are considered common organisms in fermented Thai foods and certainly play important roles during fermentation. As a result of this study, isolates were preserved in the BCC, which then deposited them in the NBRC to make available for the public. Accordingly, cooperative researches through culture collections from different countries should be useful in enriching biological resources and in promoting their conservation and sustainable use.

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タイ産発酵食品から分離した乳酸菌の16S rRNA 遺伝子解析

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タイの発酵食品に存在する乳酸菌の多様性を調べるために、タイの東北部、中部、および北部南寄りの地域から114の様々な発酵食品を収集し、945株の乳酸菌を分離した。更なる解析に供するため、決定した16S rRNA 遺伝子配列と分離源情報に基づいて410株を選抜した。16S rRNA 遺伝子配列に基づく解析の結果、分離株は *Aerococcus* 属, *Enterococcus* 属, *Lactobacillus* 属, *Pediococcus* 属, *Tetragenococcus* 属, *Weissella* 属の6属に近縁な50の配列グループに分かれた。*Aerococcus* 属に近縁だった1株は、*A. viridans* の配列と100%の相同性を示した。46株が *Enterococcus* 属に含まれ、それぞれ *E. avium*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. thailandicus*, *E. gilvus*/*E. raffinosus* に近縁な6グループに分かれた。*Lactobacillus* 属に含まれた309株は34の配列グループに分かれた。殆どの分離株は既知種の配列と100%の相同性を示したが、8つのグループに属する12株は99.0%以下のやや低い相同性を示し、新種である可能性が示唆された。*Pediococcus* 属に含まれたのは3グループの38株で、その1つは *P. acidilactici* に、他の2グループは *P. pentosaceus* に近縁だった。4株が *Tetragenococcus halophilus* に近縁だった。*Weissella* 属に含まれた12株は *W. cibaria*, *W. confusa*, *W. paramesenteroides*, *W. thailandensis*, *W. viridescens* の5種のいずれかに近縁だった。この研究で分離した株は、タイのBIOTEC culture collection (BCC) と NITE Biological Resource Center (NBRC) の両コレクションから入手することができる。

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