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Isolation, Characterization, and Evaluation of Wild Isolates of *Lactobacillus reuteri* from Pig Feces

Deog Yong Lee[#], Yeon-Soo Seo, Nabin Rayamajhi, Mi Lan Kang, Su In Lee, and Han Sang Yoo[*]

Department of Infectious Diseases, College of Veterinary Medicine, KRF Zoonotic Priority Research Institute and Brain Korea 21 Program for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea
[#]Present Address: Division of Enteric Bacterial Infections, Center for Infectious Diseases, National Institute of Health, Seoul 122-701, Republic of Korea

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Lactic acid bacteria (LAB) are a well-used probiotics for health improvements in both humans and animals. Despite of several benefits, non-host-specific LAB showed poor probiotics effects due to difficulty in colonization and competition with normal flora. Therefore, the feasibility of porcine LAB isolates was evaluated as a probiotics. Ten of 49 *Lactobacillus* spp. isolates harbored 2~10 kb plasmid DNA. Seven strains were selected based on the safety test, such as hemolytic activity, ammonia, indole, and phenylalanine production. After safety test, five strains were selected again by several tests, such as epithelial adherence, antimicrobial activity, tolerance against acid, bile, heat, and cold-drying, and production of acid and hydrogen peroxide. Then, enzyme profiles (ZYM test) and antibiotics resistance were analyzed for further characterization. Five *Lactobacillus reuteri* isolates from pig feces were selected by safety and functional tests. The plasmid DNA which was able to develop vector system was detected in the isolates. Together with these approaches, pig-specific *Lactobacillus* spp. originated from pigs were selected. These strains may be useful tools to develop oral delivery system.

***Keywords*:** probiotics, *Lactobacillus* spp., safety, characteristics, tolerance

Lactic acid bacteria (LAB) are an important probiotic organisms for restoring the intestinal ecosystem (Sandine, 1979). LAB inhibit growth of pathogenic bacteria such as *Listeria monocytogenes* (Sandine, 1979; Ashenafi and Busse, 1991; Aymerich *et al*., 2006), *E. coli*, *Salmonella* spp. (Hudault *et al*., 1997; El-Ziney and Debevere, 1998) and others (Axelsson, 1989; Coconnier *et al*., 1997), due to the production of inhibitory compounds like organic acids, hydrogen peroxide, bacteriocin (Juven, 1992), and reuterin (Axelsson, 1989), and competition for epitheilium adhesion. Especially, the genus *Lactobacillus* is widespread in nature. Several applications were found in the food industry (Amoa-Awua *et al*., 1996; Menendez *et al*., 2000; du Plessis *et al*., 2004) and they are associated with mucosal membranes such as the oral cavity, gastrointestinal tract, and vagina of human and animals (Pryde *et al*., 1999; Mercenier *et al*., 2000; Almstahl and Wikstrom, 2005; Delgado *et al*., 2006). Therefore, we selected *Lactobacillus* spp. as host strains to develop new oral delivery system for pig.

Although LAB used as a probiotics have several benefits for health, they still have problems such as a poor colonization and competition with normal flora. These problems may arise from the use of non-host-specific LAB. Bacteria isolated from a specific environment are not effective probiotics for people of different cultures and regions (Haddadin *et al.*, 2004). A high number of probiotic bacteria have been isolated from feces of individuals taking probiotics. However, the number of fecal probiotic bacteria sharply decreased within one week after the patients stop eating (Berg, 1998), suggesting that commercial probiotic bacteria could not compete with the normal intestinal flora, even though they sometimes can infect their host. Previous data indicate that food-borne probiotic lactobacilli and bifidobacteria are safe for human use (Adams and Marteau, 1995; Reid, 2002). Nevertheless, side effects sometimes have been reported, including rare systemic infections (Rautio *et al*., 1999; Marteau, 2002).

To establish selection guidelines for safe probiotic organisms, a joint FAO/WHO working group recommended a series of tests including metabolic activity, toxin production, hemolytic activity, infectivity in immunocompromised animal models, antibiotics resistance patterns, and adverse incidents in consumers (Organization, 2002). Recent *in vivo* (Makelainen *et al*., 2003) and *in vitro* (Choi *et al*., 2005) studies recommended additional tests to assess the safety of probiotics. These include plasmid transfer, enzyme profile, acute or subacute toxicity of ingestion of extremely large amount of the bacteria, intestinal mucus degradation by infection, and various side effects (Reid *et al*., 2003).

In this study, we integrated general and specific guidelines and applied them to isolate and select porcine *Lactobacillus* spp. from pig feces. Applicability of those strains was evaluated and various tests were demonstrated as effective and safe probiotic candidates for pig.

* To whom correspondence should be addressed.
(Tel) 82-2-880-1263; (Fax) 82-2-874-2738
(E-mail) yoohs@snu.ac.kr

Materials and Methods

Bacterial strains and culture conditions

This study was performed with 49 strains of *Lactobacillus* spp. which were kindly provided by professor Wan-Kyu Lee, ChungBuk National University, Korea. Fecal samples collected from pig farms located in the central Korea. They were diluted and plated on *Lactobacillus* spp. selective media, LBS (Fluka, Schweiz) and non-selective media, BS (Fluka). The plates were anaerobically incubated at 37°C for 48 h. The typical colonies of *Lactobacillus* spp. were picked and anaerobically cultured on Blood Liver (BL) media (Nissui Pharm, Japan) at 37°C for 48 h. These were then identified using aerobic growth, Gram-staining, catalase activity, colony morphology, and cell morphology. Non-LAB controls were also isolated from pig farms and their identities were confirmed by an automated bacterial identification system, Vitek® system (bioMeriuex, Inc., France). The bacterium were stored at -70°C (Table 1). The *Lactobacillus* spp. were anaerobically grown in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) at 30°C and non-LAB controls were grown in LB broth (Difco, USA) at 37°C for 24 h. For long-term storage, the bacterium were kept at -70°C in 10% glycerol. All strains were subcultured twice prior to testing.

Analysis of plasmid DNA

Plasmid DNA in isolated *Lactobacillus* spp. was extracted using the O'sullivan and Klaenhammer's method (1993) after brief modification. The extracted DNA was analyzed by running on a 0.7% agarose gel at 50 V for 4 h.

Identification and genetic characterization

The identification of *Lactobacillus* spp. which were selected by plasmid profiling was performed by phenotypic and genotypic methods. The 16S rRNA sequencing (Flint and Angert, 2005) and API CH50L kit were used for genotypic and phenotypic characterizations, respectively. The 16S rRNA sequence was analyzed using the NCBI in NIH, DNassist, and Megalign program (www.ncbi.nlm.nih.gov).

Random primer # 5; 5'-AACGCGCAAC-3' and primer # 6; 5'-CCCGTCAGCA-3' were used to amplify genomic DNA to confirm genotypic characteristics by random amplified polymorphic DNA. Genomic DNA was prepared using commercial kit (Wizard® Genomic DNA Purification kit, Promega, USA). PCR was performed with two steps. First, the reaction was performed at 4 cycles of 94°C for 5 min, 34°C for 1 min, and 72°C for 5 min. Second, of the reactions were incubated for 30 cycles of 94°C for 1 min, 34°C for 1 min, 72°C for 2 min. Random amplified DNA was analyzed on a 1.5% agarose gel and compared with other strains (Tynkkynen *et al*., 1999).

Safety assessment

Hemolysis and the production of harmful metabolites were evaluated to determine the safety of selected *Lactobacillus* spp. The hemolysis assay was performed by culturing the bacteria on the sheep blood agar containing 5% defibrinated sheep red blood cells. The production of indole, ammonia, and phenylalanine was also assayed for medias compared to control strains and the safety level was determined (Cappucino and Sherman, 1987; Isenberg, 1992; Ishibashi and Yamazaki, 2001).

Functional characteristics

Adhesion assay

Enterocyte-like Caco-2 cells were cultured in 8 well plates (Nunc, Denmark) at 1.5×10^5 cells/well and incubated until a confluent monolayer formed. Cells were washed twice with phosphate-buffered saline (PBS) and 200 μl of α-MEM (Gibco, USA) was added. Plates were incubated for 30 min and then inoculated with 200 μl of *Lactobacillus* spp. at 1×10^5 cells. After 1 h incubation at 37°C, the plates were washed four times with PBS to release unbound bacteria. The Caco-2 cells were then fixed with 200 μl of methanol and stained with 300 μl of Giemsa stain solution (1:20) (Merck) for 30 min. The plates were washed until no color was observed in the washing solution and dried in an incubator overnight. The adherent lactobacilli in 5 random microscopic fields (magnification, ×1000) were counted for each test. Each adhesion assay was performed in duplicate with cells from three successive passages.

Table 1. Bacterial strains used in this study

Type	Purposes			Strains	Sources	Remark
LAB	Probiotic Candidates			*Lactobacillus* spp.	Isolated	49 strains
	Antimicrobial Assay			*E. coli*	SNU[a]	
				S. Typhi	ATCC 19430	
				S. Typhimurium	ATCC 14028	
				P. multocida A	SNU[a]	
Non-LAB	Safety Test	Hemolysis	Positive control	*Streptococcus agalactiae*	SNU[a]	
		Ammonia Production	Positive control	*Proteus vulgaris*	SNU[a]	
			Negative control	*E. coli*	SNU[a]	
		Indole Production	Positive control	*E. coli*	SNU[a]	
			Negative control	*Pseudomas aeruginosa*	SNU[a]	
		Phenyl-alanine Production	Positive control	*Proteus vulgaris*	SNU[a]	
			Negative control	*E. coli*	SNU[a]	

[a] SNU; these strains were isolated from pigs in Korea and stored at the Laboratory of Infectious Diseases, College of Veterinary Medicine, Seoul National University, Korea.

Antimicrobial activity assay

Lactobacilli were cultured overnight and supernatants were harvested by centrifugation. The culture supernatants were serial twofold diluted with LB broth until reaching a 1:64 dilution and seeded into a 96 well plate (Nunc). Pathogens and normal habitant of the gastrointestinal tract were used as indicators for antimicrobial activity. *Salmonella* Typhi (*S*. Typhi, ATCC 19430) and *Salmonella* Typhimurium (*S*. Typhimurium, ATCC 14028) were used as pathogenic indicators and *E. coli*, isolated in our lab, was used as the non-pathogenic indicator. *Pasteurella multocida* (*P. multocida*) type A was also used as comparison for GI and non-GI originating bacteria. Cultured bacteria were inoculated into the 96 well plates containing the serially diluted culture supernatant. These were incubated for 24 h. The optical density of each well was measured every 2 h at 620 nm (GeneQuant *pro*, *Amershampharmacia* biotech, England).

Tolerance to pH and bile acid and acid production

Tests were performed in round-bottom 96 well micro plates (Nunc). Each well was inoculated with late-log phase *Lactobacillus* spp. at a level of 1×10^6 cells/ml in either acidic MRS (pH 2.0, pH 3.0, pH 4.0 adjusted with 1.0 N HCl), MRS containing 0.3% oxgall, and normal MRS. Changes in optical density were measured at 620 nm (GeneQuant *pro*) after 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation at 37°C. Test cells were cultured on MRS agar to monitor survival after 4 h of incubation under the different conditions. Colony formation on MRS agar was assayed after 24 h at 37°C to indicate survival. Culture pH was measured using a pH meter to assay acid production by *Lactobacillus* spp.

Heat and cold-drying tolerance

Lactobacillus spp. were cultured overnight and adjusted 1×10^8 cells/ml. Each *Lactobacillus* spp. was distributed into an eppendorf tube and the tubes were dipped incubated in a water bath for 10 min at 50, 55, 60, 65, 70, 75, and 80°C. One hundred microliter of heated bacteria were plated on MRS media and incubated at 30°C for 24 h. The number of colonies was then counted.

These cultures were mixed with 20% skim milk and freeze-dried. Freeze-dried cultures were stored at 4°C. Survival was assayed every month for six months. Freeze-dried cultures were resuspended in 5 ml of MRS medium, which was equivalent to the culture volume at the point of freeze-drying. Suspensions were then diluted by ten-fold serial dilutions with MRS medium, plated on MRS agar plates, and incubated at 30°C for 24 h. The number of resulting colonies was counted.

Hydrogen peroxide production

Tetramethyl-benzidine (TMB) medium was used to detect

Table 2. Oligonucleotide sequences of primers and PCR conditions for the detection of antibiotics resistant genes

Target genes	Primer pairs	Sequence of primers (5'→3')	PCR conditions and cycles	Size of amplicon (bp)	References
aph(2")-*Id*	GMhl-Id-1 GMhl-Id-2	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	95°C for 1 min, 55°C for 1 min, 72°C for 2 min; 30 Rx.	641	Kastner *et al.* (2006)
erm(B)	ErmB-FW ErmB-RV	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	94°C for 1 min, 55°C for 1 min, 72°C for 2 min; 30 Rx.	405	Gevers *et al.* (2003a)
lnu(A)	lina1 lina2	GGTGGCTGGGGGGTAGATGTATTAACTGG GCTTCTTTTGAAATACATGGTATTTTTCGATC	95°C for 30 sec, 55°C for 30 sec, 60°C for 4 min; 25 Rx.	323	Kastner *et al.* (2006)
*Mef*A	mefAfw mefArv	CTATGACAGCCTCAATGCG ACCGATTCTATCAGCAAAG	95°C for 40 sec, 52°C for 40 sec, 72°C for 2 min; 35 Rx.	1,400	Kastner *et al.* (2006)
*Mef*E	mefE-f mefE-r	ATGGAAAAATACAACAATTGGAAACGA TTATTTTAAATCTAATTTTCTAACCTC	95°C for 40 sec, 52°C for 40 sec, 72°C for 2 min; 35 Rx.	1,191	Kastner *et al.* (2006)
tet(K)	TetK-FW1 TetK-RV2	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAAACTCTTGAAA	95°C for 1 min, 55°C for 1 min, 72°C for 2 min; 30 Rx.	348	Kastner *et al.* (2006)
tet(M)	DI TetM-R	GAYACNCCNGGNCAYRTNGAYTT CACCGAGCAGGGATTTCTCCAC	94°C for 1 min, 55°C for 1 min, 60°C for 2 min; 30 Rx.	1,513	Gevers *et al.* (2003a)
tet(W)	tetW-FW tetW-RV	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	95°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec; 30 Rx.	168	Kastner *et al.* (2006)
*van*A	vanA-F vanA-R	GCAAGTCAGGTGAAGATGG ACCTCGCCAACAACTAACGC	95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; 30 Rx.	393	Rautio *et al.* (1999)
*van*B (ligase)	VANB VANB1	CCCGAATTTCAAATGATTGAAAA CGCCATCCTCCTGCAAAA	95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; 30 Rx.	439	Miele *et al.* (1995)
*van*C1 (ligase)	VANC VANC1	GCTGAAATATGAAGTAATGACCA CGGCATGGTGTTGATTTCGTT	95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; 30 Rx.	831	Miele *et al.* (1995)
*van*E	VANE1 VANE2	TGTGGTATCGGAGCTGCAG GTCGATTCTCGCTAATCC	95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec; 30 Rx.	513	Kastner *et al.* (2006)
*vat*C	vatc1 vatc2	GAAATGGTTGGGAGAAGCATACC CAGCAATCGCGCCCGTTTG	95°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec; 30 Rx.	392	Kastner *et al.* (2006)
*vat*E	satG1 satG2	CTATACCTGACGCAAATGC GGTTCAAATCTTGGTCCG	95°C for 40 sec, 52°C for 40 sec, 72°C for 1 min; 35 Rx.	490	Kastner *et al.* (2006)

hydrogen peroxide-production (Ruiz-Barba *et al*., 1991; du Plessis *et al*., 2004). One hundred ml of MRS agar was prepared with 5.5 g MRS powder and 1.5 g agar. The autoclaved media was maintained at 50°C to add 25 mg TMB and 1 mg horseradish peroxidase. Ten microliter of the solution was poured in each petridish. *Lactobacillus* spp. test stains were inoculated onto the media and anaerobically incubated at 30°C for 24 h or 48 h. The production of hydrogen peroxide was visualized by dark-purple colonies.

Enzyme profile (ZYM test)

The metabolic end products of *Lactobacillus* spp. were assayed with the API ZYM test (bioMeriex, USA). *Lactobacillus* spp. were prepared in API suspension medium with a turbidity of 5~6 McFarland and inoculated onto API ZYM test strips. After inoculation, strips were incubated for 4 h at 37°C. One drop of ZYM reagent A and B were serially added to each strip and incubated until color developed.

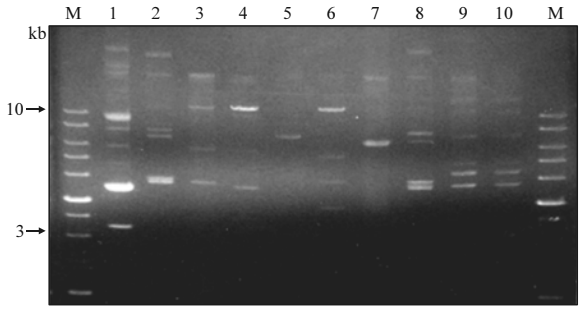

Fig. 1. Electrophoretic analysis of plasmids extracted from *Lactobacillus* spp. isolates. Forty-nine strains of *Lactobacillus* spp. were screened by a modified version of the O'Sullivan and Klaenhammer's plasmid DNA preparation method. Lanes: M, 1 kb DNA marker; 1, L9; 2, L28; 3, L36; 4, L42; 5, L43; 6, L44; 7, L45; 8, L46; 9, L48; 10, L49.

Antibiotics resistance

Resistance to antibiotics was determined by measuring minimum inhibitory concentration (MIC) and detection of antibiotic resistance genes. MIC of probiotic candidates were assayed with fifteen antibiotics using a micro-dilution plate following recommendations of the Clinical and Laboratory Standards Institute (CLSI). Briefly, antibiotics were serially diluted from 0.5 to 500 μg/ml in 96 well plates. One hundred microliter of each *Lactobacillus* spp. at 1×10^5 CFU/ml were inoculated into the diluted antibiotics and cultured

Table 3. Characteristics of selected *Lactobacillus* spp. isolated from pigs

	Characteristics	Selected *Lactobacillus* spp.									
		L9	L28	L36	L42	L43	L44	L45	L46	L48	L49
Safety	Hemolysis	-	-	-	-	+[a]	-	+[a]	-	-	-
	Ammonia production	-	-	-	+[a]	-	-	-	-	-	-
	Indole test	-	-	-	-	-	-	-	-	-	-
	Phenylanine product	-	-	-	-	-	-	-	-	-	-
	Adherent activity (Bacterium/×1000 magnitude)	≥ 100	27±2.6	70±26	NT[e]	NT[e]	14±3.5	NT[e]	19±6.6	18±2	20±16
	Antimicrobial activity										
	E. coli (Isolate)	-	-	-	NT[e]	NT[e]	±	NT[e]	-	-	-
	S. Typhi (ATCC 19430)	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
	S. Typhimurium (ATCC 14028)	+	+[b]	+[b]	NT[e]	NT[e]	+[b]	NT[e]	+[b]	+[b]	+[b]
	P. mutocida typeA	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
Function	pH tolerance										
	pH 2.0	+	+	+	NT[e]	NT[e]	+	NT[e]	-[c]	+	+
	pH 3.0	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
	pH 4.0	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
	Bile tolerance	+	+	+	NT[e]	NT[e]	+	NT[e]	-[c]	+	+
	Heat tolerance	65°C	60°C	60°C	NT[e]	NT[e]	60°C	NT[e]	65°C	60°C	60°C
	Cold-dry tolerance	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
	Acid production	4.03	4.41	4.32	NT[e]	NT[e]	4.06	NT[e]	4.29	4.49	4.51
	Hydrogen production	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
	Aerobic growth	+	+	+	NT[e]	NT[e]	+	NT[e]	+	+	+
Identification[d]	16S rRNA	Lr	Lr	Lr	Lr	Uk	Lr	Ls/p	Lr	Lr	Lr
	API CH50L kit	Lf	Lf	Lf	Lf	Uk	Lf	Ll	Lf	Lf	Lf

[a] The positive strains of the safety test were excluded from candidate probiotics and not tested for further study
[b] *S.* Typhimurium (ATCC 14028) did not grow at 1/4 dilution of culture supernatants
[c] L46 strain was not grown on MRS agar after treatment of pH 2 and 0.3% oxgal media
[d] The strain names were shortened: Lr, *Lactobacillus reuteri*; Uk, unknown; Ls/p, *Lactobacillus salivarius* or *Lactobacillus pentosus*; Lf, *Lactobacillus fermentum*; Ll. *Leuconostoc lactis*
[e] NT means Not Tested

overnight. Aminoglycoside (kanamycin, gentamycin), penicillin (ampicillin, amoxicillin), cephalosporins (cefixitin, cefotaximo), glycopeptide (vancomycin), chloramphenicol (chloramphenicol, florfenicol), macrolide (erythromycin, tylosin), novobiocin, nystatin, and rifampicin were antibiotics used in antibiotics resistance test.

The antibiotic resistance genes *aph*(2")-*I*d, *erm*(B) *tet*(K), *tet*(M) *tet*(W), *lnu*(A), *mef*A, *van*E, *vat*C, and *vat*D were amplified by PCR as previously reported (Gevers *et al*., 2003a; Kastner *et al*., 2006) (Table 2). As previously reported, amplication of *van* A, B, and C confirmed the presence of vancomycin resistant genes in *Lactobacillus* spp. lacking other tested resistance genes (Miele *et al*., 1995; Tynkkynen *et al*., 1998).

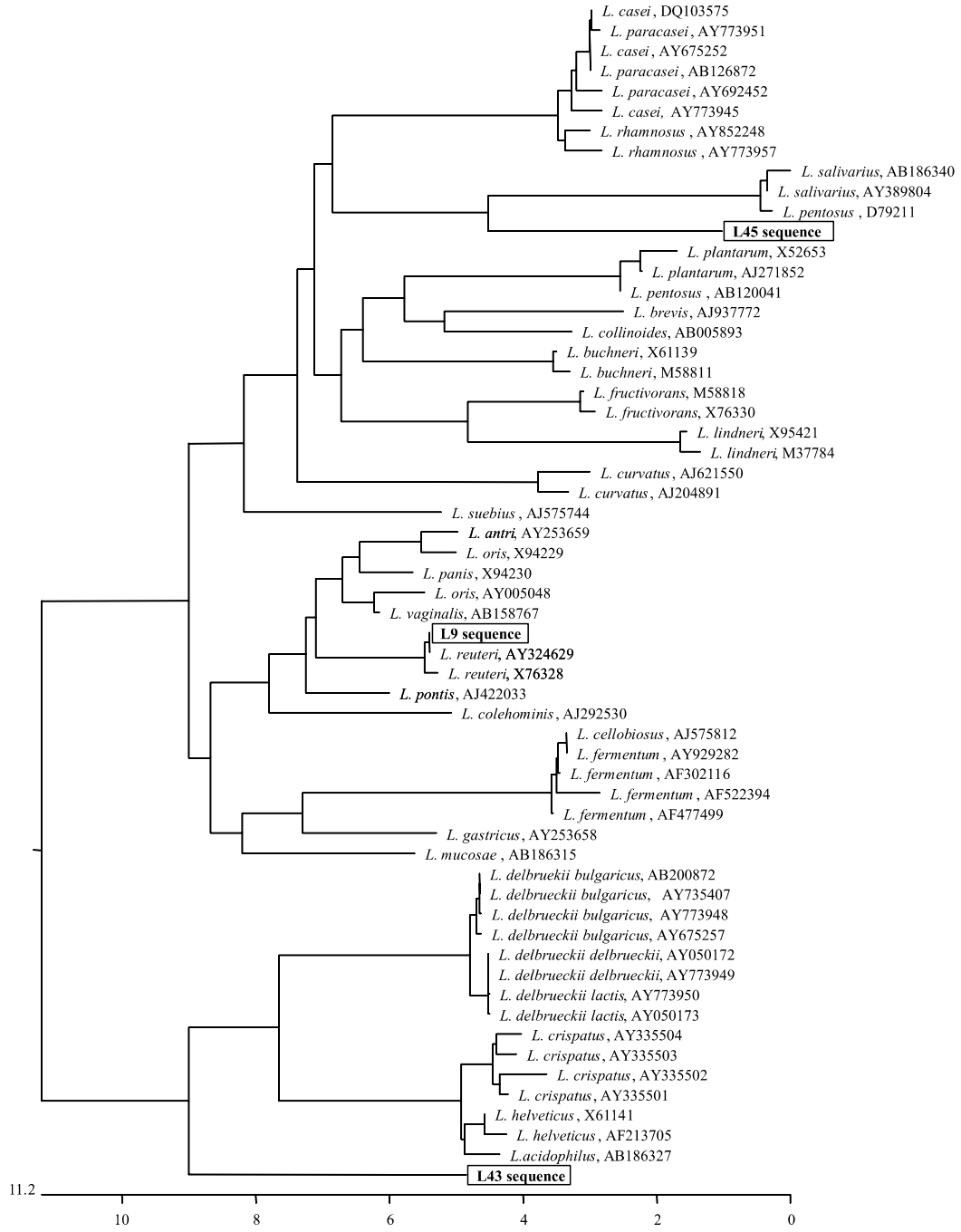

Fig. 2. Phylogenetic analysis of selected *Lactobacillus* spp. by 16S rRNA sequencing. 16S rRNA from 10 LAB strains was amplified and sequenced. The phylogenetic tree was arranged using Megalign program. L9 sequence is representative of eight strains (L9, L28, L36, L42, L44, L46, L48, and L49 strains) and similar to *L. reuteri*. L43 and L45 sequence show different 16S rRNA sequence and may be unknown or unidentified microorganisms.

Results

Plasmid DNA profile

Of the forty-nine strains of *Lactobacillus* spp., ten strains harbored the plasmid DNA ranging from 2 to above 10 kb of size (Fig. 1). The number of plasmid in each strains was various from 1 to 9. More detailed information on the plasmid was described in our previous report (Lee *et al*., 2008).

Identification and genetic characterization identification: API CH50L kit and 16S rRNA sequencing

The identity of ten strains of *Lactobacillus* spp. was analyzed by physiological characteristics using the API CH50L kit. We identified strains L9, L28, L36, L42, L44, L46, L48, and L49 as *Lactobacillus fermentum* and strain L45 as *Leuconostoc lactis*. L43 could not be identified because it had characteristics of both *Lactobacillus fermentum* and *Leuconostoc lactis* (Table 3).

In the sequence analysis of 16S rRNA of ten selected *Lactobacillus* spp., eight strains, which was already identified as *Lactobacillus fermentum*, were had the same 16S rRNA sequence and identified *Lactobacillus reuteri* (*L. reuteri*). The DNA sequence of 16S rRNA of L09 was 99.6% identical to that of *L. reuteri* (AY324629), possessing only 5 nucleotide differences over 1,346 nucleotides compared, and was 99.2% identical to that of *L. reuteri* (X76328), 11 nucleotides differences over 1,346 nucleotides. L43 was identified with *Lactobacillus johnsonii* and L45 was *Lactobacillus murinus* (Table 3).

In the phylogenetic analysis, the nearest strain of L9 was *L. reuteri*, which represented the eight strains and it also neighbored to *L. antri*, *L. oris*, *L. panis*, *L. vaginalis*, and *L. pontis.* L43 did not have the close strains and L45 showed proximity with *L. salivarius* and *L. pentosus* (Fig. 2).

Random amplified polymorphic DNA

Genomic DNA of *Lactobacillus* spp. was amplified with random primers and common bands were shown at 700 bp and 1,400 bp. Eight strains were already identified as the same strains except L43 and L45, but the different amplified band pattern is found shown as the plasmid profile (Fig. 3).

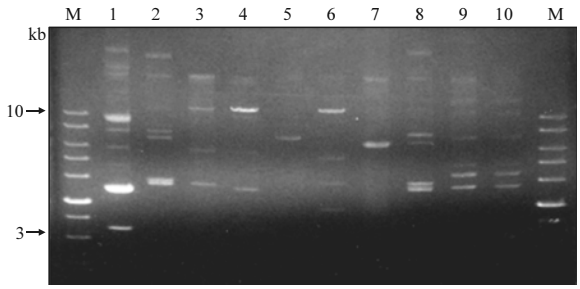

Fig. 3. Random amplified polymorphic DNA analysis of selected *Lactobacillus* spp. isolates. Genomic DNA was amplified using random primers. Lanes: M, marker; 1, L9; 2, L28; 3, L36; 4, L42; 5, L43; 6, L44; 7, L45; 8, L46; 9, L48; 10, L49; 11, W6-14 (reference genes); 12, blank.

Safety assessment

L43 and L45 strains showed weak α-hemolytic activity on sheep blood agar. Only L42 produced ammonia. Indole production and phenylealanine dissolution was not detected on Lactobacilli strains (Table 3). L9, L28, L36, L44, L46, L48, and L49 were selected as they possess no harmful characteristics *in vitro*.

Functional characteristics

Based on adhesion assays, antimicrobial assays, acidic and bile-salt tolerance, heat and cold-drying tolerance, and hydrogen peroxide production, strains L9, L36, L44, L48, and L49 were selected as good probiotic candidates.

Adherent activity; Of the seven candidates, L9 and L36 were strongly adhesive to Caco-2 cells, while the rest were only moderately adhesive (Table 3). However, the adhesiveness of all strains was sufficient for probiotic use, compared with previous study (Jacobsen *et al*., 1999).

Antimicrobial activity; All seven *Lactobacillus* strains had antimicrobial properties, though there were different antimicrobial patterns against the tested bacteria (Table 3). All *Lactobacillus* strains showed no inhibition of the normal inhabitant, *E. coli* and actually mildly induced *E. coli* growth. The growth of pathogens, *S. typhi* and *S.* Typhimurium, were mildly suppressed. *S.* Typhimurium was inhibited at only 1/4 dilution of all *Lactobacillus* strain culture supernatants. The growth of *P. multocida* A was also dramatically inhibited by all *Lactobacillus* strains.

pH and bile tolerance; All *Lactobacillus* strains, except for strain L46, were tolerant to acidic- and bile salt (Table 3). Although growth was suppressed, compared to the positive control, all stains except L46 survived pH 2 and the presence of bile salt.

Heat and cold-dry tolerance, Hydrogen peroxide production; Most *Lactobacillus* strains tolerated 10 min incubation at 60°C. L9 and L46 tolerated temperatures up to 65°C (Table 3).

When testing for cold-dry tolerance, most strains survived until 6 months without a change in cell number, but L28 and L46 numbers sharply decreased after 4 months (Table 3). All tested *Lactobacillus* strains generated hydrogen peroxide, as assayed on TMB medium (Table 3).

Enzyme profile (ZYM test); Enzyme profiles of *Lactobacillus* strains were screened with the API ZYM test (Table 4). All strains had leucine arylamidase, esterase (C4), and acid phosphatase. All strains lacked lipase, trypsin, α-chymotrypsin, α-mannosidase, and α-fucosidase. L28 and L46 lacked esterase lipase (C8) and valine arylamidase. L43 lacked naphthol-AS-BI and β-galactosidase. L44 and L45 lacked α-glucosidase. L44 also lacked alkaline phosphatase, and L9 lacked β-glucuronidase. L43 lacked β-glucosidase and N-acetyl-β-glucosaminidase. Only L43 and L45 had crystine arylamidase.

Antibiotics resistance; LAB, in general, have a high prevalence of antibiotic resistance in order to survive in intestine for preventive antibiotics treatment. In this study, fifteen antibiotics were screened, and high MIC values were shown for kanamycin, vancomycin, erythropmycin, nalidixic acld, tylosin, and nystatin. However, *Lactobacillus* strains were susceptible to penicillin (ampicillin, amoxicillin) and chlor-

Table 4. Enzyme profiles of selected *Lactobacillus* spp. isolated from pigs

Test	Selected *Lactobacillus* spp.										Remark
	L9	L28	L36	L42	L43	L44	L45	L46	L48	L49	
Control	-	-	-	-	-	-	-	-	-	-	
Alkaline phosphatase	-	-	-	-	-	+	(+)	-	-	(+)	
Esterase (C4)	+	+	+	+	+	+	+	+	+	+	
Esterase Lipase (C8)	+	-	+	+	(+)	+	+	-	+	+	
Lipase (C14)	(+)	-	-	-	-	(+)	(+)	-	-	(+)	
Leucine arylamidase	+	+	+	+	+	-	+	+	+	+	
Valine arylamidase	+	-	+	+	+	(+)	(+)	-	+	(+)	
Crystine arylamidase	(+)	-	-	(+)	+	(+)	+	-	+	-	
Trypsin	-	-	-	-	-	-	-	-	-	-	
α-Chymotrypsin	-	-	-	-	-	-	-	-	(+)	-	
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	-	+	+	+	+	+	
α-Galactosidase	+	+	+	+	+	-	+	+	+	+	
β-Galactosidase	+	+	+	+	+	-	+	+	+	+	
β-Glucuronidase	+	-	-	(+)	-	-	-	-	-	-	
α-Glucosidase	+	+	+	+	+	-	-	+	+	+	
β-glucosidase	-	-	-	-	+	-	-	-	-	-	
N-Acetyl-β-glucosaminidase	-	-	-	-	(+)	-	-	-	-	-	
α-Mannosidase	-	-	-	-	-	-	-	-	-	-	
α-Fucosidase	-	-	-	-	-	-	-	-	-	-	

amphenicol (chloramphenico, florfenicol) (Table 5). The *tet* (W) and *lnu*(A) genes were only detected in L36 and L44 but *erm*(B) gene was detected all strains (Table 6).

Discussion

In this study, screened *Lactobacillus* strains contained 2∼10 plasmids from 2∼10 kb of size, regardless the multiplication or state of plasmid DNA. Generally, lactobacilli have 1∼10 plasmids varying from 1.2 to 169 kb (Mayo *et al*., 1989). In the case of *Lactobacillus plantarum* LPC25, 16 plasmids have been identified (Ruiz-Barba *et al*., 1991). Our analysis was similar to previous reports regarding the number and size of plasmids (Mayo *et al*., 1989).

The identified *Lactobacillus* strains had different phenotypic and genotypic phenomena, possibly due to limited phenotypic data-base entries. Variations in phenotype were not consistent with genotype variations. The API CH50L data-base did not contain information for the near *Lactobacillus* spp. such as *L reuteri*, *L. antri*, *L. oris*, *L. panis*, *L. vaginalis*, and *L. pontis.* Therefore, genotyping was critical to identify the bacteria strains.

Strain L9, which was representative of eight strains, was identified as *L. reuteri*, commonly found in the intestines

Table 5. Minimum inhibitory concentrations of selected *Lactobacillus* spp. isolated from pig

Antibiotics		L09	L36	L44	L48	L49
Aminoglycoside	Kanamycin	128	128	128	128	128
	Gentamycin	16	16	32	16	16
Penicillin	Ampicillin	4	4	4	4	4
	Amoxicillin	1	1	1	2	1
Cephalosporins	Cefoxitin	56	56	56	56	56
	Cefotaximo	32	32	32	32	32
Glycopeptide	Vancomycin	≥500	≥500	≥500	≥500	≥500
Chloramphenicol	Chloramphenicol	8	8	8	8	8
	Florfenicol	16	8	16	8	8
Macrolide	Erythromycin	≥500	≥500	≥500	≥500	≥500
	Tylosin	≥500	≥500	≥500	≥500	≥500
Quinolones	Nalidixic acid	≥500	≥500	≥500	≥500	≥500
	Novobiocin	4	2	8	4	4
Nystatin	Nystatin	≥500	≥500	≥500	≥500	≥500
Rifampin	Rifampicin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5

Each value represents the breakpoint of antibiotics (μg/ml)

Table 6. Antibiotic resistant genes of selected *Lactobacillus* spp. isolated from pigs

Genes	*Lactobacillus* spp. strains					Remarks
	L09	L36	L44	L48	L49	
Aph(2")-Id	-	-	-	-	-	Aminoglycoside
erm(B)	+[a]	+[a]	+[a]	+[a]	+[a]	Erythromycin
lnu(A)	-	-	+[a]	-	-	Lincosamide
*Mef*A	-	-	-	-	-	Macrolide efflux
*Mef*E	-	-	-	-	-	
tet(K)	-	-	-	-	-	Tetracycline
tet(M)	-	-	-	-	-	
tet(W)	-	+[a]	-	-	-	
*van*A	-	-	-	-	-	Glycopeptide
*van*B	-	-	-	-	-	
*van*C	-	-	-	-	-	
*van*E	-	-	-	-	-	
*vat*C	-	-	-	-	-	Streptogramin A
*vat*E	-	-	-	-	-	

[a] Resistant genes were detected by PCR

and the mucus layers of pigs and chickens. Neighboring strains in phylogenetic tree were also related by name to mucus layers; these include *L. antri*, *L. oris*, *L. panis*, *L. vaginalis*, and *L. pontis*. The isolated strain of *L. reuteri* may have originated from pig intestine but not the other, similar to commercial probiotics. Thus *L. reuteri* may effectively colonize treated animals and compete with the normal porcine flora (Berg, 1998; Haddadin, 2004).

Strains L28 and L46 grew slowly compared with the other identified strains. Those had no esterase lipase and valine arylamidase in enzyme profiles, and had similar plasmid and RAPD patterns with 300 bp fragments, coincidently. L48 and L49, which were well grown, had similar plasmid profiles, enzyme profiles, and RAPD patterns. L43, which produced ammonia, did not have naphthol-AS-BI and β-galactosidase in enzyme profiles, alone. These characteristics may serve as indicators for the prediction of culture conditions or treatment safety (Tynkkynen *et al*., 1999; Nigatu, 2000; Yeung *et al*., 2002; Yeung *et al*., 2004). L36 and L44 also generated a small band by RAPD analysis and it may show growth failure with small influences, similar to L28 and L46.

The same strains of *Lactobacillus* spp. showed little difference in adherent activity, as previously observed (Chauviere *et al*., 1992; Tuomola and Salminen, 1998). However, the observed variations were somewhat dependent on the culture medium (Sarem *et al*., 1996). In this study, the differences are due to their own characteristics because the experiments were performed in triplicate.

All *Lactobacillus* strains were inhibitory towards pathogens but not towards the normal inhabitant, *E. coli*. Instead, it seemed that the growth of the normal flora was induced by *Lactobacillus* culture supernatants. The test strains may produce antimicrobial compounds, such as reuterin (El-Ziney and Debevere, 1998), which is produced by *L. reuteri*. Reuterin inhibits pathogens like *P. multocida* A, which is a pathogen to respiratory system. However, other factors produced from *L. reuteri* may promote the growth of the normal flora. Thus, *Lactobacillus* spp. may not only have antimicrobial activity as well as modulate intestinal milieu for the maintenance of the intestinal microflora (Jacobsen *et al*., 1999).

All strains of *L. reuteri* showed resistance to kanamycin, vancomycin, erythromycin, nalidixic acid, tylosin, and nystatin. Furthermore, the MIC of these antibiotics exceeded 500 μg/ml, even though resistance genes were not detected except erythromycin resistant gene, *erm*(B). These results are similar with previous reports (Florez *et al*., 2005) which suggest that resistance mechanisms involve effects on cell-permeability (Florez *et al*., 2005), cytochrome-mediated electron transports (Codon, 1983), multi-drug-transporters (Putman *et al*., 2001), and defective cell wall autolytic systems (Kim *et al*., 1982). However, the resistance of *Latobacillus* spp. to vancomycin was due to the presence of D-Ala-D-Lac dipeptide in their peptidoglycans (Klein *et al*., 2000). For these reasons, the extreme antibiotics resistance patterns of *L. reuteri* may result from their structural components. Therefore, general guidelines might consider the adjustment of the MIC break point (Florez *et al*., 2005). And, the presence of resistance genes must also be considered and must be investigated for their transferability, even though the structure of *L. reuteri* regarded as important roles of antibiotic resistance (Table 6).

Although more tests (Isenberg, 1992; Tynkkynen *et al.*, 1998; Ishibashi and Yamazaki, 2001; Gevers *et al*., 2003b) are required to eliminate the possibility that some LAB enzymes may cause disease or illness (Mastromarino *et al*., 1976; Ishibashi and Yamazaki, 2001; Rafii *et al*., 2003), the strains selected from this experiment represent possible and considerable candidates for pig probiotics.

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