Chapter 4

Results and discussions

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 among them there were 38 isolates of *Lactobacillus 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 (Appendix E). The most frequently species detected in this study was *L. fermentum*, which is obligately heterofermentative. It was isolated from milk products, sourdough, fermenting plant material, manure, sewage and human mouth and feces. Nowadays, *L. fermentum* is being used as commercial probiotics (Wood and Holzapfel, 1995). *L. plantarum* is homofermentative and commonly found in many fermented food products. It has one of the largest genomes known among the lactic acid bacteria and is a very flexible and versatile species (Wood and Holzapfel, 1995). Meanwhile, *L. brevis* can be found in many different environments and in fermented foods. It is one of the most common causes of beer spoilage because its major metabolites are lactic acid and ethanol. *L. halotolerans* can grow in medium containing 12-14% NaCl, therefore, can be isolated from Pla-som and Nham which having high salt concentration. Eighteen isolated of other lactobacilli could not be identified by the conventional methods due to the complexity of the genus *Lactobacillus*.

Therefore, molecular biology techniques were used for further identification of lactobacilli such as 16S rRNA sequencing.

2. Isolation of plasmids

Twelve of the 105 *Lactobacillus* spp. were shown to contain plasmids (11.43%). They were isolated from Nham, Pak-kad-dong and Kimchi (Table 4.1). After the identification of bacterial isolates by conventional method, these isolates were repeatly confirmed by 16S rRNA gene sequence determination. Phylogenetic analysis based on 16S rRNA gene sequencing showed that isolates namely A15, F31, F32, F33, F34 and F35 were *Lactobacillus plantarum* while D11, D13, E6, E36, E37 and G20 were *Lactobacillus brevis* (Figure 4.1, Table 4.2). According to Wang and Lee (1997), plasmids were mostly found in *L. plantarum, L. acidophilus, L. casei* and *L. helveticus*.

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 lactobacilli isolated showed similar plasmid profiles; D11 and D13, E36 and E37, and F31 to F35 (Figure 4.2) but their macroscopic morphologies were different. 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 4.3). Furthermore, the species of *Lactobacillus* spp. were not correlated to the pattern of plasmid profiles and not associated to the source of isolation. Thus, plasmid profiles could not be useful for identification of lactobacilli.

According to the 3 different topological isomeric forms (linear, nicked-circular and supercoiled) of plasmid, the number of bands detected on agarose gel were not correlated with the number and size of plasmids because the standard marker DNA used in this study was linear.

It is interesting that the *L. plantarum* A15 strain showed high copy number of plamids, up to eight different molecules. In accordance with the report by Ruiz-Barba (1991), *L. plantarum* had high copy number of plasmids.

Table 4.1 Summary of 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, F33, F34, F35
	Pak-kad-dong	D1-2, D2, D5, G7, G8, G9	
	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	
	Kimchi	E36, E37, Y11, Y12	E36, E37
	Nham	G20, G25, T2, T13	G20
	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	

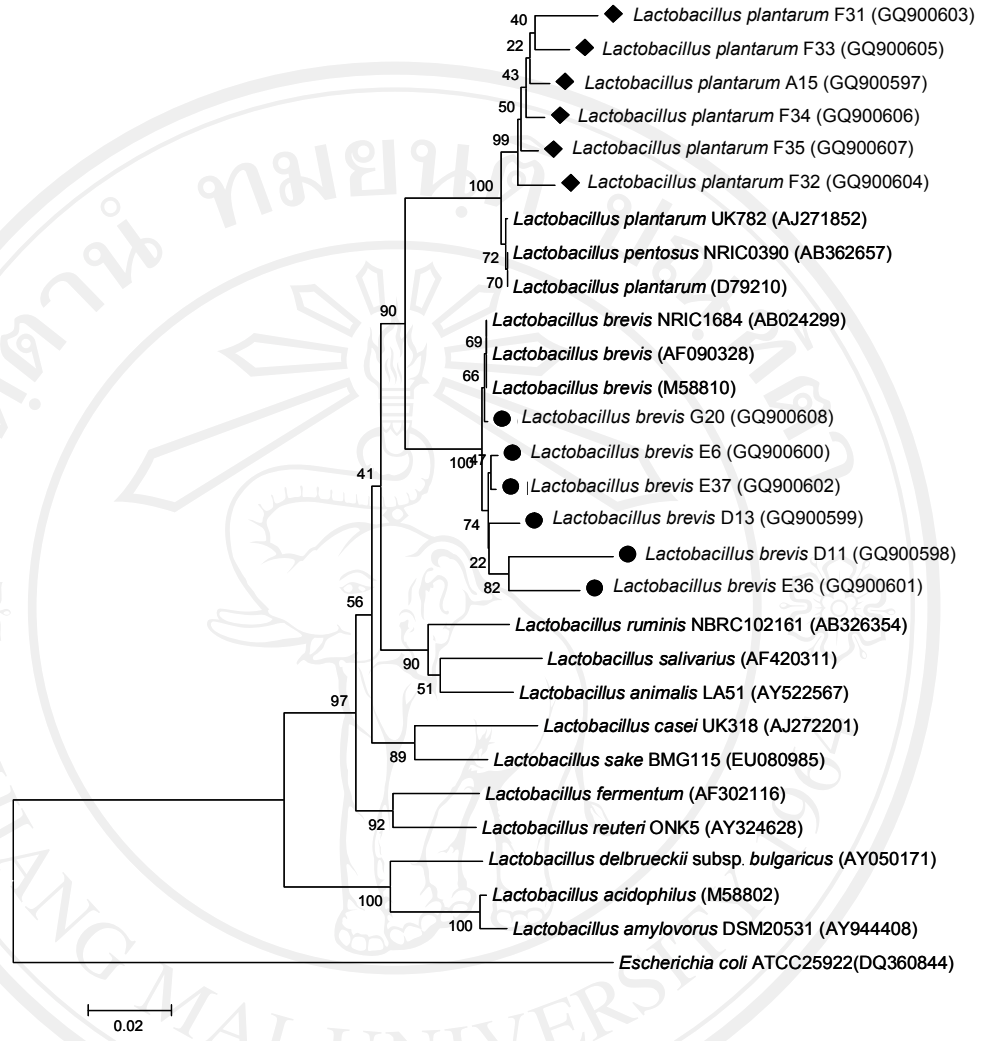

Figure 4.1 Neighbour-joining tree based on 16S rRNA gene sequences showing the position of 12 plasmid containing *Lactobacillus* spp. and related strains. The sequence of *Escherichia coli* ATCC25922 was used as an outgroup. Bootstrap values were calculated from 1,000 re-samplings and the represented 0.02 bar showed substitution per nucleotide position. The accession numbers were in parentheses.

Table 4.2 Blast results of 12 plasmid containing *Lactobacillus* spp.

Isolate	Description	Max Identity	Accession number
A15	*L. plantarum* strain DSPV 354T	98%	FJ751793.1
F31	*L. plantarum* strain DSPV 354T	98%	FJ751793.1
F32	*L. plantarum* strain LP-01	98%	HQ441200.1
F33	*L. plantarum* strain KW30	98%	GU552552
F34	*L. plantarum* strain DSPV 354T	99%	FJ751793.1
F35	*L. plantarum* strain NM174-1	99%	HM218707.1
D11	*L. brevis* strain NM101-1	96%	HM218421.1
D13	*L. brevis* strain SC2A	98%	GU220024.1
E6	*L. brevis* strain KLDS1.0411	99%	HM067023.1
E36	*L. brevis* strain BFE 8325	96%	EU147301.1
E37	*L. brevis* strain KLDS1.0411	99%	HM067023.1
G20	*L. brevis* strain IMAU80083	99%	GU125505.1

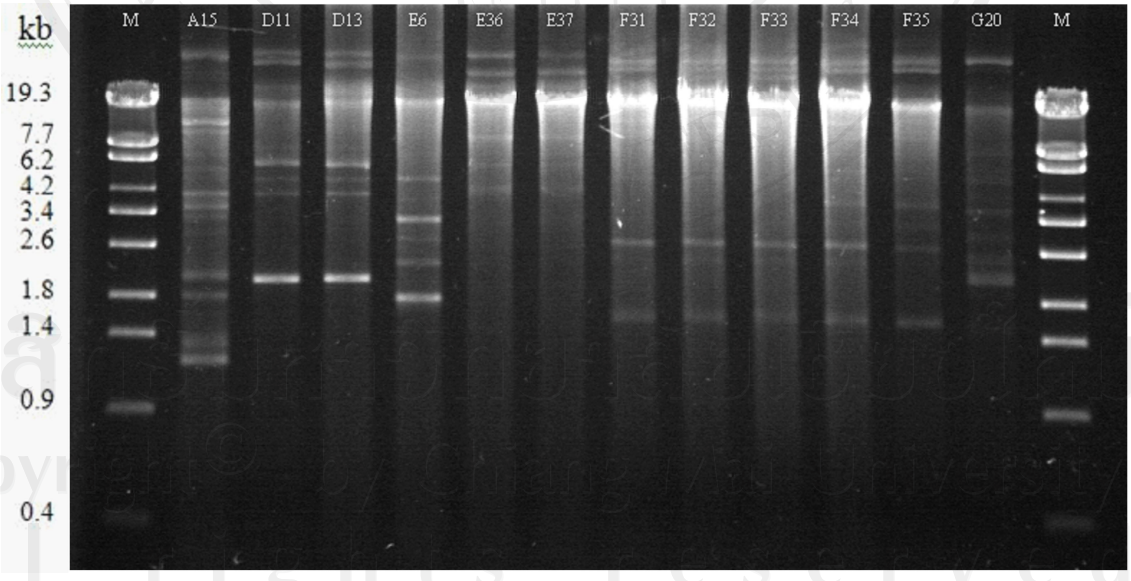

Figure 4.2 Plasmid profiles of *Lactobacillus* spp.[a];
[a] A15: *L. plantarum* A15; D11: *L. brevis* D11; D13: *L. brevis* D13; E6: *L. brevis* E6; E36: *L. brevis* E36; E37: *L. brevis* E37; F31: *L. plantarum* F31; F32: *L. plantarum* F32; F33: *L. plantarum* F33; F34: *L. plantarun* F34; F35: *L. plantarum* F35; G20: *L. brevis* G20; M = λDNA/*Eco*130I (*Sty*I)

Table 4.3 Numbers of plasmid bands in tested *Lactobacillus* spp.

Strains	Number of plasmid bands
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

3. Characterization of plasmid containing *Lactobacillus* spp.

3.1 Antibiotic susceptibility test

Antibiotic susceptibility of the 12 plasmid-containing *Lactobacillus* spp. was conducted by a disc diffusion method. All bacterial isolates were resistant to bacitracin, ciprofloxacin, fusidic acid, kanamycin, nalidixic acid, norfloxacin, streptomycin and vancomycin (Table 4.4, Figure 4.3). 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). 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 of them were sensitive to ampicillin, cefotaxime, chloramphenicol, erythromycin and rifampicin which were grouped in β-Lactam antibiotic, gram-positive spectrum antibiotics and broad spectrum antibiotics, respectively, which was consistent with the report of Zhou *et al*. (2005). Various antibiotic susceptibility profiles were found when tested lactobacilli were measured against cefoxitin, cephalothin, clindamycin, penicillin G, polymyxin B and tetracycline. Moreover, only *L. brevis* G20 was gentamycin sensitive and nitrofurantoin resistant while the other strains showed opposite results. *L. brevis* E6 was the only one isolate that was sensitive to cephalothin and penicillin G. Plasmid profiles of *L. brevis* E6 and G20 shared no similarity with any other isolate in this study (Figure 4.2). 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 bacterial chromosome.

Table 4.4 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
Bacitracin	R	R	R	R	R	R	R	R	R	R	R	R
Cefotaxime	S	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin	R	R	R	R	R	MS	R	MS	R	R	R	R
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
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R
Clindamycin	S	MS	S	MS	MS	S	S	S	S	R	R	MS
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
Nalidixic acid	R	R	R	R	R	R	R	R	R	R	R	R
Nitrofurantoin	S	S	S	S	S	S	S	S	S	S	S	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
Polymyxin B	R	R	R	R	R	R	R	R	MS	MS	MS	R
Rifampicin	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	R	R	R	R	R	R	R	R	R	R	R
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

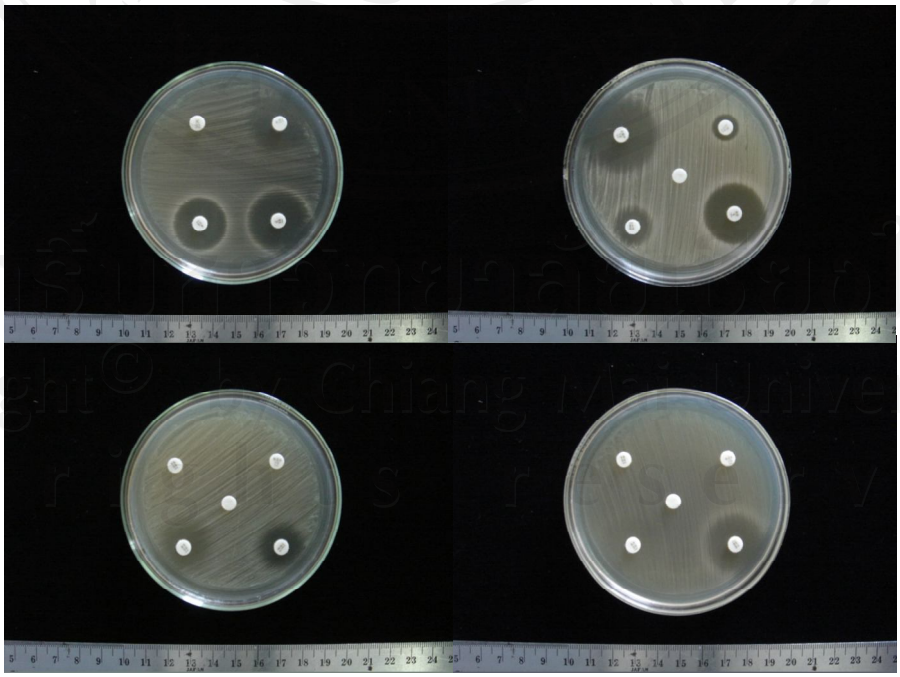

Figure 4.3 The examples of inhibition zones caused by antibiotic susceptibility test

3.2 Bacteriocin production test

Twelve plasmid containing *Lactobacillus* spp. were determined for their bacteriocin production by the well-diffusion technique. After incubation at 37°C for 24-48 hours, the results showed that all tested lactobacilli could inhibit growth of indicator strains except *L. monocytogenes* when used untreated supernatant. For the supernatant that neutralized to pH 6.0 following by ultrafiltration, all tested lactobacilli could not inhibit growth of all indicator strains. The results indicated that the inhibition zone might be caused by the acid in the culture broth, not from bacteriocins.

In contrast with the report from Lash *et al*. (2005), *L. plantarum* cell-free supernatant exhibited an antibacterial effect on a broad range of bacterial species. Cell free supernatant from *L. plantarum* inhibited the Gram-negative bacteria: *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Shigella flexneri* and *Salmonella typhimurium*. In addition, *L. plantarum* supernatant inhibited the Gram-positive bacteria: *Staphylococcus aureus, S. epidermidis, Micrococcus luteus, Listeria innocua* and *Bacillus cereus*.

Many researcher reported that *Lactobacillus* spp. can produce bacteriocins such as *L. pentosus* B96 (Delgado *et al*., 2005), *L. plantarum* C19 (Atrih *et al*., 1993), *L. plantarum* LPCO10 (Jimenez-Diaz *et al*., 1993) and *L. brevis* P319 (Li *et al*., 2008). The bacteriocins produced by LAB have been classified into four groups according to their biochemical characteristics (Klaenhammer, 1993). Small and heat-stable peptides containing thioether amino acids are classified to Class I bacteriocins (lantibiotics) with molecular weight ≤ 5 kDa (Nes *et al*., 1996). Small, heat-stable, non-lantibiotic peptides are Class II bacteriocins with molecular weight ≤ 10 kDa

(Nes and Halo, 2000). Some large, heat-stable proteins belong to Class III bacteriocins with molecular weight ≥ 10 kDa and Class IV which are large, complex bacteriocins containing lipid or carbohydrate groups (Joerger and Klaenhammer, 1990). Class I and II bacteriocins are most likely to be used as bio-preservatives in the food industry to substitute for chemical preservation in all these four groups (Elotmani *et al*., 2002). However, in this study, bacteriocins from plasmid containing *Lactobacillus* spp. could not identified, possibly they had no gene to encode for bacteriocin production.

3.3 Substrates utilization

Twelve isolates of plasmid containing *Lactobacillus* spp. were evaluated their ability to utilize several substrates including soluble starch, CMC, colloidal chitin, inulin, gelatin, citrate, phenylalanine and tryptophan. After measuring pH and turbidity of the culture broth at 660 nm every 24 hours for 3 days, the results showed that all tested *Lactobacillus* spp. could not grow and produce lactic acid when grown in the medium broths mentioned above (Table 4.5, 4.6). However, six *L. plantarum* could grow on all solid media containing each substrate while six *L. brevis* could grow only in media containing CMC, colloidal chitin, inulin, citrate and tryptophan with no change of culture pH. All *Lactobacillus* spp. showed no clear zone on any solid media tested.

Table 4.5 Changes of OD660 of 12 plasmid containing *Lactobacillus* spp. in soluble starch, cmc, colloidal chitin and inulin

Isolate / hr	soluble starch				cmc				colloidal chitin				inulin			
	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
L. plantarum A15	0.000	0.014	-0.008	0.023	0.000	0.014	-0.002	-0.007	0.000	0.021	0.026	0.009	0.000	0.028	0.015	-0.008
L. plantarum F31	0.000	0.022	0.025	0.011	0.000	0.011	0.019	0.020	0.000	0.003	0.014	0.016	0.000	0.013	0.022	0.017
L. plantarum F32	0.000	0.054	0.017	0.022	0.000	0.010	0.012	0.002	0.000	0.032	0.021	0.021	0.000	0.022	0.041	0.019
L. plantarum F33	0.000	0.018	-0.003	0.026	0.000	0.015	0.001	-0.010	0.000	0.024	0.029	-0.011	0.000	0.011	0.027	0.032
L. plantarum F34	0.000	0.035	0.010	0.034	0.000	0.011	0.004	0.012	0.000	0.019	0.034	0.009	0.000	0.025	0.015	0.001
L. plantarum F35	0.000	0.024	0.007	0.025	0.000	0.015	0.000	-0.009	0.000	0.023	0.029	0.003	0.000	0.027	0.015	0.008
L. brevis D11	0.000	-0.028	0.079	0.058	0.000	0.050	-0.008	0.007	0.000	0.021	0.098	0.020	0.000	0.041	0.049	0.012
L. brevis D13	0.000	0.011	0.023	0.035	0.000	0.034	0.013	0.001	0.000	0.002	0.023	0.017	0.000	0.031	0.023	0.022
L. brevis E6	0.000	0.059	0.019	0.024	0.000	0.020	0.030	0.004	0.000	0.002	0.002	0.003	0.000	0.025	0.055	-0.004
L. brevis E36	0.000	0.046	0.019	0.028	0.000	0.033	0.032	0.024	0.000	0.007	0.016	0.011	0.000	0.022	0.010	0.001
L. brevis E37	0.000	0.043	0.063	0.018	0.000	0.046	0.030	-0.009	0.000	0.055	-0.025	0.036	0.000	0.046	0.062	0.002
L. brevis G20	0.000	0.023	0.083	0.008	0.000	0.046	0.010	0.011	0.000	0.055	-0.014	0.034	0.000	0.045	0.052	-0.007

Table 4.6 Changes of OD660 of 12 plasmid containing *Lactobacillus* spp. in gelatin, citrate, phenylalanine and tryptophan

Isolate / hr	gelatin				citrate				phenylalanine				tryptophan			
	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
L. plantarum A15	0.000	-0.007	0.004	-0.018	0.000	0.025	0.032	0.018	0.000	0.032	0.014	-0.02	0.000	0.032	0.013	-0.003
L. plantarum F31	0.000	0.023	0.031	0.004	0.000	0.002	0.016	0.011	0.000	0.025	0.036	0.014	0.000	0.011	0.023	0.016
L. plantarum F32	0.000	-0.014	0.003	0.024	0.000	0.023	0.054	0.004	0.000	0.004	0.021	0.035	0.000	0.047	0.023	0.019
L. plantarum F33	0.000	0.012	0.003	0.024	0.000	0.027	0.028	0.019	0.000	0.030	0.018	0.012	0.000	0.030	0.012	0.007
L. plantarum F34	0.000	-0.002	0.003	0.017	0.000	0.028	0.026	0.020	0.000	0.018	0.036	0.020	0.000	0.028	0.012	-0.009
L. plantarum F35	0.000	-0.004	0.003	-0.018	0.000	0.026	0.029	0.019	0.000	0.032	0.017	-0.022	0.000	0.030	0.013	0.006
L. brevis D11	0.000	0.068	0.021	-0.015	0.000	0.009	0.009	-0.003	0.000	0.058	-0.001	-0.003	0.000	0.055	-0.007	-0.004
L. brevis D13	0.000	0.057	0.032	0.011	0.000	0.022	0.033	0.005	0.000	0.046	0.003	0.014	0.000	0.044	0.028	0.009
L. brevis E6	0.000	0.030	0.047	-0.010	0.000	0.007	0.027	0.022	0.000	0.035	0.032	0.007	0.000	0.024	0.028	0.007
L. brevis E36	0.000	0.057	0.016	0.034	0.000	0.036	0.024	0.012	0.000	0.012	0.030	0.011	0.000	0.036	0.017	0.004
L. brevis E37	0.000	0.064	0.004	0.046	0.000	0.021	0.012	0.045	0.000	0.088	0.005	-0.001	0.000	0.064	0.032	0.031
L. brevis G20	0.000	0.043	0.018	0.052	0.000	0.006	0.015	0.092	0.000	0.085	0.008	0.001	0.000	0.055	0.034	0.043

Wang and Lee (1997) reported that only few *Lactobacillus* plasmid-encoding functions are known. For the metabolism of carbohydrates, *Lactobacillus* plasmids are known to be associated with lactose, galactose, maltose and sorbitol metabolisms (Liu *et al*., 1988; Ahrne *et al*., 1989; Kanatani *et al*., 1991; Kanatani and Oshimura, 1994; Mayo *et al*., 1994). The carbohydrates used in this study were soluble starch, CMC, colloidal chitin and inulin. All were complex carbohydrates. No report demonstrated that *Lactobacillus* containing plasmid could utilize these complex carbohydrates. In addition, *Lactobacillus*-plasmid is related to amino acid metabolism such as cysteine uptake system (Shay *et al*., 1988) and associated with citrate fermentation (Nakamura *et al*., 1991). However in this study, no *Lactobacillus* spp. can utilize gelatin, phenylalanine, tryptophan and citrate. The results suggested that plasmids of *Lactobacillus* spp. in this study might not encoding genes for enzymes production to utilize substrates mentioned above.

4. Characterization of plasmid

4.1 Digestion of plasmids by restriction enzymes

Plasmids from 12 *Lactobacillus* spp. were digested with restriction enzymes; *Bam*HI, *Eco*RI and *Hin*dIII (Figure 4.4-4.6). To determine plasmid profiles and dendrogram construction, all digested products were analyzed by using SPSS program (Version 16.0, SPSS Inc.). The results were shown in Figure 4.7-4.9.

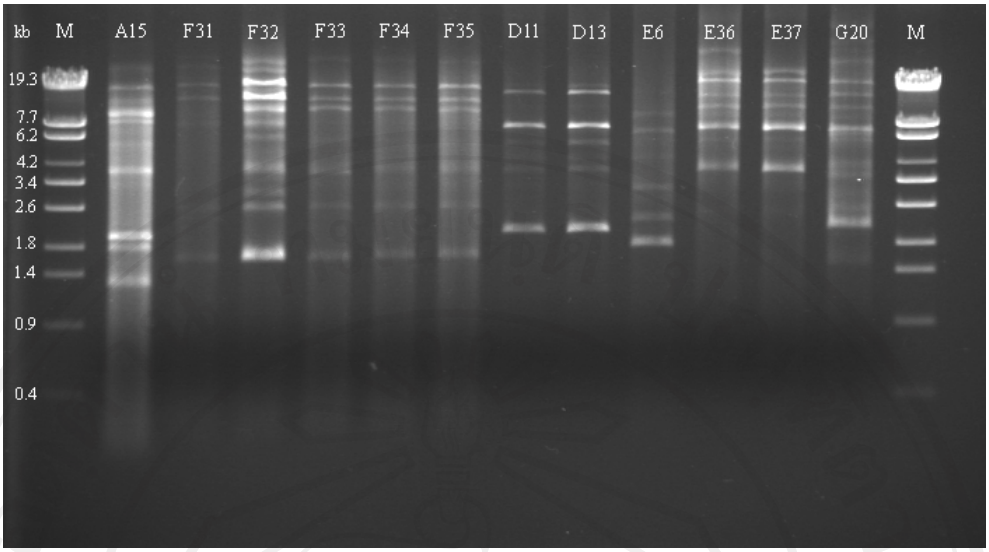

Figure 4.4 *Bam*HI digestion of 12 plasmids isolated from *Lactobacillus* spp. [a]

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; M = λDNA/*Eco*130I (*Sty*I)

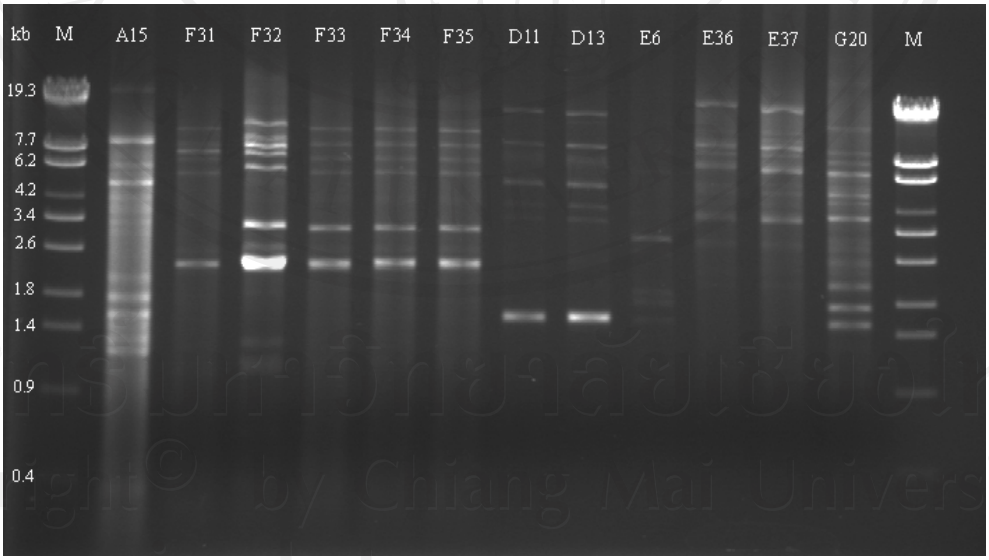

Figure 4.5 *Eco*RI digestion of 12 plasmids isolated from *Lactobacillus* spp. [a]

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; M = λDNA/*Eco*130I (*Sty*I)

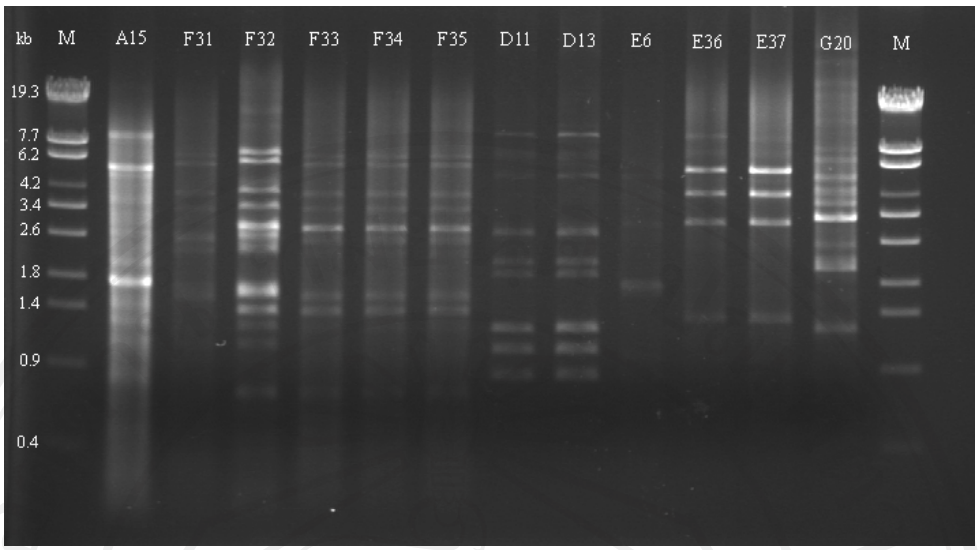

Figure 4.6 *Hin*dIII digestion of 12 plasmids isolated from *Lactobacillus* spp. [a]

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; M = λDNA/*Eco*130I (*Sty*I)

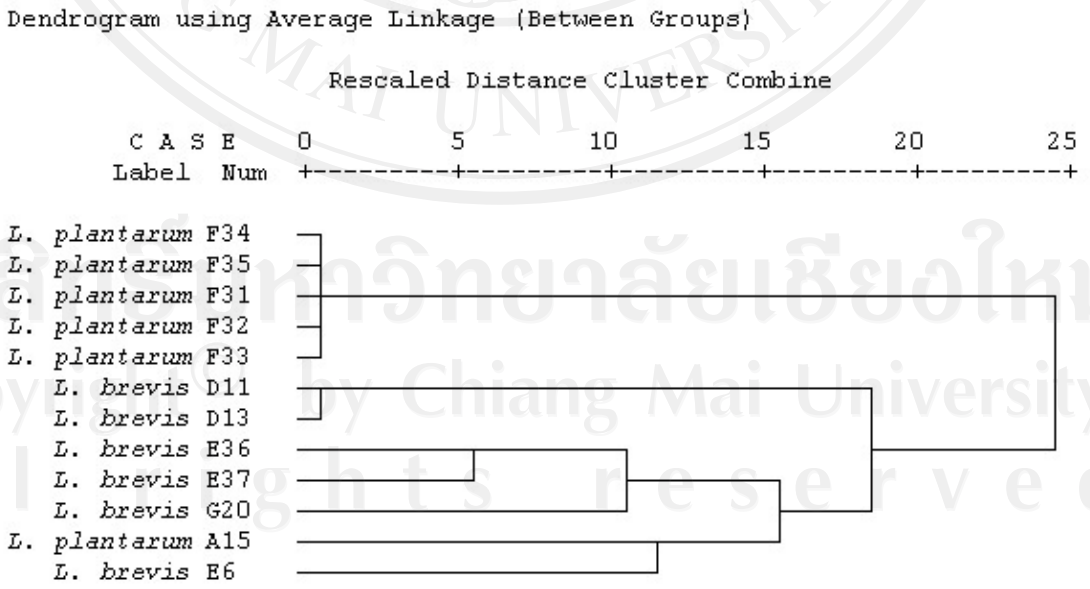

Figure 4.7 Dendrogram of plasmids digested with *Bam*HI

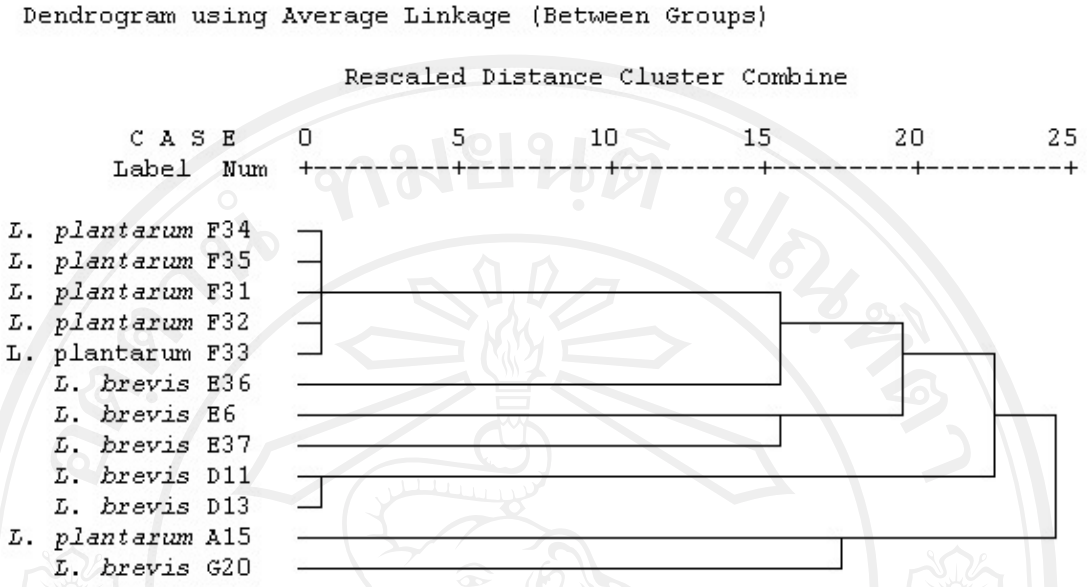

Figure 4.8 Dendrogram of plasmids digested with *Eco*RI

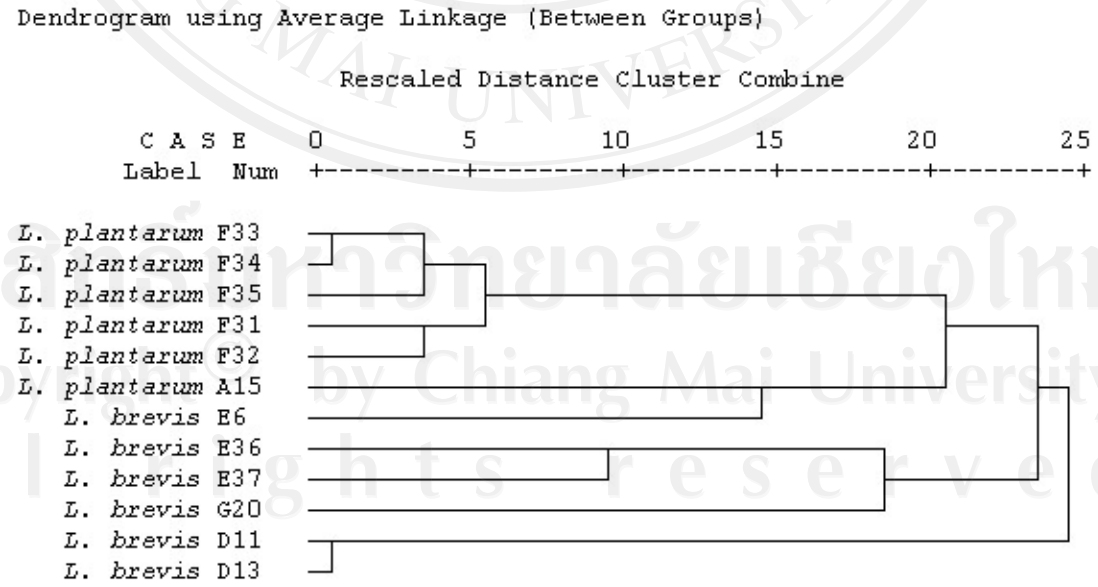

Figure 4.9 Dendrogram of plasmids digested with *Hin*dIII

The dendrogram constructions showed that the species subjected to analyze were not grouped in correct except for *Hin*dIII. This indicated that plasmid profiles were not related to the species in *Lactobacillus* since this study used few isolates of *Lactobacillus* spp. and grouping of the species were dependent on the type of restriction enzymes. However, some *Lactobacillus* species have been identified from the environments by using profiles of *Lactobacillus* plasmids (Wang and Lee, 1997). Many researchers reported the use of probes based on some specific DNA sequences from *Lactobacillus* plasmids to detect *Lactobacillus* spp. from many environments such as silage and digestive tract of animals (Tannock *et al*., 1990; Duffner and O'Connell, 1995). Therefore, plasmid profiles could not be used for identification to species in lactobacilli but can be used for detection *Lactobacillus* spp. in environments.

4.2 Plasmid DNA sequencing

The plasmid pSD11, the smallest fragment of plasmid in *Lactobacillus brevis* D11, was selected to perform DNA sequencing. pSD11 was extracted from agarose gel followed by digestion with *Eco*RI. The 1.7 kb fragment digested from *Eco*RI was cloned into the vector pUC19 (2.6 kb) and was transformed into a cloning host *E. coli* DH5α. The result showed that after purified the pSD11 from agarose gel, two bands were observed. Possibly, due to their different conformation of plasmid (Figure 4.10; Lane 3). In lane 4, when digested the pSD11 with *Eco*RI, there were two bands with 3.2 and 1.7 kb sizes. The 3.2 kb fragment was formed by *Eco*RI that could partially digest the pSD11 at one restriction site. The 1.7 kb fragment was cloned into pUC19 vector. After isolation of recombinant plasmid from white colony, restriction analysis with *Eco*RI was used to confirm the 1.7 kb fragment (Figure 4.10; Lane 6)

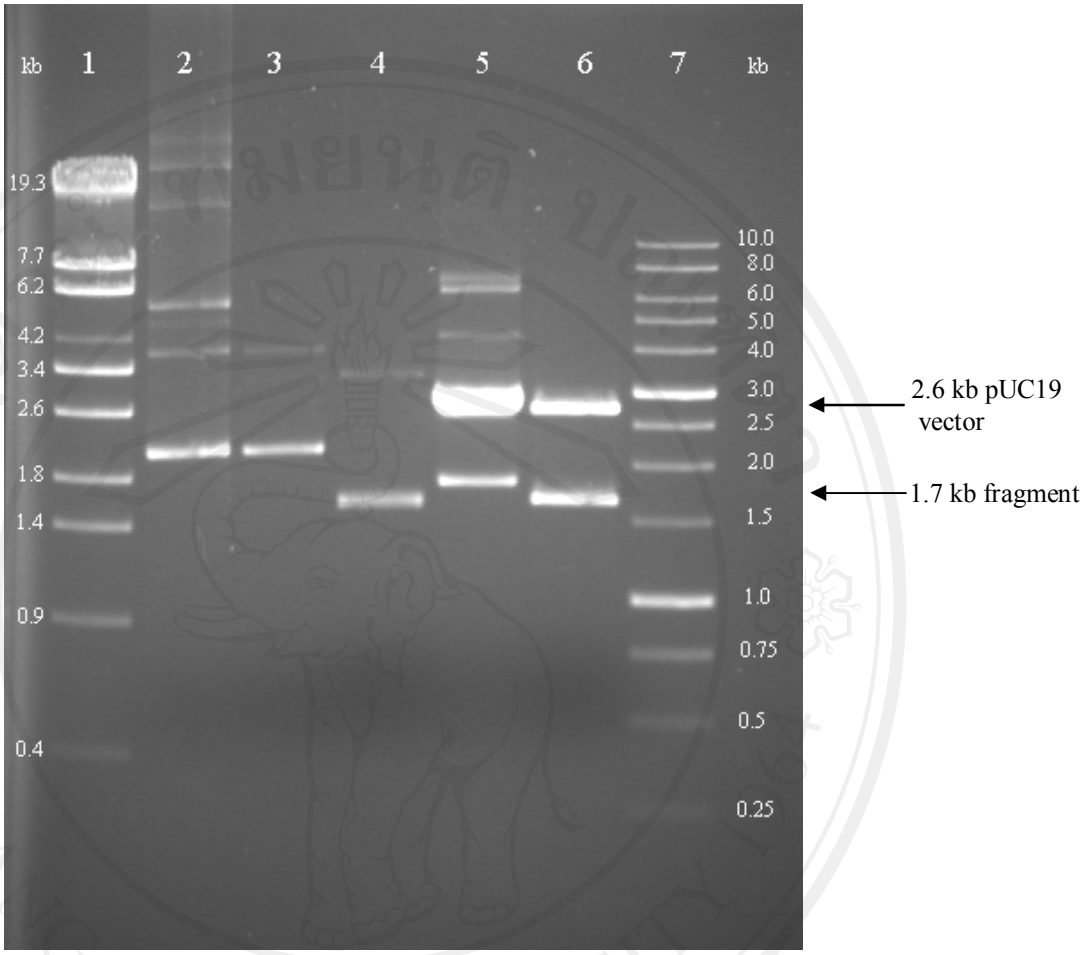

Figure 4.10 Preparation of plasmid pSD11 and cloned into pUC19 vector

Lane 1 = marker λDNA/*Eco*130I (*Sty*I)
Lane 2 = plasmid from *L. brevis* D11
Lane 3 = purified pSD11
Lane 4 = purified pSD11/*Eco*RI
Lane 5 = recombinant plasmid of 1.7 kb fragment
Lane 6 = recombinant plasmid/*Eco*RI
Lane 7 = marker 1 kb ladder

DNA sequence analysis using primer walking indicated that pSD11 was a circular DNA molecule of 3325 bp in length with a G+C content of 38.32 mol%, whereas G+C content of *L. brevis* ATCC 367 chromosome is 46.2% (Makarova *et al*., 2006). Eight putative ORFs with at least 100 nucleotides in length were found in the pSD11 DNA sequence. However, only two ORFs were predicted to encode putative proteins. The two ORFs were in the same orientation and located at positions 714-1340 and 2140-3225, respectively. The two putative proteins encoded by ORF1 and ORF2 were designated as Rep and Mob on the basis of protein sequence similarity (Figure 4.11).

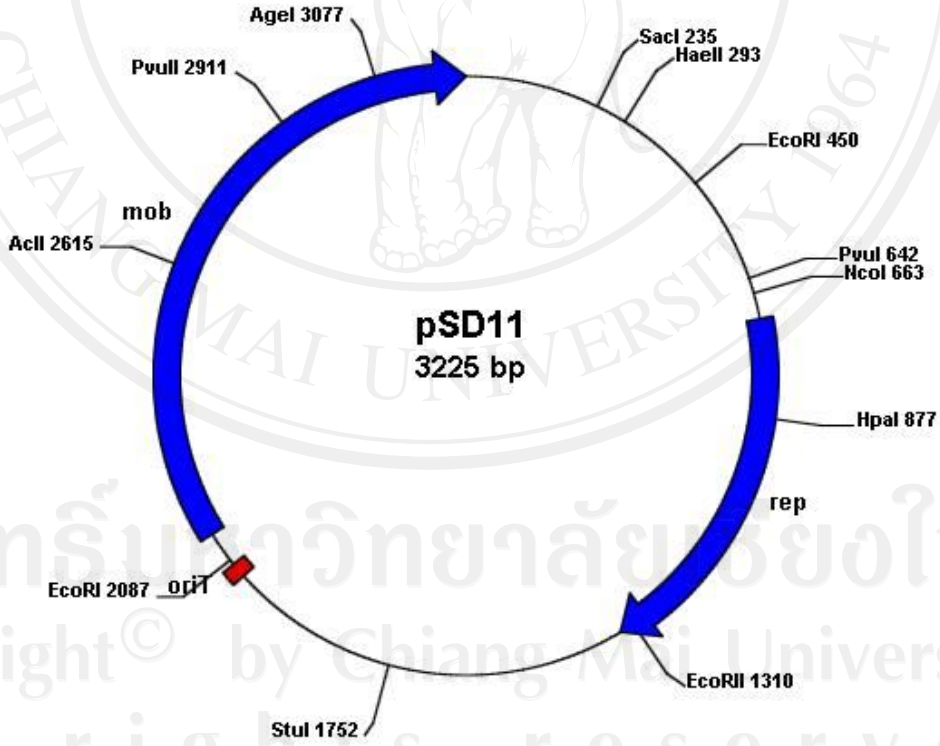

Figure 4.11 Physical map of plasmid pSD11 from *Lactobacillus brevis* D11. Some restriction enzyme sites were shown. Two ORFs were indicated by closed arrows. The position of the putative *oriT* was indicated by box.

ORF1, designated as Rep, encoded a replication initiation protein of 208 amino acids. Sequence analysis showed that ORF1 shared 100%, 98% and 97% similarities with the Rep proteins of plasmids pM4, pF8801 and pWCFS101, respectively, which belonged to the RCR plasmid family (Yin *et al*., 2008; Walling *et al*., 2005; van Kranenburg *et al*., 2005). Therefore, pSD11 was likely to be a member of RCR plasmid.

Based on similarity of the Rep protein and *dso* sequence, plasmid was classified into at least five families: pT181, pE194/pLS1, pC194/pUB110, pSN2 and pIJ101/pJVI (Khan, 1997). Yin *et al.* (2008) reported that sequence analysis also revealed the presence of three highly conserved motifs in pC194/pUB110 family including motif I: FLTLTVE/KN, motif II: HH/QHMHVLLF and motif III: TAKYQVKSKD, which also observed in pSD11 Rep protein (Figure 4.12). Motif III included the putative active Tyr residue which possessed nicking-closing activity necessary for DNA ligation (Ilyina and Koonin, 1992; Khan, 2005). However, a typical *dso* was not observed in this sequence. Therefore, pSD11 could be classified in the RCR pC194/pUB110 family, but the plasmid control system has not yet identified (del Solar *et al*., 1998).

The replication of the lagging strand of RCR plasmids initiated from their single strand origin (*sso*), which was generally located a short distance upstream of the double-strand origin (*dso*). As in the case of pSD11, it did not contain a typical *sso*, but had several inverted repeats designated as IR1 and IR2, from which it was possible to generate stem-loop structures that were significant for the lagging-strand initiation (Figure 4.13).

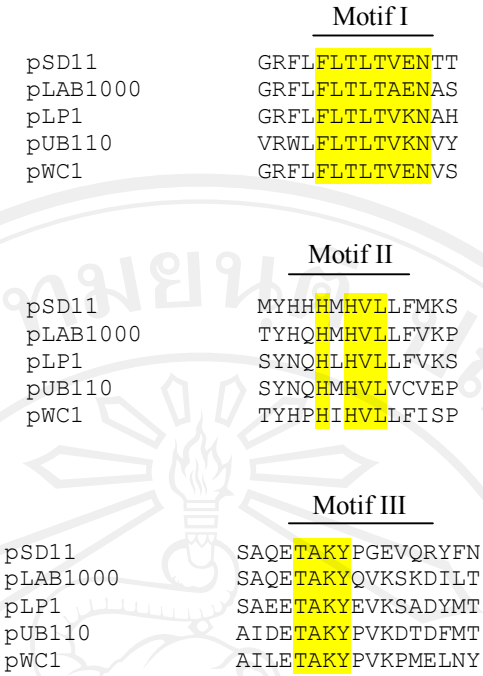

Figure 4.12 Multiple sequence alignment of the amino acid sequences of Rep protein. Conserved motifs (I, II, III) detected in Rep proteins of the pC194/pUB110 group were highlighted.

Figure 4.13 Detailed DNA sequence of *rep* gene from pSD11. Inverted repeats (IR) were indicated by small horizontal arrows, shade boxes and marked with arabic numerals. The Rep protein sequence was shown.

```
 361  CATTTTAAAAAACCGGGATAAACCCGTGACCCAACTGGGCTTAGGCGTATTATGAGTTTA

 421  TAAAATGAATAAAGAAAAAACCCACGTGAGAATTCCTAGTTTGGCGACCCGGAACACGTG

 481  AGTTATCTTGAATATTCGTATTTACTAGACATAGTTTAAAGCTTGAGTTAGCAAGCGTCA

 541  AGCCCTTGGCTTTAGTAAATACATAAAAGATTAGCTCTTCTCACGTGGCTGAATGAGGGG

 600  AGCTTTTTAGTTTGGCTGATAGAAAAGTTTTAGTTGATCGATCGCAGTCGGGCAAAGTAC

 661  GACCATGGCGAGAACATAAGTTAGAAAATTTACAGTATGGTGATTATTTACAAATGTTGC
                                                        M   L

 721  ACTACAAGAAAGCCCATCGAGTTAAAGAGTGTGGTGAAGTATTACGTTTTGTGGAAGATA
       H   Y   K   K   A   H   R   V   K   E   C   G   E   V   L   R   F   V   E   D

 781  AAAATGGTCACAAAAAACTGGCTCAGACTTGGTTTTGCCATTCCCGTTTGTGTCCGTTAT
       K   N   G   H   K   K   L   A   Q   T   W   F   C   H   S   R   L   C   P   L

 841  GTAATTGGCGACGGTCAATGAAACAATCTAACCAGTTAACTCAAATTTTGACAGAAGCAG
       C   N   W   R   R   S   M   K   Q   S   N   Q   L   T   Q   I   L   T   E   A

 901  TTAAACAGCGAAAAACGGGTCGGTTCTTGTTTTTAACATTGACGGTAGAGAATACTACAG
       V   K   Q   R   K   T   G   R   F   L   F   L   T   L   T   V   E   N   T   T

 961  GGGATTTGTTGAAGAGTGAATTACGGCAGATGGGACGAGCTATTGCAAAGATCTTTCAGT
       G   D   L   L   K   S   E   L   R   Q   M   G   R   A   I   A   K   I   F   Q

1021  ATAAAAAAGTGGCTAAAAATTTGTTGGGCTATGTACGTTCAACTGAGGTTACCATTAATC
       Y   K   K   V   A   K   N   L   L   G   Y   V   R   S   T   E   V   T   I   N

1081  ACGAAGCGGATCAGCCGATGTATCACCACCATATGCATGTTTTGCTTTTTATGAAATCTA
       H   E   A   D   Q   P   M   Y   H   H   H   M   H   V   L   L   F   M   K   S

1141  GTTATTTTACAGGAACTGATAATTATATTTCACAAACAGAATGGACTAGATATTGGCAAC
       S   Y   F   T   G   T   D   N   Y   I   S   Q   T   E   W   T   R   Y   W   Q

1201  GAGCGATGAAATTAGCTTATGTGCCGGTTGTGAATGTTGAAGCGGTTAAACCGAATGTGA
       R   A   M   K   L   A   Y   V   P   V   V   N   V   E   A   V   K   P   N   V

1261  AACGCCAGAAAAATTCTTTACTGGCTAGTGCCCAAGAAACGGCTAAGTATCCAGGTGAAG
       K   R   Q   K   N   S   L   L   A   S   A   Q   E   T   A   K   Y   P   G   E

1321  TCCAAAGATATTTTAACTAATAATCAAGAACAAGATTTACAAGTAATTGATGATTTGGAA
       V   Q   R   Y   F   N   *
```

Figure 4.13 Detailed DNA sequence of *rep* gene from pSD11. Inverted repeats (IR) were indicated by small horizontal arrows, shade boxes and marked with arabic numerals. The Rep protein sequence was shown (continued).

A mobilization protein (Mob) of 361 amino acids was encoded by ORF2. Sequence analysis showed that ORF2 exhibited 96%, 95% and 71% similarities with the Mob proteins of plasmids pM4, pLB925A02 and pSMA23, respectively (Yin *et al*., 2008; Wada *et al*., 2009; Sudhamani *et al*., 2008).

Francia *et al*. (2004) proposed a classification scheme for plasmids based on the homology of the mobilization protein and *oriT* region that including MOB_Q family, ColE1-superfamily, pMV158-superfamily and CloDF13 family. Mobilization proteins normally demonstrate relaxase activity and are thus essential in preparation of the plasmid for transfer. Mobilization plasmids usually carry a mobilization gene (*mob*) encoding a specific relaxase and the origin of transfer (*oriT*) (Francia *et al*., 2004).

Three highly conserved motifs (motif I: HxxR, motif II: NY(D/E)L and motif III: HxDExxPHxH) were also detected in plasmid pSD11 (Figure 4.14). In addition, a 25-bp nucleotide sequence (5′-TAAAGTATATTGGGCTATACCTTGC-3′) located at position 2049-2073 upstream of the *mob* gene was found to possess a highly conserved part of the transfer origin sequence (*oriT*) of pMV158-superfamily (Priebe and Lacks, 1989) (Figure 4.15). Furthermore, similarity was revealed between the mobilization protein of pSD11 and those of pM4, pLB925A02 and pSMA23, which are relaxase number of the pMV158-superfamily (Francia *et al*., 2004). Therefore, it had sufficient reason to indicate that pSD11 was a new number of the pMV158-superfamily of mobilizable plasmids.

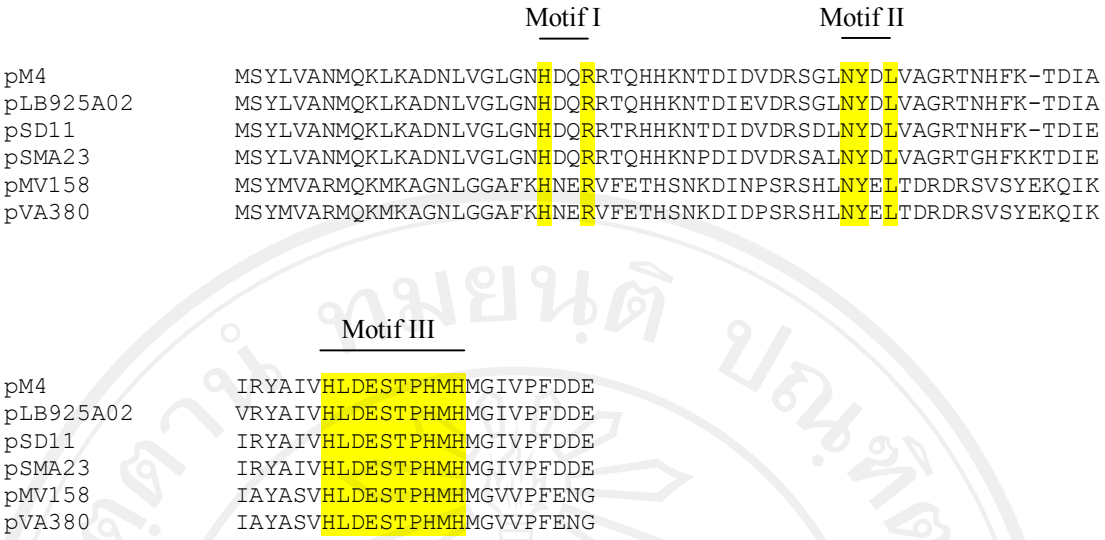

Figure 4.14 Multiple sequence alignment of the amino acid sequences of Mob protein. Conserved motifs (I, II, III) detected in Mob proteins of the pMV158-superfamily were highlighted.

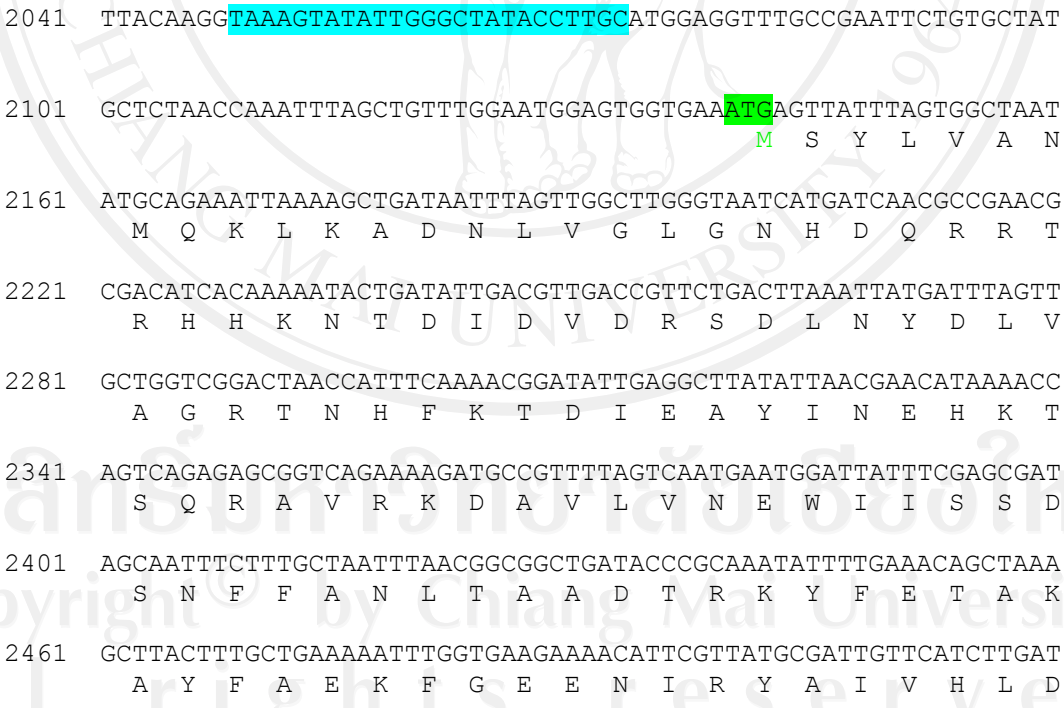

Figure 4.15 Detailed DNA sequence of *mob* gene from pSD11. Origin of transfer (*oriT*) was highlighted. The Mob protein sequence was shown.

```
2521  GAGAGTACTCCCCATATGCATATGGGAATTGTTCCATTTGATGATGAGTATAAATTATCG
        E  S  T  P  H  M  H  M  G  I  V  P  F  D  D  E  Y  K  L  S

2581  GCTAAACGGGTGTTTAATCGTGCGGCTTTGCAAAACGTTCAAGATCAATTGCCAGTTTAT
        A  K  R  V  F  N  R  A  A  L  Q  N  V  Q  D  Q  L  P  V  Y

2641  TTACGACAACGCGGATTTGCTGTTGAACGTGGTATTCAAGAATCGCAACATAAAAGTTTA
        L  R  Q  R  G  F  A  V  E  R  G  I  Q  E  S  Q  H  K  S  L

2701  ACGGTACCAGAATACAAAGCTATGCGGGAAGATTTGAAAAAGGCGACGCTTCAAAAACGA
        T  V  P  E  Y  K  A  M  R  E  D  L  K  K  A  T  L  Q  K  R

2761  GAAATACAAGCTGAACTTGAAGATGCCAGAAAACACCTTGCTGAACTTAAACCTCGTGAT
        E  I  Q  A  E  L  E  D  A  R  K  H  L  A  E  L  K  P  R  D

2821  CAGCAGGAAATTGAGAGCAAACCTACTTTTTTAAGCAAGGATAAAGTGGTTGTTAGAAAA
        Q  Q  E  I  E  S  K  P  T  F  L  S  K  D  K  V  V  V  R  K

2861  AGTGATCTTCATGACTTAGAATCTCGAGCAGCTGTCAGTGATATTTATAATCAACAACAG
        S  D  L  H  D  L  E  S  R  A  A  V  S  D  I  Y  N  Q  Q  Q

2921  AACCGTTTAAAACTTGATAATCAAAGCCTAAATTATCAACTGCTTGAAGTTAAAGACAAT
        N  R  L  K  L  D  N  Q  S  L  N  Y  Q  L  L  E  V  K  D  N

3001  AATTATGATTTAAGCAAGAAAAATGAGAAGCTCCAAAAATTAGTGGATACGTTACAAGGA
        N  Y  D  L  S  K  K  N  E  K  L  Q  K  L  V  D  T  L  Q  G

3061  ATTGTTCGGAGCGTTGACCGGTTCTTACAGCGCAAATTAGGTGTTGGCTTACCAAATGAG
        I  V  R  S  V  D  R  F  L  Q  R  K  L  G  V  G  L  P  N  E

3121  TGGCTAGAGCGAGCTGGACTAAAAGAACCGTCTAAAAAAGCCCCTCAGAGGCCACAAGAG
        W  L  E  R  A  G  L  K  E  P  S  K  K  A  P  Q  R  P  Q  E

3181  CGTTCGGAGGGACAACATGATGAATTAGATGGTCCAAGCCTTTGA
        R  S  E  G  Q  H  D  E  L  D  G  P  S  L  *
```

Figure 4.15 Detailed DNA sequence of *mob* gene from pSD11. Origin of transfer (*oriT*) were highlighted. The Mob protein sequence was shown (continued).

pSD11 was appropriate to construct the vectors including shuttle vectors and expression vectors because pSD11 had the Rep protein that necessary for plasmid replication in lactobacilli. To construct the shuttle vectors, the replicon for *E. coli* from commercial vectors such as pBluescript SK+ was required. In addition, cassettes carrying different antibiotic resistance gene were cloned into the construct to

provide cloning flexibility. For *mob* gene, Sudhamani *et al*. (2008) repoted that the region carrying *mob* was almost entirely deleted during the construction of shuttle vector, indicating that *mob* being non-essential for replication in *Lactobacillus*.