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Genomic Fingerprinting of Antituberculosis Agents-Resistant *Lactobacillus ruminus* SPM0211 Using the Microbial Uniprimer™ Kit

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A *Lactobacillus* isolate was collected from the feces of a healthy Korean individual and named as *Lactobacillus ruminus* SPM0211. It was further characterized by subjecting it to an antibiotic resistance test and genetic analysis. In the antibiotic resistance test, all tested *Lactobacillus* spp. were classified as "high resistance" for multiple antibiotics, such as isoniazid, ethambutol, cycloserine, and vancomycin. *L. ruminus* SPM0211 was classified as "high resistance" for streptomycin also, while the other tested *Lactobacillus* spp. were classified as low resistance. This suggests that the antimicrobial spectra may be a good indicator in the discrimination of this strain among the tested *Lactobacillus* spp. In a polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD) analysis using the Microbial Uniprimer kit, *L. ruminus* SPM0211, and *L. suebicus* were clustered as a group with a 74.3% similarity level, suggesting that these two species are genetically related. Thus, our data suggest that the PCR-RADP method using the Microbial Uniprimer kit may be valuable in discriminating *L. ruminus* SPM0211 from other *Lactobacillus* spp.

Key words: Dendrogram, *Lactobacillus*, Streptomycin

INTRODUCTION

Lactic acid bacteria are crucial microorganisms in the bio-industry, for the production of dairy products, such as cheeses and yogurt (Drake *et al.*, 1996). Among those, *Lactobacillus* spp. is found throughout the whole gastrointestinal tract of humans (Klein *et al.*, 1998). It's also widely used as a starter or probiotic in dairy fermentation. The taxa of this genus have gram positive, catalase negative, non-spore-forming and rod-shaped characteristics (Roy *et al.*, 2000).

In this study, a *Lactobacillus ruminus* isolate, which was named as SPM0211, was collected from the feces of a healthy Korean individual. Establishing this isolate's antibiotic resistance profile and genetic background would be essential, in order to utilize it as a probiotic in the food industry. Thus, we evaluated the antibiotic resistance profile of this isolate and subjected it to PCR-RAPD (polymerase chain reaction-random amplified polymorphic DNA) analysis to discriminate it from other *Lactobacillus* spp.

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MATERIALS AND METHODS

Bacteria samples

Seven *Lactobacillus* spp. were registered in this study. Six *Lactobacillus* spp. were purchased from the Korean Collection for Type Culture (KCTC), while *L. ruminus* SPM0211 was isolated from the feces of a healthy Korean individual at Sahmyook University. The seven *Lactobacillus* spp. analyzed in this study are listed in Table I.

Measurement of minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) for the 7 *Lactobacillus* spp. were measured by using the solid medium dilution method according to the guidelines of the NCCLS (National Committee for Clinical Laboratory Standards, 1993). In this study, isoniazid, ethambutol,

Table I. List of *Lactobacillus* spp. and isolates used in this study

Strain	Source
Lactobacillus plantarium KCTC1048	Commercial[1]
Lactobacillus casei KCTC2180	Commercial
Lactobacillus fermentum KCTC3112	Commercial
Lactobacillus suebicus KCTC3549	Commercial
Lactobacillus reuteri KCTC3594	Commercial
Lactobacillus ruminis KCTC3601	Commercial
Lactobacillus ruminus SPM0211	Isolate[2]

Abbreviations: [1]purchased from Korean Collection for Type Culture and [2]isolated from human feces.

rifampicin, streptomycin, cycloserine, clindamycin, ciprofloxacin, and vancomycin were used as antituberculosis and antibiotic agents.

PCR-RAPD analysis

The complete genomic DNA of all 7 *Lactobacillus* spp. was isolated by using the Wizard genomic DNA purification kit (Promega, Co. Ltd., Madison, U.S.A.). Four primers of 20 mer (Microbial Uniprimer™ Kit) were purchased from Seoulin Bioscience Institute, Seoulin Bioscience, Co. Ltd., Seoul, Korea (Patent No. 10-0248906, Table II). PCR reactions were carried out in 30 μL reaction mixtures containing the DNA template (50 to 100 ng of purified DNA), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 200 ng primer, and 2.5 unit *Taq* DNA polymerase (Promega, Co. Ltd., Madison, U.S.A.). The reaction mixture was overlaid with a thin layer of sterile mineral oil to prevent evaporation. DNA amplification was performed in a programmable PTC-200 thermal cycler (MJ research, U.S.A.). The used cycling condition was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C (Kang *et al.*, 2002). After the final cycle, the PCR tubes were incubated at 72°C for 7 min and then kept at 4°C. Amplified PCR products were electrophoresed in a 1.5% agarose gel in TAE buffer and visualized by staining them with ethidium bromide. Amplification reaction was performed twice to establish the reproducibility of this method.

Table II. Four Primers of Microbial Uniprimer Kit

Primers	Sequence (5'-3')
Microbial Uniprimer1	5'-ATCCAAGGTCCGAGACAACC-3'
Microbial Uniprimer2	5'-CCCAGCAACTGATCGCACAC-3'
Microbial Uniprimer3	5'-GTGTGCGATCAGTTGCTGGG-3'
Microbial Uniprimer4	5'-AGGACTCGATAACAGGCTCC-3'

Data analysis

Each PCR product of the 7 *Lactobacillus* spp. was scored for its presence (value = 1) or absence (value = 0). The similarity matrix was calculated by using Nei's method (1972). A dendrogram was constructed based on a similarity matrix, using the unweighed pair-group method for arithmetric mean (UPGMA).

RESULTS AND DISCUSSION

A total of seven *Lactobacillus* spp. were tested for resistance to 8 antibiotics by using the broth microdilution method (Table III). All the tested *Lactobacillus* spp. were highly resistant to isoniazid, ethambutol, cycloserine, and vancomycin. Only *L. plantarium*, *L. casei*, and *L. luminis* were resistant to clindamycin and susceptible to rifampicin and ciprofloxacin. In the case of *L. ruminus* SPM0211, a unique pattern of antitiotics resistance was detected in this study. In *L. ruminus* SPM0211, the MIC for streptomycin exceeded 100 μg/mL, and thus, highly resistant to this antibiotic, while the other tested *Lactobacillus* spp. were not highly resistant. Therefore, the antibiotic resistance profile measured by the solid medium dilution method may provide an useful tool in identifying and characterizing *L. ruminus* SPM0211. Altogether, the results from

Table III. Minimum inhibitory concentrations (MICs) for 7 *Lactobacillus* spp.

Strain	Antibiotics/MICs (mg/mL)							
	INAH[1]	ETM[2]	RIF[3]	SM[4]	CYCS[5]	CLIN[6]	CIF[7]	VAN[8]
L. plantarum	>100	>100	0.4	25	>100	50	1.6	>100
L. casei	>100	>100	0.4	12.5	>100	25	1.6	>100
L. fermentum	>100	>100	0.4	12.5	>100	0.8	12.5	>100
L. suebicus	>100	>100	1.6	25	>100	6.25	25	>100
L. reuteri	>100	>100	0.05	1.6	>100	1.6	12.5	>100
L. ruminis	>100	>100	0.2	12.5	>100	25	1.6	>100
L. ruminus	>100	>100	0.4	>100	>100	12.5	1.6	>100

Abbreviations: [1]Isoniazid, [2]ethambutol, [3]rifampicin, [4]streptomycin, [5]cycloserine, [6]clindamycin, [7]ciprofloxacin, and [8]vancomycin.

the antibiotic resistance test revealed that *L. ruminus* SPM0211 has a wide range of antibiotic resistance. The reason for this multidrug resistance remains unclear, but may in part be explained by previous exposure to various antibiotics in the microflora. Further studies are needed to clarify the acquisition mechanism of multidrug resistance in this strain, because in the bio-industry the antibiotic resistance profile of *Lactobacillus* spp. would be a major consideration.

In determining the genetic background of bacterial species, the PCR-RAPD method has been widely employed, because of its convenience in use and stability against environmental factors (Welsh and McClelland, 1990; Mazurier *et al.,* 1992; McMillin and Muldrow, 1992; MacGowan *et al.,* 1993; Sandery *et al.,* 1994). However, this method requires very low annealing temperatures (about 35~37°C) as well as relatively short primer sequences (about 10 mer). As a result, non-specific bands are frequently formed by PCR reaction and that would lead to low reproducibility (Wu *et al.,* 1991). On the other hand, fingerprinting technique using the Microbial Uniprimer kit can solve this problem by using a relatively long primer and high annealing temperature (Kang *et al.,* 2002). Therefore, we selected this technique as a method for discriminating *L. ruminus* SPM0211 from the other tested *Lactobacillus* spp..

In order to establish the reproducibility of the Microbial Uniprimer, all fingerprinting experiments were performed twice. The repeated experiments produced identical results. Therefore, our results using the Microbial Uniprimer kit indicated very high reproducibility in detecting the genetic polymorphism of *Lactobacillus* spp..

When the six standard *Lactobacillus* spp. from KCTC and an isolate from *L. ruminus* SPM0211 were analyzed with all four primers and their RAPD profiles were compared (Fig. 1), three oligonucleotide primers (Microbial Primer 2, 3, and 4) among the four primers tested were found to generate the discriminatory RAPD profiles from all the tested *Lactobacillus* spp. The remaining primer (Microbial Uniprimer 1) generated the relatively common RAPD banding patterns among the tested *Lactobacillus* spp. PCR amplification, using the Microbial Uniprimer 1, produced bands varying in size from 1,500 bp to 200 bp. Furthermore, all tested *Lactobacillus* spp. produced two

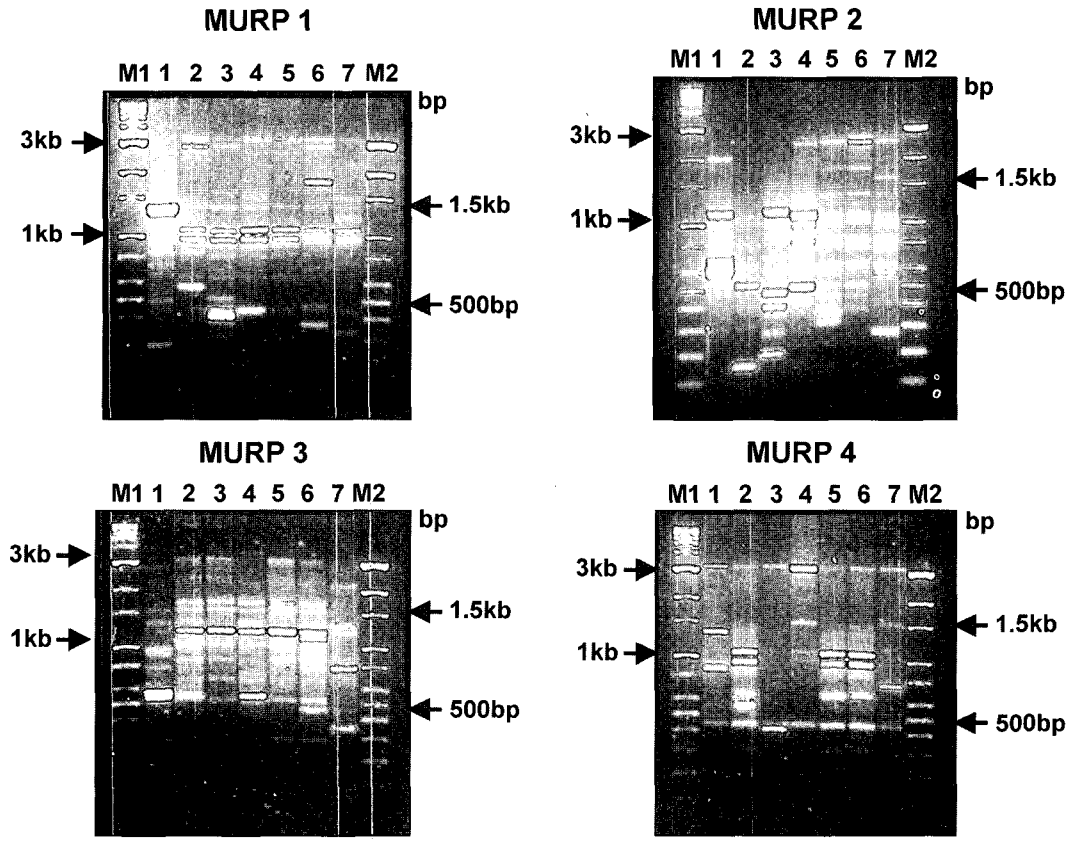

Fig. 1. Genomic fingerprinting patterns of several *Lactobacillus spp.* by using the microbial uniprimer kit. Lane M1, 1 kb ladder size marker; lane 1, *Lactobacillus plantarum*, KCTC1048; lane 2, *Lactobacillus casei* KCTC 2180; lane 3, *Lactobacillus fermentum* KCTC3112; lane 4, *Lactobacillus suebicus* KCTC3549; lane 5, *Lactobacillus reuteri* KCTC5394; lane 6, *Lactobacillus ruminis* KCTC3601; lane 7, *Lactobacillus rminus* SPM0211; lane M2, 100 bp ladder size marker.

Table IV. Similarity matrix (%) among 7 *Lactobacillus spp.*

	L. plantarum	*L. casei*	*L. fermentum*	*L. suebicus*	*L. leuteri*	*L. ruminis*	*L. ruminus*
L. plantarum	100.0						
L. casei	54.3	100.0					
L. fermentum	64.3	54.3	100.0				
L. suebicus	57.1	74.3	64.3	100.0			
L. leuteri	52.9	75.7	60.0	75.7	100.0		
L. ruminis	48.6	74.3	58.6	71.4	90.0	100.0	
L. ruminus	60.0	54.3	55.7	74.3	58.6	54.3	100.0

common bands of approximately 850 bp and 750 bp, suggesting that their genetic makeup may be related in a genus level.

Unlike the Microbial Uniprimer 1, the PCR-RAPD analysis using the Microbial Uniprimer 2~4 produced the polymorphic band patterns. Especially, the presence of 550 bp and 250 bp bands by the Microbial uniprimer 2, or 700 bp and 350 bp bands by the Microbial Uniprimer 3, clearly discriminated between *L. ruminus* SPM0211 and the other tested *Lactobacillus* spp. In addition, PCR amplification using the Microbial Uniprimer 4 differentiated *L. ruminus* SPM0211 from the other tested *Lactobacillus* spp. by the presence of a 650 bp band. Our finding that these species-specific bands were detected in the RAPD profiles using the Microbial Uniprimer kit, could serve as a basis for further studies involving the development of a sequence-characterized amplified region (SCAR) marker through DNA sequencing and database search.

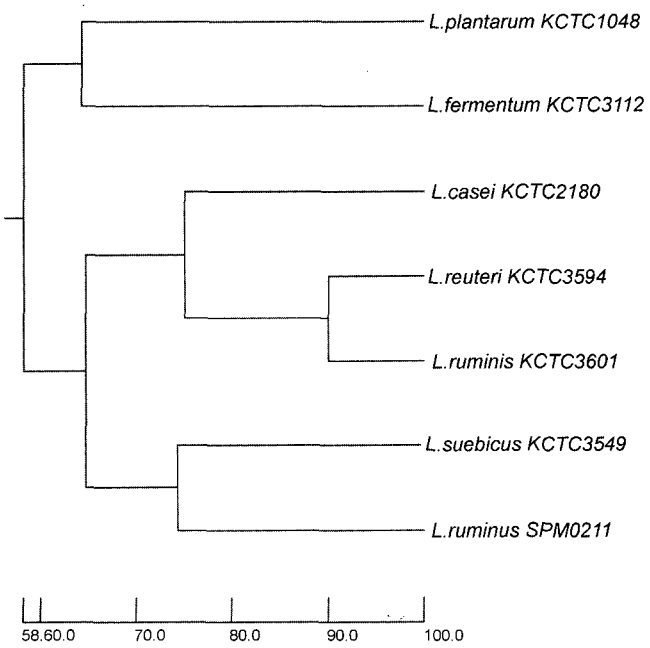

Fig. 2. Dendrogram of 7 *Lactobacillus* spp. constructed from similarity index. The scale represents similarity (%).

Based on the data from the PCR-RAPD analysis, a similarity matrix was calculated to construct a dendrogram for the seven tested *Lactobacillus* spp. (Table IV). All tested *Lactobacillus* spp. were clustered into three groups at the 0.75 similarity level (Fig. 2). *L. ruminus* SPM0211 and *L. suebicus* are clustered as a group at the 74.3% similarity level, suggesting that these two species are closely related to each other. Thus, our results suggest that PCR-RAPD analysis using the Microbial Uniprimer 2~4 may be useful tool in analyzing the phylogenetic relationship among *Lactobacillus* spp.

In conclusion, our results from the antibiotic resistance test using the solid medium dilution method and genetic analysis using the Microbial Uniprimer kit showed that these methods could help in discriminating *L. ruminus* SPM0211 from the other *Lactobacillus* spp. examined.

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