

Identification and Comparison of the Nucleotide Sequence of 16S-23S rRNA Gene Intergenic Small SR(Spacer Region) of *Lactobacillus rhamnosus* ATCC 53103 with Those of *L. casei*, *L. acidophilus* and *L. helveticus*

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ABSTRACT : Reliable PCR based identification of *lactobacilli* has been described utilizing the sequence of 16S-23S rRNA intergenic spacer region. Those sequence comparisons showed a high degree of difference in homology among the strains of *L. rhamnosus*, *L. casei*, *L. acidophilus* and *L. helveticus* whose 16S-23S rRNA intergenic small SR's sizes were 222 bp, 222 bp, 206 bp and 216 bp respectively. The sequence of 16S-23S rRNA intergenic spacer region of *L. rhamnosus* ATCC 53103 revealed the close relatedness to those of *L. casei* strains by the homology ranges from 95.4% to 97.2%. 16S-23S rRNA intergenic spacer region nucleotide sequence of *L. acidophilus* showed some distant relatedness with *L. rhamnosus* ATCC 53103 with the homology ranges from 40.3% to 41.8% and that with *L. helveticus* was shown to be 30% of homology, which exists at the most distant phylogenetic relatedness. The identification of species and strain of *lactobacilli* was possible on the basis of these results. The common sequences among the 17 strains were CTAAGGAA located in the initiating position of the DNA and some discrepancies were found between the same strains based on these results. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 12 : 1816-1821)

Key Words : 16S-23S Intergenic Spacer Region, Identification, *L. rhamnosus*

INTRODUCTION

Molecular biological identification methods are a powerful alternative to the conservative differentiation of bacteria. There are several reports on species specific PCR identification system for *lactobacilli*, mainly based on ribosomal genes (Drake et al., 1996, Yeung et al., 2002) and the ribosomal intergenic region (Tilsala and Alatossava, 1997). The rRNA genetic locus is a genetic unit found in prokaryotic and eukaryotic organisms: in prokaryotes the RNA genetic loci contain the genes for all three rRNA species of 16S, 23S, and 5S genes. These genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the levels of genus and species. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacers (Loughney et al., 1982). The most rapid method to visualize the polymorphic character of internal spacers is to carry out PCR amplification of the spacer regions by using primers from highly conserved flanking sequences. The length and sequence polymorphisms present in the PCR product can be used for the recognition of genera and species. Additional information inherent in the polymorphic character of the amplified product is utilized by several means: i) digestion with restriction enzyme and visualization with fragment size pattern. ii) hybridization with probe with unique sequence. iii) DNA amplification by using primer sequences and amplification conditions that

discriminate for the organism of interest.

Oligonucleotide DNA probes that mainly target variable regions of the 16S or 23 S rRNA genes have been widely used for species identification and strain detection. However, in the case of closely related species, such rRNA probes cannot be used because of the high similarity of rRNA sequences (Fox et al., 1992). These probes do not distinguish between the *L. plantarum*, *L. pentosus* and *L. paraplantarum* strains which are closely related to *L. plantarum* (Bringel et al., 1996). The variation and sequence of the 16S-23S rRNA regions (SRs) have been used to identify species and to type strains at the species level. 16S-23S SRs exhibit a larger variation than rRNA genes (Moreira and Amils, 1996). The SRs also show variations which made it possible to discriminate between strains within some species. The SRs frequently display variable length within a single genome. Nagakawa et al. (1994) reported that the SR sequences were identical among different species but also diverse within species. Very few data are available on the SR of *Lactobacillus* strains.

The aim of this study was to sequence the SRs of a large collection of well-characterized *Lactobacillus* strains and to analyze the variations among them. The second objective was to use the species -specific sequence variation of the SRs to develop a rapid molecular based method for their identification at the species level.

MATERIALS AND METHODS

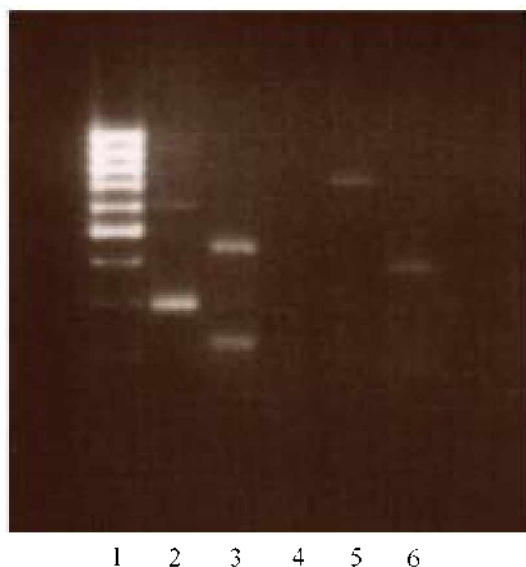
Bacterial strains and media

Lactobacillus spp. were cultured in MRS broth (Difco,

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Table 1. Sequences of the oligonucleotide primers and MgCl₂ concentration used for PCR amplification and sequencing

Species	Primer	Target	Sequence	PCR annealing temp (°C)	MgCl ₂ conc. (mM)
<i>L. helveticus</i>	Hel I	16'-end of spacer	GAAGTGATGGAGAGTAGAGATA	62	2.0
	Hel II	23'-end of spacer	CTCTTCTCGGTTCGCCTTG		
<i>L. johnsonii</i>	Ljon I	16'-end of spacer	AAGGAAGGCGAAAGATGATGGAGAGTGCGA	55	1.5
	Ljon II	23'-end of spacer	TATTTCAAGTTGAGTTTCTC		
<i>L. rhamnosus</i>	Rha I	16'-end of spacer	CAGACTGAAAGTCTGACGG	55	3.0
	Rha II	23'-end of spacer	GCGATGCGAATTTCTATTATT		
<i>L. acidophilus</i>	Aci I	16'-end of spacer	TCTAAGGAAGCGAAGGAT	55	1.5
	Aci II	23'-end of spacer	CTCTTCTCGGTCTCTA		
<i>L. casei</i>	Pr I	16'-end of spacer	CAGACTGAAAGTCTGACGG	55	1.5
	Pr II	23'-end of spacer	GTACTGACTTGCGTCAGCGG		

**Figure 1.** PCR products of amplified 16S-23S rRNA gene spacer regions from different lactobacilli using primer Hel, Ljon, Rha, Aci, and Pr. 1) 1kb DNA ladder 2) *L. helveticus* CU631 3) *L. johnsonii* C-4 4) *L. rhamnosus* ATCC 53103 5) *L. acidophilus* ATCC 4356 6) *L. casei* YIT 9018.

USA) at 37°C and maintained in 11% skim milk containing 0.75 M adonitol at -70°C.

Polymerase chain reaction

Overnight cultures of *Lactobacillus rhamnosus* were pelleted and washed twice with 50 mM EDTA. Chromosomal DNA was isolated by using the Wizard genomic DNA purification kit (Promega USA), described by Tilsala and Alatosava (1997) and Yoon and Won (2002). PCR was performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk) with an AccuPower PCR premix (Bioneer, Korea). Oligonucleotide primers used for amplifying the 16S-23S ribosomal RNA gene spacer region were shown in Table 1. A reaction mixture (50 µl) for PCR of the 16S-23S ribosomal RNA gene spacer region consisted of reaction

buffer (end concentrations MgCl₂ were 3.0 mM), 200 µM each dNTP, 1 µM of Rha I and Rha II primer, 50 ng of bacterial DNA and 0.6 U of Bioneer DNA polymerase. The amplification profile was at 92°C for 30s, 55°C for 30s, 72°C for 30s. This was repeated for 30 cycles. The program also included a preincubation at 92°C for 2 min before the first cycle and an incubation at 72°C followed by a cooling step down to 4°C after last cycle. Amplification products were analyzed with an agarose gel electrophoresis. Amplified DNA products were then purified free from primers and nucleotide with an AccuPrep PCR purification kit (Bioneer, Korea). The amplification products were purified by utilizing the PCR purification kit (Biolabs USA) and the sizes were estimated at agarose gel electrophoresis system.

Electrophoresis and imaging

A 5 µl aliquot of the reaction mixture was combined with 2 µl of loading buffer and the preparation was electrophoresed on 1.0% agarose gel. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

16S-23S rDNA spacer region sequencing and analysis

For each sample two sequencing mixtures were prepared. One contained 4 µl of purified PCR product, 4 µl of BigDye Terminator Reaction Mix (Perkin Elmer /Applied Biosystem Division), 1.6 µl of primer and 0.4 µl of H₂O. The sequencing reactions were performed in a GeneAmp PCR System 9,600 (Perkin Elmer) and the sequencing products were purified through a column comprised of G-50 Sephadex Sigma (Sigma St.Louis, MO). The sequence was determined on a 373 automated DNA sequencer (Perkin Elmer/Applied Biosystem Division) according to the manufacturer's instructions. Sequences determined by the automated sequencer were edited by Factura (Perkin Elmer/Applied Biosystem Division). The relationships of the strains based on 16S-23S rRNA spacer region sequences were determined by Genrunner Package.

		10	20	30	40	50	60	
C	YIT 9018	1	CTAAGGAAACAGACTTTAAGTCTGACGGAAACCTGCACACACGAAACTTTGTTTAGTTTT					
C	ATCC334	2 GA					
C	JCM1134	3					
C	ATCC7422	4 GA					
C	ATCC7217	5 GA					
C	ATCC393	6 TT C					
C	ATCC7128	7 GA					
R	ATCC7469	8 GA					
R	U32966	9 GA					
R	ATCC53103	10 GA					
A	ATCC4356cu	11 G . . A . . . A . . T . GAGAGTA . . . TACTA . G T . T . CAGAGCA . . CGGA					
A	ATCC4356	12 G . . A . . . A . . T . GAGAGTA . . . TACTA . G T . T . CAGAGCA . . CGGA					
A	z75472	13 G . TA . . . A . . T . GAGAGTA . . . TACTA . G T . T . CAGAGCA . . CGGA					
A	JCM1132	14 G . . A . . . A . . T . GAGAGTA . . . TACTA . G T . T . CAGAGCA . . CGGA					
H	JCM1120	15 G . GAAGC - . G . A GTGATG . AG . GTAGAG . T . . TAAG . GAAG . CACA . AAGCA					
H	ATCC15308	16 G . - - - - - . G . A GTGATG . AG . GTAGAG . T . . TAAG . GAAG . CACA . AAGCA					
H	ATCC15009	17 G . TA - - - - - G . A GTGATG . AG . GTAGAG . T . . TAAG . GAA . G . . CGG . CCG .					

		70	80	90	100	110	120	
C	YIT 9018	1	GAGGGGACGA CCCTCAAGCA CCCTAGCGGGTCCGACTTTG TTCTTTGAAA ACTGGATATC					
C	ATCC334	2 TC A G					
C	JCM1134	3					
C	ATCC7422	4 TC G					
C	ATCC7217	5 TC A G					
C	ATCC393	6					
C	ATCC7128	7 TC G C					
R	ATCC7469	8 TT G					
R	U32966	9 TT G					
R	ATCC53103	10 TT G					
A	ATCC4356cu	11	. . . T T GGT . G . AC . . CAAAA . . GT TAGTACAT . .					
A	ATCC4356	12	. GGT . G . AC . . CAAAA . . GT TAGTACAT . .					
A	z75472	13	. CG . GGT . G . AC . . CAAAA . . GT TAGTACAT . .					
A	JCM1132	14	. GGT . G . AC . . CAAAA . . GT TAGTACAT . .					
H	JCM1120	15	AGC . . A . GC . . A . . G . GAA . . TT . GTTTA . . TTTGAGGGT AGTACCTC . . . GA . CTAG . A					
H	ATCC15308	16	AGC . . A . GC . . A . . G . GAA . . TT . GTTTA . . TTTGAGGGT AGTACCTC . . . GA . CTAG . A					
H	ATCC15009	17	. A . TA A . . G . GAA . . TT . GTTTA . . TTT . GCCGC CAAAGCTC . . . GA . CTAG . A					

Figure 2. i) Sequence of 16S-23S rRNA intergenic spacer region and comparison with those sequences of other strains. Position in the alignments that are preserved in the other sequences are indicated by "...". Gaps introduced to maintain alignment are indicated by "-". Arrows indicate the first and the last nucleotide of the SRs. Symbol of species of *Lactobacillus* spp in strain column; C-*L. casei*, R-*L. rhamnosus*, A-*L. acidophilus*, H-*L. helveticus*.

		130	140	150	160	170	180
C YIT 9013	1	ATTGTTGTAA ATGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTAATTTA					
C ATCC334	2AT.....T.....G.AAA.					
C JCM1134	3					
C ATCC7422	4AT.....T.....G.AAA.					
C ATCC7217	5AT.....T.....G.AAA.					
C ATCC393	6					
C ATCC7128	7AT.....T.....G.AAA.					
R ATCC7469	8AA.					
R U32966	9AA.					
R ATCC53103	10AA.					
AATCC4356cu	11	.AAAC.....AA.CC..G CAAAAAACCG AGACAATC.A AGAG.ACAGA ..G..GACCG					
A ATCC4356	12	.AAAC.....AA.CC..G CAAAAAACCG AGACAATC.A AGAG.ACAGA ..G..GACCG					
A 275472	13	.AAAC.....AA.CC..G CAAAAAACCG AGACAATC.A AGAG.ACAGA ..G..GACCG					
A JCM1132	14	.AAAC.....AA.CC..G CAAAAAACCG AGACAATC.A AGAG.ACAGA ..G..GACCG					
H JCM1120	15	CA.TGAAA.C TGAA.A...T CCAAG..A..A...A.A.A A.CA.A...A ACAG.T.GC.					
H ATCC15308	16	CA.TGAAA.C TGAA.A...T CCAAG..A..A...A.A.A A.CA.A...A ACAG.T.GC.					
H ATCC15009	17	CA.TGAAA.C TGAA.A...T CCAAG..A..A...A.A.A A.CA.A...A ACAG.T.GC.					

		190	200	210	220
C YIT 9013	1	GAATTTTCGCA TCGCATAACG GTGACGCAAG TCA-----			
C ATCC334	2C.....GTACA--			
C JCM1134	3C.....--			
C ATCC7422	4C.....GG			
C ATCC7217	5C.....--			
C ATCC393	6C.....--			
C ATCC7128	7C.....--			
R ATCC7469	8C.....--			
R U32966	9C.....--			
R ATCC53103	10C.....GG			
AATCC4356cu	11	ACCGAGAAGA GAATTCTTG..TAAGG			
A ATCC4356	12	ACCGAGAAGA GAATTCTTG..TAAGG			
A 275472	13	ACCGAGAAGA GAATTCTTG..TAA--			
A JCM1132	14	ACCGAGAAGA GAATTCTTG..TAA--			
H JCM1120	15	AGGCGACCG..GAAG.G..TTC.TGACT.GA GT.AGGTCAA A			
H ATCC15308	16	AGGCKACCG..GAAG.G..TTC.TGA-----GT.AGG----			
H ATCC15009	17	AGGCGACCG..GAAG.G..TTC.TGA-----GT.A-----			

Figure 2. ii) Sequence of 16S-23S rRNA intergenic spacer region and comparison with those sequences of other strains. Position in the alignments that are preserved in the other sequences are indicated by ‘.’. Gaps introduced to maintain alignment are indicated by ‘-’. Arrows indicate the first and the last nucleotide of the SRs. Symbol of species of *Lactobacillus* spp in strain column; C-*L. casei*, R-*L. rhamnosus*, A-*L. acidophilus*, H-*L. helveticus*.

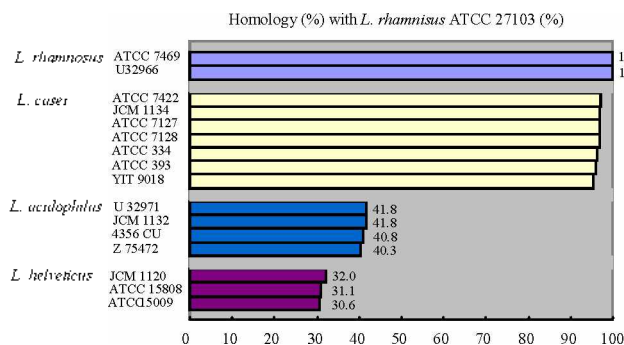


Figure 3. Homology of the 16S-23S rRNA intergenic small SR nucleotide sequence between *L. rhamnosus* ATCC 53103 and *Lactobacillus* spp.

RESULTS AND DISCUSSION

Sequence analysis and comparison of the small 16S-23S rRNA SRs

Primers Rha I and Rha II for *L. rhamnosus* ATCC 53103, and primers Pr I and Pr II for *L. casei* YIT 9018 were used to amplify 16S-23S rRNA gene spacer region from genomic DNA. Primers Aci I and Aci II were used to amplify the 16S-23S rRNA gene spacer region from genomic DNA of *L. acidophilus* ATCC 4356, primers Hel I and Hel II were used for *L. helveticus* CU 631. The PCR products obtained from all *Lactobacilli* were shown in Figure 1. The sizes of 16S-23S rRNA intergenic small SR of *L. rhamnosus*, *L. casei*, *L. acidophilus* and *L. helveticus* were shown to be 222 bp, 222 bp, 206 bp and 216 bp respectively.

Sequences of 16S-23S rRNA intergenic spacer region of 17 *Lactobacillus* strains were aligned to give maximum homology in Figure 2 and comparisons of homology of sequences between 16 strains of *Lactobacilli* and *L. rhamnosus* ATCC 53103 were given in Figure 3 and the sequence of the 16S-23S rRNA intergenic spacer region of the industrial strain of *L. casei* YIT 9018 was determined and aligned with the other two sequences of *L. casei* ATCC 334 strain (AF121200) and JCM 1134 in Figure 2 and Table 3.

16S-23S rRNA intergenic spacer region nucleotide sequence of *L. rhamnosus* ATCC 53103 were homologous with those of two strains of *L. rhamnosus* by 100% homology and *L. rhamnosus* ATCC 53103 revealed the closest relatedness with *L. casei* strains with the homology ranges from 95.4% to 97.2%. The sequences of *L. casei* and *L. rhamnosus* revealed close similarity but differentiation was possible through comparison of the sequences of 16S-23S rRNA intergenic spacer region. The sequence homology between *L. rhamnosus* ATCC 53103 and those of 6 strains of *L. casei* were compared and ranged from 95.4% to 97.2% and the homologies between *L. rhamnosus* ATCC

53103 and *L. acidophilus* strains were shown to be 40.3% to 41.8%.

The homology of the sequences between *L. rhamnosus* ATCC 53103 and *L. helveticus* was shown to range from 30.8% to 32.0% but the homology between three *L. helveticus* strains was shown to be 89.7% to 97.6%. Identification of species of *Lactobacilli* is capable on the basis of these results.

L. casei ATCC 334 was identical with *L. casei* ATCC 7217 in its 16S-23S rRNA intergenic spacer region nucleotide sequence and *L. casei* ATCC 7422 was identical with *L. casei* ATCC 7128 in its 16S-23S rRNA intergenic spacer region nucleotide sequence. That of *L. casei* YIT 9018 was very closely related with that of *L. casei* JCM 1134 with the homology of 99.1%, and it showed most distant relatedness with *L. casei* ATCC 7217.

The sequence of 16S-23S rRNA intergenic spacer region of *L. acidophilus* ATCC 4356 determined in this study was identical with that preserved in the gene bank with the accession number of u32971 but revealed discrepancies with the sequence of accession number z75472. The sequence of 16S-23S rRNA intergenic spacer region of *L. acidophilus* ATCC 4356 determined in this study and compared with that sequence the same strains of *L. acidophilus* from gene bank (U32971) were revealed to be almost identical with the similarity of 98.6%. The sequence of *L. acidophilus* ATCC 4356 and JCM 1132 were aligned in Figure 2 and revealed a homology of 98.1% and similarity between reference strain and JCM 1132 found to be 98.9%.

Ribosomal RNA genes have been generally accepted as a potential tool for the identification and phylogenetic analysis of bacteria. In the case of closely related species, 16S rRNA probes have not been used due to little variation. The sequence of the 16S-23S rRNA intergenic spacer region exhibits greater variation than that of the 16S rRNA structural gene and hence is more suitable for designing specific primers or to identify closely related species or strains (Barry et al., 1991).

The conserved region of 16S-23S rRNA intergenic spacer region of all the tested *Lactobacilli* consisted of CTAAGGAA as an initiating sequence and had some variable nucleotide (s) in those closely related sequences of *L. casei* and *L. rhamnosus* at the position numbers 16, 17, 68, 69, 91, 126, 127, 175-180, 184 and 200.

Tannock et al. (1999) stated a similarity of 97.5% or greater was considered to provide species identification and spacer region sequencing provided a rapid and accurate identification of *Lactobacillus* isolates from gastrointestinal, yoghurt and silage samples. The comparison of 16S-23S rRNA intergenic spacer regions of all the tested *Lactobacilli* in this study revealed variable homology ranges depending on the species of *Lactobacilli*; the minimum homology

values were greater than 92.7%, 96.7% and 89.7% in *L. casei*, *L. acidophilus* and in *L. helveticus* respectively, which was lower than the value proposed by Tannock et al. (1999) as a species identity. A similarity of 90% or greater could provide an identification of species based on this study.

16S-23S rRNA intergenic spacer regions of *lactobacilli* are about 200 bp in length. These relatively short sequences can be easily sequenced on both polynucleotide strands and provide reliable information for comparative work. The use of 16S-23S rRNA intergenic spacer regions of *lactobacilli* in identification promises to be a valuable aid in advancing our knowledge of the species composition of *Lactobacillus* populations. The generic character of the amplification process provides for a simple and direct genetically based diagnostic procedure for the identification of *lactobacilli*.

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