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Short Communication

Profiles of Plasmids in Lactobacilli Isolated from Fermented Foods

Panitnart Auputinan, Yingmanee Tragoolpua, Sumalee Pruksakorn and Narumol Thongwai*
Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200 Thailand.
*Author for correspondence; e-mail: nthongw@hotmail.com

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ABSTRACT

Biochemical tests were used for identification of 105 isolates of lactobacilli isolated from 24 fermented foods. The dominant species found were *Lactobacillus fermentum, L. plantarum* and *L. brevis*. Plasmids profiles of these isolated lactobacilli were further investigated. Interestingly, only twelve bacterial isolates containing plasmids were detected (11.43 %). Their plasmid patterns were variable with plasmid bands ranging from six to eight. Furthermore, these plasmid harboring lactobacilli were evaluated by 16S rRNA gene sequencing..

Keywords: *Lactobacillus* sp., plasmid DNA, fermented foods, lactic acid.

1. INTRODUCTION

Lactobacilli are members of the lactic acid bacteria, whose primary fermentation end product is lactic acid. They are commercially important bacteria with a wide variety of applications, both in food and non-food industries. Due to their "Generally regarded as safe" (GRAS) status, lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics [1].

Most *Lactobacillus* strains harbor one or more indigenous plasmids. These plasmids interfere not only the stability of the recombinant plasmid, but also harbor undesirable traits such as antibiotic resistance. Many cryptic plasmids originating from *Lactobacillus* have been isolated, characterized and used for construction of cloning vectors or for heterologous gene expression in lactobacilli.

For the last 20 years, different aspects of *Lactobacillus* plasmids have been studied. Nowadays, with the development of molecular tools and genetic characterization, novel species of lactobacilli were discovered as well as novel native plasmids. *Lactobacillus* plasmids were first isolated from *Lactobacillus casei* [2] and were detected mostly in *L. plantarum*, *L. acidophilus, L. casei* and *L. helveticus*. *Lactobacillus* appeared to contain one or more (usually from 1 to 10) different plasmids except for *L. plantarum* LPC25 which had 16 plasmids. The size of plasmids was variable as it was 1.2 kb in *L. plantarum* LL31 and was 169 kb in *L. plantarum* LL2. To this date, many lactobacilli plasmids have been found, but most remain cryptic. However, some functions have been found to be plasmid-encoded that relate to lactose metabolism, antibiotic resistance, bacteriocin production and immunity, DNA restriction or modification (R-M), exopolysaccharide production, N-acetyl glucosamine fermentation and cysteine transport [2]. In this study, plasmid diversity, sizes and numbers in

lactobacilli isolated from fermented foods were investigated.

2. MATERIALS AND METHODS

2.1 Isolation of *Lactobacillus* spp.

Fermented foods (1 g) were inoculated into 5 ml of MRS broth (Merck, Germany) containing bromocresol green as a pH indicator. After 24-48 h of incubation at 37°C, only samples that changed color of media from green to yellow were streaked on MRS agar plate containing bromocresol green and incubated at 37°C for 24-48 h. All Gram-positive rods, catalase negative and non-motile isolates were subcultured to obtain pure cultures. Each pure isolate was maintained at 4 and -20°C for further studies.

2.2 Isolation of Plasmids

The alkaline extraction procedure was modified from a technique described by O'Sullivan and Klaenhammer [3]. Briefly, 10 ml overnight culture was harvested and pellets were resuspened in 25% (w/v) sucrose containing 30 mg/ml lysozyme. After incubation for 1 h at 37°C, cells were lysed with alkaline SDS solution and ice-cold 3M sodium acetate (pH 4.8) was added. After centrifugation, the supernatant were extracted with phenol-chloroform and precipitated by ethanol prior to dissolve plasmid DNA in distilled water. Only plasmid containing isolates were repeatly extracted by a plasmid extraction kit (HiYield™ Plasmid Mini Kit, RBC Bioscience, Taiwan). For DNA detection, agarose gel electrophoresis was performed. Plasmid DNA was maintained at -20°C for further studies.

2.3 Bacterial Identification

2.3.1 Conventional method

All bacterial isolates were evaluated by their macroscopic and microscopic morphologies, and biochemical characterizations e.g. catalase test, motility test, growth at 15 and 45°C, gas production and fermentation of 22 carbohydrates.

2.3.2 Molecular identification by 16s rRNA gene determination

Genomic DNA of each bacterial isolate containing plasmid were extracted by the method of Martin-Platero *et al.* [4] as follows: 1 ml of overnight culture was harvested and resuspened in 100 µl of TES buffer (10% w/v sucrose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mg/ml freshly made lysozyme and 40 µg/ml RNaseA) for 30 min at 37°C. The protoplast cells were immediately lysed by adding 600 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10mM NaCl and 1% w/v SDS) for 15 min at room temperature. The lysates were treated with 10 µl of proteinase K (10 mg/ml) for 15 min at 37°C. After incubation at 80°C for 5 min and cooling down to room temperature for 5-10 min, 200 µl of sodium acetate (3 M, pH 5.2) were added, chilled on ice for 15 min and centrifuged. The supernatant was taken to a new tube and 600 µl of isopropanol were added to precipitate the DNA. Finally, genomic DNA was dissolved in distilled water and maintained at -20°C for further studies.

The PCR of 16S rRNA gene was amplified using the LacbF/LacbR primer pair following the method of Corsetti *et al.* [5]. LacbF primer was 5′-TGCCTAATACAT GCAAGT-3′ while LacbR primer was 5′-CTT GTTAC GACTTCACCC-3′.

PCR products (1,500 bp) were separated by gel electrophoresis and were sequenced (Macrogen, Korea). The identities of nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis with the NCBI database (http://www.ncbi.nlm.nih.gov). Sequences were aligned with representative *Lactobacillus* spp. 16S rRNA gene sequences and phylogenetic tree was constructed using the Molecular Evolution Genetics Analysis (MEGA) software version 4.0.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of *Lactobacillus* spp.

One hundred and five bacterial isolates were obtained from 24 samples of fermented

foods. They were Gram positive, rod-shaped, catalase negative and non-motile. The conventional identification method revealed that there were 38 isolates of *L. fermentum*, 25 isolates of *L. plantarum*, 20 isolates of *L. brevis,* 3 isolates of *L. halotolerans*, 1 isolate of *L. collinoides* and 18 isolates of other lactobacilli (data not shown). Among these lactobacilli, only 12 isolates contained plasmids (Table 1).

Table 1. Isolation of *Lactobacillus* spp.

Species	Origin	Strain	Plasmid containing isolates
L. fermentum	Pickling fish	D10, F22, F23, F24	-
	Nham	E20	
	Pak-kad-dong	F3, F4, F9, F10, X26, X27, X30	
	Nor-mai-dong	F5, F7, F8, F14, F15, F16, F17, X35	
	Tao-jeaw	F19, F21	
	Yogurt	X1, X2, X3, X36, X37, Y5, Y7	
	EM	X4, X6-2, X31, X34, Y1	
	Drinking yogurt	X38, X45	
	Sausages	Y3	
	Kimchi	Y4	
L. plantarum	Nham	A15, F31, F32, F33, F34, F35	A15, F31, F32,
	Pak-kad-dong	D1-2, D2, D5, G7, G8, G9	F33, F34, F35
	Pickling crab	D7-2, D8-2	
	Pla-Ra	D16, D18, T3	
	Miang	G10, G11	
	EM	T1, T8	
	Kimchi	T7, Y8	
	Sausages	Y2, Y6	
L. brevis	Pak-kad-dong	D11, D13, D3-1, D4-1, E6, T14	D11, D13, E6,
	Nor-mai-dong	D20, G16, G17	E36, E37, G20
	Kimchi	E36, E37, Y11, Y12	
	Nham	G20, G25, T2, T13	
	Yogurt	T11, T12, T17	
L. halotolerans	Pla-Som	G3, G4	-
	Nham	G24	
L. collinoides	Sausages	A9	-
Other lactobacilli	Pak-kad-dong	A1, A4, A5, T19	-
	Nham	F30, G22, T16	
	Miang	G12	
	Nor-mai-dong	G15	
	Drinking yogurt	X6-1, X9	
	Kimchi	T4, T5, T15	
	Pla-Ra	T6	
	Yogurt	T9, T10, T18	

3.2 Distribution of plasmids in *Lactobacillus* spp.

Twelve isolates of the 105 *Lactobacillus* spp. were shown to bear plasmids (11.43%). These plasmid bearing isolates were isolated from Nham, Pak-kad-dong and Kimchi. The plasmid containing isolates were further determined their 16S rRNA gene sequencing and conducted the phylogenetic analysis. It was found that isolates A15, F31, F32, F33, F34 and F35 were identified as *L. plantarum* while D11, D13, E6, E36, E37 and G20 were *L. brevis* (Figure 1). Furthermore, the plasmid profiles of these 12 isolates were variable with 6 different patterns (Figure 2). Six isolates of *L. brevis* namely D11, D13, E6, E36, E37 and G20 showed 4 patterns of plasmid profiles. *L. brevis* D11 and D13 had a similar plasmid profile while 6 isolates of *L. plantarum* including A15 and F31-F35 had 2 plasmid profiles, and F31-F35 had a similar profile (Figure 2). Plasmid bands detected were ranging from six to eight bands (data not shown). Due to the fact that plasmid DNA has at least 3 different topological isomeric forms (linear, nicked-circular and supercoiled), therefore, number of bands detected on agarose gel was not correlated with number of plasmids. The sizes of plasmids could not be determined because the marker DNA used was only in linear forms. However, it is interesting that *L. plantarum* A15 showed higher number of plamids, up to eight different molecules. Furthermore, the species

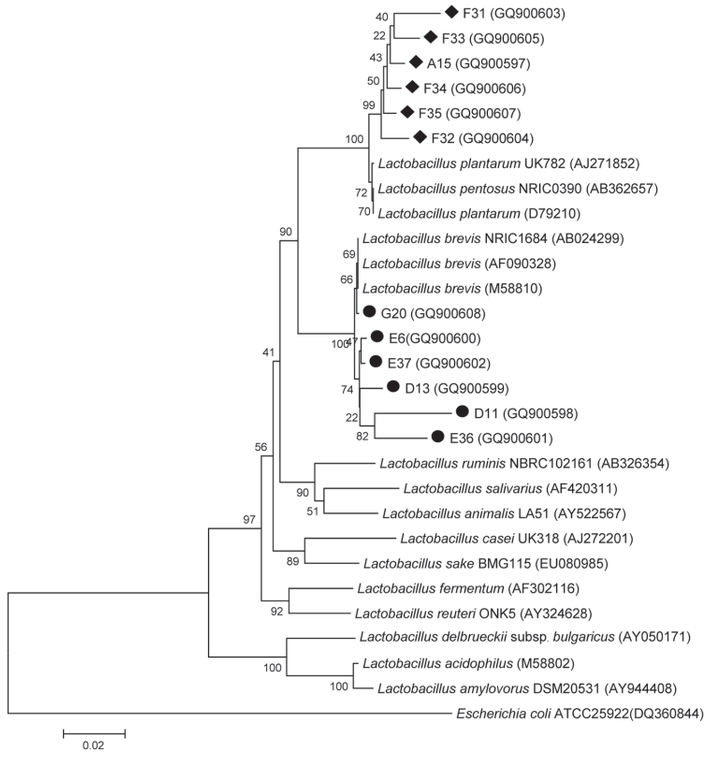

Figure 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of 12 *Lactobacillus* spp. containing plasmids and related strains. The sequence of *Escherichia coli* ATCC25922 was used as an outgroup. Bootstrap values were calculated from 1,000 re-samplings and the bar represents 0.02 showed substitution per nucleotide position. The GenBank accession numbers were in parentheses.

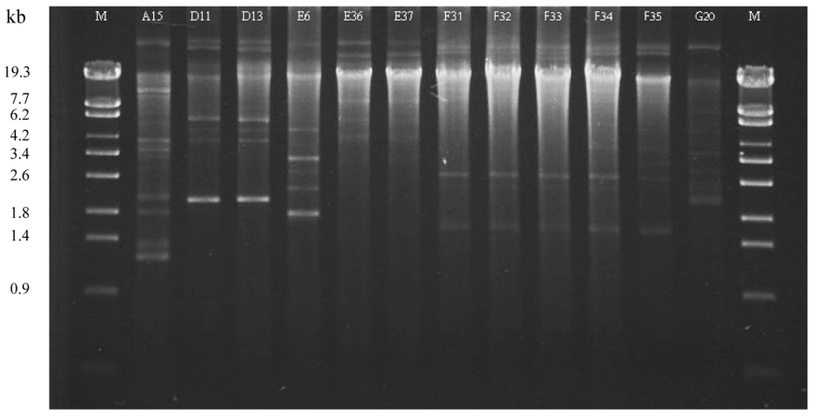

Figure 2. Plasmid profiles of *Lactobacillus* spp.[a]; M = Marker lDNA/*Eco*130I (*Sty*I).

[a] A15: *L. plantarum* A15; F31: *L. plantarum* F31; F32: *L. plantarum* F32; F33: *L. plantarum* F33; F34: *L. plantarun* F34; F35: *L. plantarum* F35; D11: *L. brevis* D11; D13: *L. brevis* D13; E6: *L. brevis* E6; E36: *L. brevis* E36; E37: *L. brevis* E37; G20: *L. brevis* G20.

of *Lactobacillus* spp. were not correlated to the pattern of plasmid profiles which were not correlated to the source of isolation as each isolate had different plasmid profiles. Thus, it was comfirmed that plasmid profiles could not be useful for identification of lactobacilli.

4. CONCLUSION

Plasmids could be extracted from *Lactobacillus* spp. isolated from fermented foods. However, the results revealed that not all lactobacilli isolated harbored plasmids. For those harboring plasmids, high level of plasmid diversity was presented. These characteristics were not typical to the species. Therefore, further studies on these molecules will permit to understand the role of these cryptic plasmids in lactobacilli including structural and functional properties.

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