การตรวจหาพลาสมิดของ *Lactobacillus* spp. ที่แยกได้จากอาหารหมักดอง
Detection of Plasmids from *Lactobacillus* spp. Isolated from Fermented Foods

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บทคัดย่อ

Lactobacillus spp. จำนวน 105 ไอโซเลทถูกแยกจากอาหารหมักดอง เมื่อนำไปจำแนกชนิดโดยวิธีทางชีวเคมีและการหาลำดับเบสของ 16S rRNA gene พบว่า สปีชีส์เด่นคือ *Lactobacillus fermentum, L. plantarum* และ *L. brevis* เมื่อศึกษาการแพร่กระจายของพลาสมิดใน *Lactobacillus* spp. ทั้งหมดพบว่า มีเพียง 12 ไอโซเลทที่มีพลาสมิด (11.43%) ซึ่งมี plasmid profiles ที่หลากหลาย *Lactobacillus* spp. แต่ละไอโซเลทมีแถบ DNA ของพลาสมิดระหว่าง 6 ถึง 8 แถบ *Lactobacillus* spp. ที่มีพลาสมิดนี้ถูกนำไปทดสอบความไวต่อยาปฏิชีวนะ 12 ชนิด พบว่า ทุกไอโซเลทดื้อต่อ fusidic acid, kanamycin, vancomycin, norfloxacin และไวต่อ rifampicin, ampicillin, erythromycin และ chloramphenicol

Abstract

Biochemical tests and 16s RNA gene sequencing were used for identification of 105 isolates of lactobacilli isolated from fermented foods. The dominant species were *Lactobacillus fermentum, L. plantarum* and *L. brevis.* To investigate the distribution of plasmids in lactobacilli, isolation of plasmids was performed. Twelve bacterial isolates containing plasmids were detected (11.43 %). Plasmid profiles of tested lactobacilli showed variable patterns. Several plasmids could be differentiated on the basis of their numbers and molecular weights. The plasmid bands detected were ranging from six to eight. To characterize these plasmid containing bacterial isolates, an antibiotic susceptibility test was performed with twelve antibiotics. The results showed that all bacterial isolates were resistant to fusidic acid, kanamycin, vancomycin and norfloxacin, and were sensitive to rifampicin, ampicillin, erythromycin and chloramphenicol.

คำสำคัญ: *Lactobacillus* spp., พลาสมิดดีเอ็นเอ, ความไวต่อยาปฏิชีวนะ

Keywords: *Lactobacillus* spp., *plasmid* DNA, *antibiotic susceptibility*

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Introduction

Lactic acid bacteria (LAB) are one of the most industrially important groups of bacteria. These organisms are used in a variety of ways, including food production, health improvement and production of macromolecules, enzymes and metabolites (Pfeiler and Klaenhammer, 2007). LAB are also used as starter cultures for various food ingredients such as preservatives, flavor compounds, gums and thickeners.

Lactobacillus, a member of lactic acid bacteria, is a commercially important bacterium with a wide variety of applications, both in food and non-food industries (Conway et al. 1987; Cebeci and Gurakan, 2003). Due to its "Generally Regarded As Safe" (GRAS) status, *Lactobacillus* has been extensively studied its molecular biology in order to improve the specific beneficial characteristics (Wang and Lee, 1997).

Most *Lactobacillus* strains harbor at least one indigenous plasmid and often more (Pouwels and Leer, 1993). These plasmids may not only interfere with the stability of the recombinant plasmid, but also harbor undesirable traits such as antibiotic resistance (Posno et al., 1991). Many cryptic plasmids originating from *Lactobacillus* have been isolated and characterized (Wang and Lee, 1997). Several of these plasmids have been used for construction of cloning vectors or for heterologous gene expression in lactobacilli (Sudhamani et al., 2008).

For the last 20 years, *Lactobacillus* plasmids have been studied in several domains, for examples, plasmid detection and isolation, investigation of structure, replication, function and stability of plasmids, construction of *Lactobacillus* vectors based on plasmids and development of transformation methods (Wang and Lee, 1997). 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 and modification (R-M), exopolysaccharide production, N-acetyl glucosamine fermentation and cysteine transport (Pouwels and Leer, 1993). Hence, plasmid functions can be divided into four main groups: (1) hydrolysis of proteins, (2) metabolism of carbohydrates, amino acid, and citrate, (3) production of bacteriocins, exopolysaccharides and pigments, and (4) resistance to antibiotics, heavy metals and phages (Wang and Lee, 1997). In this study, profiles and numbers of plasmids extracted from lactobacilli isolated from fermented foods were investigated and antibiotic susceptibility of lactobacilli was performed.

Materials and Methods

Isolation of Lactobacillus *spp.*

Fermented foods (1g) were inoculated into 5 ml of MRS broth (Merck®, Germany) containing bromocresol green as a pH indicator. After 24-48 hours of incubation at 37°C, only samples that change color of media from green to yellow were streaked on MRS agar plate containing bromocresol green and incubated at 37°C for 24-48 hours. Colonies of various shapes were randomly picked from agar plates for further Gram stain evaluation. All Gram-positive rods, catalase negative and non-motile isolates were subcultured to obtain pure cultures.

Isolation of plasmids

For plasmid DNA isolation, the alkaline extraction procedure described by O'Sullivan and Klaenhammer (1993) was used. Only plasmid containing isolates were repeatly extracted by using a plasmid extraction kit (HiYield™ Plasmid Mini Kit, RBC Bioscience, Taiwan). For DNA detection, agarose gel electrophoresis was performed as described by Sambrook et al. (1989).

Antibiotic susceptibility tests

All lactobacilli isolates containing plasmid DNA were studied for their antibiotic susceptibility by using a paper disc diffusion method. Tests were performed with 12 antibiotics (Oxoid®, UK): 10 µg fusidic acid, 5 µg rifampicin, 10 µg ampicillin, 10 µg gentamycin, 30 µg kanamycin, 15 µg erythromycin, 10 µg penicillin G, 30 µg chloramphenicol, 30 µg cephalothin, 30 µg tetracycline, 30 µg vancomycin and 10 µg norfloxacin. Agar plates were inoculated and incubated according to NCCLS guidelines. These guidelines were modified for lactobacilli, where Mueller-Hinton agar was replaced by MRS agar. Inhibition zones were measured using a ruler accurate to a millimeter. The results were expressed as susceptible (S), moderately susceptible (MS) and resistant (R) and interpreted as describe by Charteris et al. (1998).

Bacterial identification

Conventional method

Macroscopic and microscopic morphologies of all bacterial isolates were evaluated. The following biochemical characterizations were tested: catalase test, motility test, growth at 15 and 45 °C, gas production and fermentation of 22 carbohydrates according to Bergey's manual of systematic bacteriology (Kandler and Weiss, 1986).

Molecular identification by 16s rRNA gene determination

Genomic DNA of each bacterial isolate containing plasmids were extracted by the method of Martin-Platero et al. (2007). All genomic DNA were maintained at -20°C for further studies.

LacbF/LacbR primers were used for the PCR of 16S rRNA gene following the method of Corsetti et al. (2004). PCR products (1.5 kb) were separated by agarose gel electrophoresis and partially sequenced (Macrogen, Korea).

Results & Discussions

Isolation and identification of *Lactobacillus* spp.

One hundred and five bacterial isolates were obtained from 24 samples of fermented foods. They were gram positive, rods-shaped, catalase negative and non-motile. The conventional identification method revealed that the dominant species were *Lactobacillus fermentum* (36.19%), *L. plantarum* (23.81%) and *L. brevis* (19.05%) (data not shown). 16S rRNA gene determination was conducted to confirm the results from the conventional method.

Distribution of plasmids in *Lactobacillus* spp.

Among 105 isolated *Lactobacillus* spp., 12 lactobacilli containing plasmids (11.43%) were found. These plasmid containing lactobacilli were *L. plantarum* and *L. brevis* but not *L. fermentum* (data not shown). There were few reports showed existence of plasmids in *L. fermentum* (Ishiwa and Iwata,1980; Fons et al., 1997; Pavlova et al., 2002; Aleshin et al., 2006). Mostly, plasmids were detected in *L. plantarum*, *L. acidophilus*, *L. casei* and *L. helveticus* (Wang and Lee, 1997).

Six different patterns of plasmids among the 12 tested isolated were observed. Six isolates of *L. brevis* namely D11, D13, E6, E36, E37 and G20 showed 4 patterns of plasmid profiles while 6 isolates of *L. plantarum*, A15 and F31-F35 had 2 plasmid profile patterns. Although some isolated lactobacilli showed similar plasmid profiles; D11 and D13, E36 and E37, and F31 to F35 (Figure 1) but their macroscopic morphologies were different. Possibly, they were the same strains.

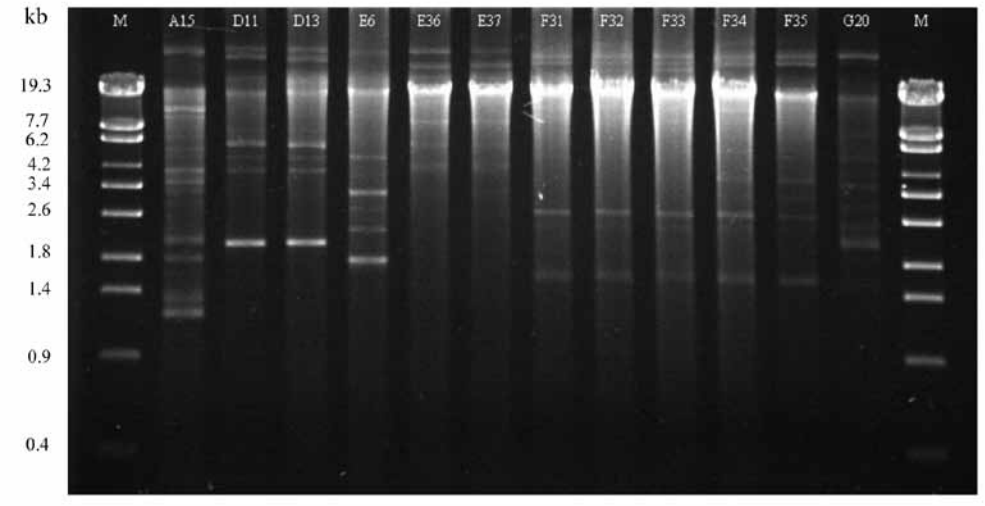

Figure 1. Plasmid profiles of *Lactobacillus* spp.[a]; M = Marker λDNA/*Eco*130I (*Sty*I)

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

The variation of plasmid profiles within species of tested lactobacilli suggested that the use of plasmid profile as an identification tool would not be reliable. In each profile, plasmid bands were ranging from six to eight (Table 1). In accordance with the report of Wang and Lee (1997), *Lactobacillus* sp. appeared to contain one or more (usually from 1 to 10) different plasmids. It is interesting that the *L. plantarum* A15 strain showed higher number of plasmids, up to eight different molecules, as also reported by Ruiz-Barba (1991) that *L. plantarum* had higher number of plasmids among *Lactobacillus* sp.

Table 1. Numbers of plasmids in tested *Lactobacillus* spp.

Strains	Numbers of Plasmids
L. plantarum A15	8
L. plantarum F31	6
L. plantarum F32	6
L. plantarum F33	6
L. plantarum F34	6
L. plantarum F35	6
L. brevis D11	8
L. brevis D13	8
L. brevis E6	7
L. brevis E36	7
L. brevis E37	7
L. brevis G20	8

Antibiotic susceptibility tests

Antibiotic susceptibility of the 12 plasmid-containing *Lactobacillus* spp. was conducted by a disc diffusion method. All bacterial isolates were resistant to fusidic acid, kanamycin, vancomycin and norfloxacin (Table 2). Lactobacilli had been reported to have a high natural resistance to vancomycin, a property that was useful to separate them from other Gram-positive bacteria (Hamilton-Miller and Shah, 1998; Simpson et al., 1988). Danielsen and Wind (2003) reported that some lactobacilli had a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamycin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole and vancomycin, which was also observed in this study. Evidently, antibiotic resistance of pathogenic bacteria had been found on plasmids. More than 10 identified *Lactobacillus* plasmids that confer resistance to antibiotics such as chloramphenicol, erythromycin, kanamycin, streptomycin and tetracycline were reported (Wang and Lee, 1997).

In contrast, all bacterial isolates were sensitive to rifampicin, ampicillin, erythromycin and chloramphenicol. Gentamycin could not arrest growth of tested lactobacilli except *L. brevis* G20 while penicillin G, cephalothin and tetracycline showed several patterns of antibiotic susceptibility profiles. *L. brevis* E6 was the only one isolate that was sensitive to cephalothin and penicillin G. Moreover, *L. brevis* G20 was also the only one isolate that could not tolerate gentamycin. Plasmid profiles of *L. brevis* E6 and G20 shared no similarity with any other isolate in this study (Figure 1). It is suggested that cephalothin, penicillin G and gentamycin resistant genes might locate on plasmid. Antibiotic resistance properties of *Lactobacillus* spp. used in this study might be correlated with those mentioned plasmids. However, plasmid DNA sequencing would be a reliable tool to confirm this hypothesis.

On the other hand, *L. brevis* E36 and E37 showed similar plasmid profile. However, *L. brevis* E36 was resistant to tetracycline while *L. brevis* E37 was moderately susceptible to tetracycline. This result indicated that tetracycline resistant gene might locate on chromosome.

Table 2. Antibiotic susceptibility profiles of tested strains[a]

Antibiotic	*L. plantarum*						*L. brevis*					
	A15	F31	F32	F33	F34	F35	D11	D13	E6	E36	E37	G20
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S
Cephalothin	MS	MS	R	MS	MS	R	MS	MS	S	R	MS	MS
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid	R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	R	R	R	R	R	R	R	R	R	R	S
Kanamycin	R	R	R	R	R	R	R	R	R	R	R	R
Norfloxacin	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin G	R	MS	R	MS	MS	MS	R	R	S	R	R	R
Rifampicin	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	MS	S	S	MS	MS	MS	S	MS	R	MS	S
Vancomycin	R	R	R	R	R	R	R	R	R	R	R	R

R, resistant; MS, moderately susceptible; S, susceptible

[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

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