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DIFFERENTIATION BETWEEN *LACTOCOCCUS* sp. AND *LEUCONOSTOC* sp. BASED ON RFLP ANALYSIS OF 16S rRNA

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Taxonomic differentiation between *Lactococcus* sp. and *Leuconostoc* sp. can sometimes be misleading due to the morphological and biochemical similarities between both genera. Therefore, several molecular techniques have been applied to identify these bacteria. Restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal RNA gene was used to generate restriction profiles of 9 strains of *Lactococcus* sp. and 5 of *Leuconostoc* sp. This method utilizes a set of universal primers for amplification of the 16S rRNA region of typical lactic acid bacteria species. The size of the amplified products was about 1500 bp and the amplicons of the different species could be differentiated from each other with four restriction endonucleases: *Taq*I, *Eco*RI, *Bam*HI and *Hin*dIII. These restriction enzymes were selected based on nucleotide sequences of 16S rRNA genes for LAB available in databases. Our study demonstrates that DNA of 16S rRNA from strains of *Lactococcus* sp. contains single restriction site for *Eco*RI and two restriction sites for *Taq*I enzymes, 16S rRNA DNA from strains of *Leuconostoc* sp. contains a single restriction site for each enzyme (*Hin*dIII, *Bam*HI) and four restriction sites for *Taq*I. This result is in good agreement with analysis *in silico* of 16S rRNA genes published in the National Center for Biotechnology Information (NCBI). These findings led to modify the classification obtained by biochemical methods for five examined strains of lactic acid bacteria. In summary, our study demonstrated that the RFLP analysis applied is a useful method for rapid differentiation between *Lactococcus* sp. and *Leuconostoc* sp.

INTRODUCTION

Lactic acid bacteria (LAB) are industrially important microbes that are used all over the world in a large variety of industrial food fermentations [Klaenhammer *et al*., 2002; Kleerebezem *et al*., 2002]. LAB are found naturally in a variety of environmental habitats, including dairy, meat, vegetable, cereal and plant environments, where lactic acid fermentation can occur. Historically, the traditional roles for many LAB have been as starter cultures for food and dairy fermentations, leading to their widespread human consumption [Klaenhammer *et al*., 2005; Yanagida *et al*., 2005].

Typical LAB are gram-positive, nonsporing, catalase-negative, devoid of cytochromes, anaerobic or aerotolerant cocci or rods that are acid-tolerant and produce lactic acid as the major end product during sugar fermentation [Axelsson, 2004].

Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, *e.g.* morphology, mode of glucose fermentation, growth at different temperatures, lactic acid optical configuration, and fermentation of various carbohydrates [Holzapfel *et al*., 2001]. Commonly applied identification system API 50CHL for diagnostics of Lactic Acid Bacteria (LAB) may lead to false classification of strains resulting from the same biochemical profiles of two organisms belonging to different genera. This may result in the non-reproducibility of the tests or difficulties in interpretation and therefore limits the use of traditional methods. These difficulties have increased the interest in molecular approaches to identification. In the past decades, several molecular techniques have been applied to identify these bacteria [Gonzalez *et al.,* 2006; Ben-Amor *et al*., 2007]. Most of these methods use PCR to amplify small-subunit rRNA genes, in particular the 16S rRNA gene, and then PCR amplicons are separated based on differences in DNA sequences of the 16S rRNA genes [Saikaly *et al.*, 2005]. These methods include ribosomal intergenic spacer analysis (RISA), single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and pulsed- field gel electrophoresis (PFGE) [Miteva *et al*., 2001; Blackwood *et al*., 2003; Rodas *et al*., 2005; Randazzo *et al*., 2009]. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of amplified 16S rRNA fragments was developed by Woese and coworkers [O'Sullivan, 2000]. This is one of the most frequent methods used to differentiate between *Lactococcus* and *Leuconostoc* species because it is more discriminatory, faster, and more cost-effective than the phenotypic tests [Randazzo *et al*., 2004]. 16S rRNA genes contain conserved regions coexisting with variable sequences that make them suitable targets for molecular identification methods. Consequently, modern molecular techniques have become increasingly important for species identification or differentiation of LAB strains.

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The objective of this study was the development of an easy-to-perform identification system based on PCR-RFLP analysis with the ability to differentiate among *Lactococcus* and *Leuconostoc* species. The additional aim of the study was taxonomic differentiation of 12 industrial strains of *Lactococcus* sp. and 5 strains of *Leuconostoc* sp. whose taxonomic identity was established on the basis of morphological properties and the API 50CHL biochemical profiles.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study are listed in Table 1. They originated from the collection of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz or were isolated from kefir grains of Polish origin. Bacteria were cultured at 30°C on simplified MRS medium supplemented with 1% glucose as a carbon source.

DNA extraction

Total DNA was isolated according to the method previously described by Anderson & McKay [1983].

PCR amplification

The alignment of 16S rRNA gene sequences of lactic acid bacteria species published in NCBI Database permitted for designing universal primers. The primers used in this study and their characteristics are listed in Table 2. Amplification of 16S rRNA was performed in a total volume of 50 μL comprising 1 μL of bacterial DNA template and 49 μL of a reaction mixture containing 40 pmol of primers, 25.0 μL Red-Taq ReadyMix DNA polymerase (Sigma-Aldrich) and supplemented with 24 μL PCR grade water. The reaction was carried out in the Uno II Thermocycler (Biometra, Germany). After an initial denaturation step at 94°C for 2 min, 39 cycles of denaturation at 94°C for 1 min, primers annealing at 55°C for 1 min and elongation at 72°C for 3 min were performed, followed by the final elongation step at 72°C for 2 min. PCR products were detected by 1% agarose gel electrophoresis in 1 × TBE buffer.

TABLE 1. Comparison of identifications obtained by biochemical and molecular methods for the strains examined.

Strain symbol	Classification based on API 50CHL tests*	Reclassified after RFLP analysis of 16S rRNA gene
A	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Lactococcus* sp.
B	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Lactococcus* sp.
C	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Lactococcus* sp.
D	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Lactococcus* sp.
E	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Lactococcus* sp.
F	*Lactococcus lactis* ssp. *lactis*	*Lactococcus* sp. (AY920468)
G	*Lactococcus lactis* ssp. *lactis*	*Lactococcus* sp. (AY920469)
H	*Leuconostoc lactis*	*Lactococcus* sp.
I	*Leuconostoc lactis*	*Lactococcus* sp.
J	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Leuconostoc* sp.
K	*Lactococcus lactis* ssp. *lactis var. diacetylactis*	*Leuconostoc* sp.
L	*Leuconostoc mesenteroides*	*Leuconostoc* sp.
M	*Leuconostoc mesenteroides*	*Leuconostoc* sp. (AY914053)
N	*Lactococcus lactis*	*Leuconostoc* sp.

* Walczak *et al.* [2003]

TABLE 2. Primers used for PCR amplification of 16S rRNA genes.

	16S rRNA genes
FORWARD	5'-AGAGTTTGATCCTGGCTCAGGA-3'
REVERSE	5'-GGAGGTGATCCAGCCGC-3'

Sequencing of the 16S rRNA gene

The amplified 16S rRNA genes of three representative strains were sequenced using ABIPRISM 3730 Gene Analyzer (Applied Biosystems) and the BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The analysis of the resultant sequences was performed using BLASTN 2.2.20+ [Zhang *et al.*, 2000] program for database searches at the NCBI Web site. The sequences obtained were compared with records for strains of lactic acid bacteria deposited in the National Center for Biotechnology Information (NCBI) and their taxonomic position was revealed.

Nucleotide sequence accession numbers

The partial sequences (507 bp) of 16S rRNA genes for strains of *Leuconostoc mesenteroides* (M) and *Lactococcus lactis* (F, G) were deposited in GenBank database with accession numbers: AY914053, AY920468 and AY920469, respectively. In this study, those strains were used as control type strains.

Restriction fragment analysis

Restriction endonuclease digestion of DNA was carried out according to the manufacturer's instructions with the restriction endonuclease *Eco*RI, *Bam*HI, *Hin*dIII (Sigma-Aldrich) for 1 h at 37°C and by *Taq*I (MBI Fermentas) for 1 h at 65°C. Restricted DNA was analysed by horizontal electrophoresis in 2% agarose gel in a 1 × TBE buffer containing 0.5 μg/mL of ethidium bromide in a Power Pack 300 (Bio Rad) apparatus and photographed under UV light.

RESULTS

The 16S rRNA gene universal primers gave good amplification for all 14 strains tested. PCR-amplified products corresponding to the 16S rRNA gene were obtained specifically from all strains. Co-migration of amplified DNA fragments from all strains indicated their identical size. The PCR products contained approximately 1500 bp (results not shown) and corresponded to the expected size of the 16S rRNA genes based on the nucleotide sequence data for lactic acid bacteria.

Three 6-base recognizing (*Bam*HI, *Eco*RI, and *Hin*dIII) and one 4-base recognizing (*Taq*I) restriction endonucleases were used to cut the amplified 16S rRNA. These restriction enzymes were selected based on the analysis of nucleotide sequences of 16S rRNA genes available in the NCBI database

(Figure 3A, 3B). RFLPs were observed and different information was obtained with each one. Differences in the restriction patterns were compared between the type strains (AY914053, AY920468, AY920469) and the analysed strains. Analysis *in silico* of the sequences of 16S rRNA genes of *Leuconostoc* sp. and *Lactococcus* sp. available in the NCBI database with our sequences showed that restriction patterns of the published sequences were similar to those obtained in our study. It was noticed that the strains analysed could be divided into two groups according to the restriction patterns obtained. For the first group of strains 16S rRNA DNA contains single restriction site for *Eco*RI and two restriction sites for *Taq*I (9 strains A – I). Two fragments that were approximately 850 and 650 bp long were observed for *Eco*RI endonuclease (Figure 1B). Digestion of amplicons with *Taq*I produced three fragments of the approximate size of 750, 550 and 200 bp (Figure 1A). In contrast, the DNA of 16S rRNA genes were not digested by *Bam*HI and *Hin*dIII.

For the second group of strains 16S rRNA DNA contains only one restriction site for enzymes: *Bam*HI and *Hin*dIII and four sites for *Taq*I (5 strains J – M). Digestion with restriction enzymes *Bam*HI and *Hin*dIII generated two different patterns of restriction fragments: 200, 1250 bp for the first endonuclease and 975, 475 bp for the second, respectively (Figure 2A, 2B). By using *Taq*I, four RFLP bands were obtained: 700, 360, 200, 150 bp. Restriction fragments shorter than 90 bp produced by endonuclease (*Taq*I) were not well resolved by electrophoresis in agarose gel (Figure 1A). Thus, the size of PCR products estimated by summing the size of the restricted fragments ranged from 1,400 to 1,500 bp. It was smaller than or equal to the size of undigested PCR products. In addition, the small differences from predicted fragments size occasionally found for measured bands were presumably due to variations in gels, buffers, ethidium bromide concentration, and electrophoresis conditions.

As expected, the four enzymes distinguished the *Lactococcus* sp. from *Leuconostoc* sp. In all of the cases, the RFLP profiles were reproducible and no variation in the restriction profiles of strains belonging to the same genera was observed. Five examined strains (H, I, J, K, N) gave discrepant identifications by biochemical testing and 16S rRNA RFLP pattern (Table 1). Strains H and I were biochemically characterized as *Leuconostoc lactis* and RFLP analysis of 16S rRNA gene

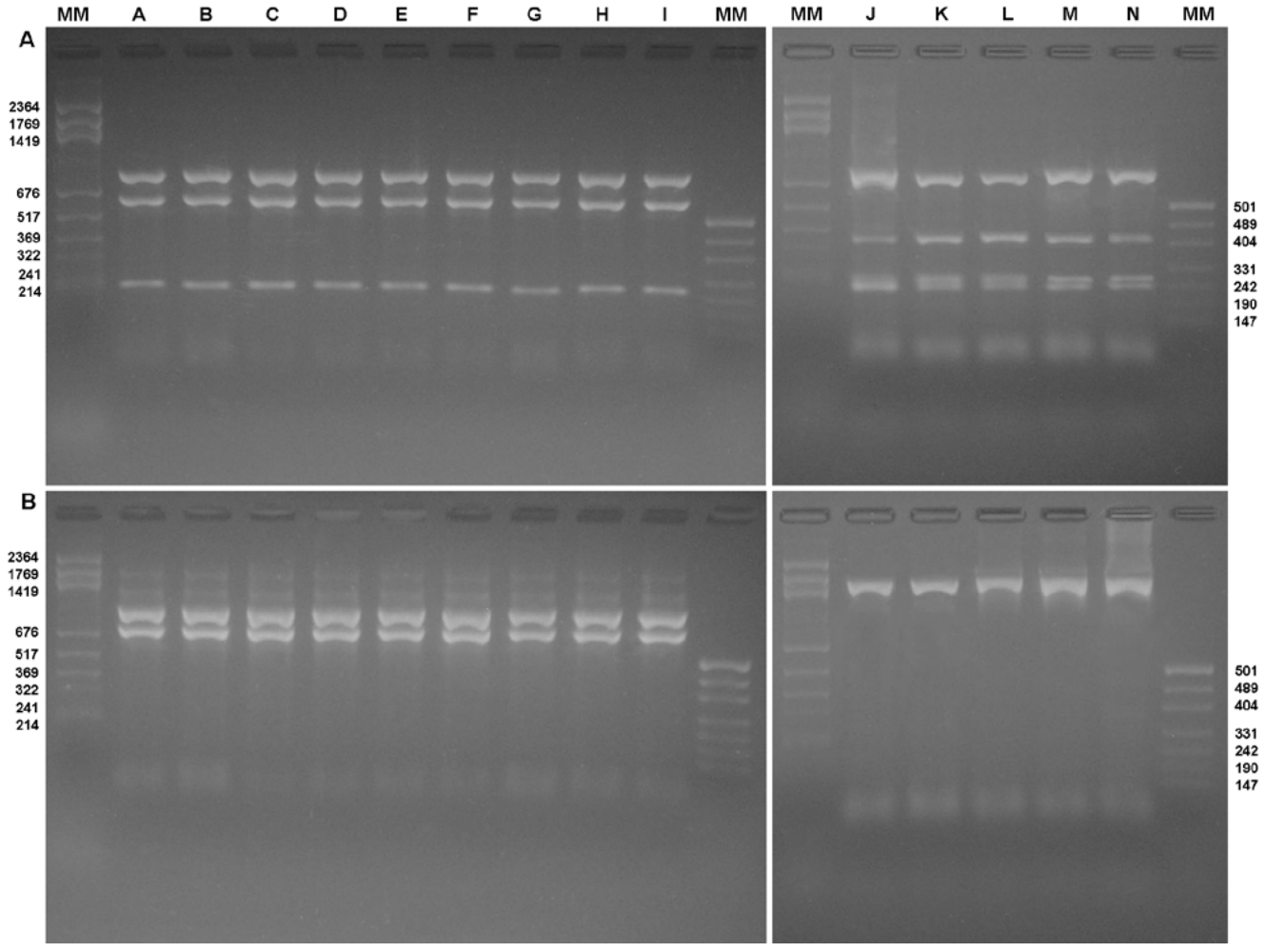

FIGURE 1. PCR-RFLP patterns derived from digestion of 16S rRNA gene PCR products with *Taq*I (A) and *Eco*RI (B).

A, B, C, D, E, H, I, J, K, L – strains of *Lactococcus* sp., N, O, P, R – strains of *Leuconostoc* sp. identified by API 50CHL biochemical tests. F, G – type strains of *Lactococcus* sp., M – type strain of *Leuconostoc* sp. MM – size marker (BTL, Poland).

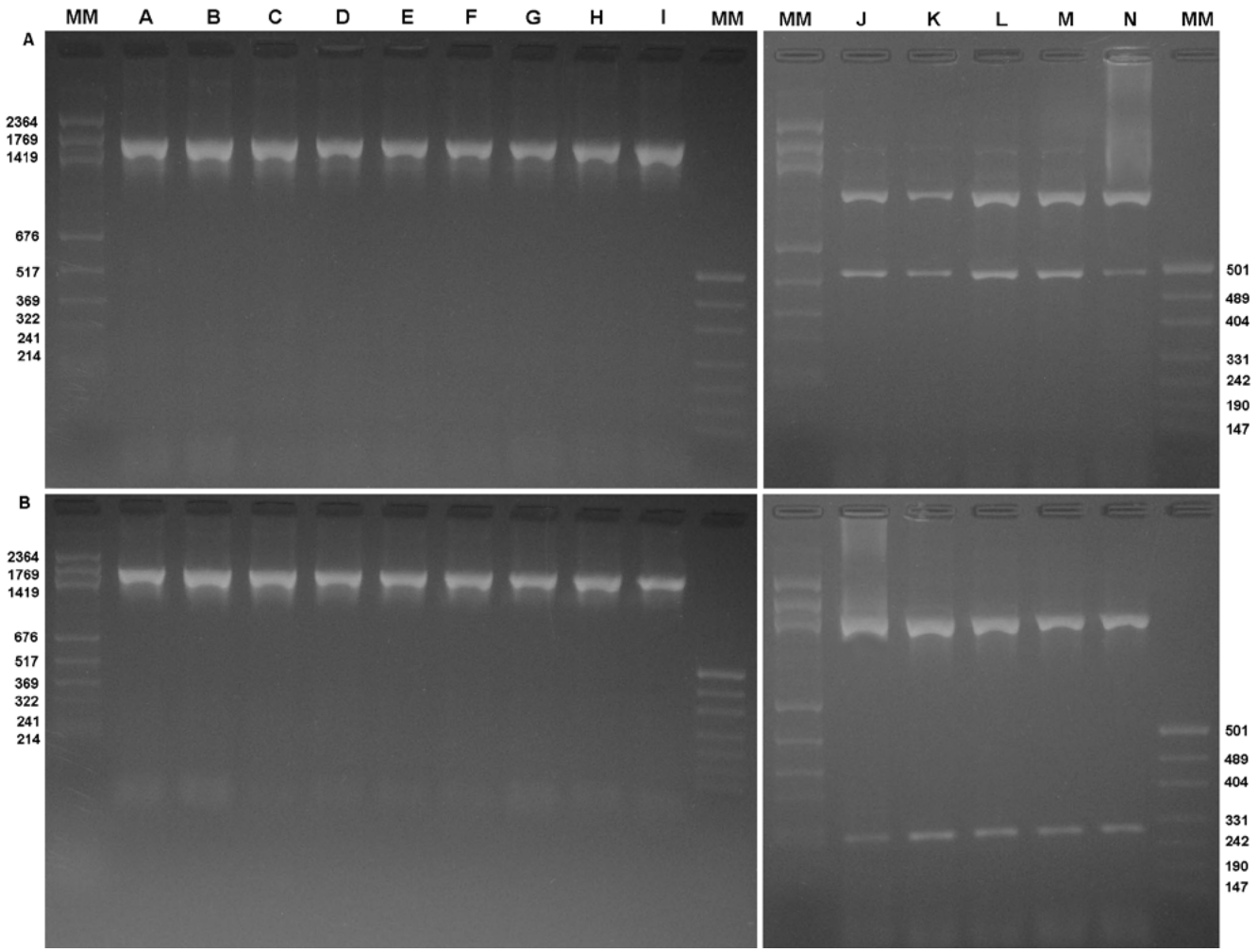

FIGURE 2. Restriction patterns of PCR-amplified fragment of 16S rRNA genes digested with *Hin*dIII (A) and *Bam*HI (B).

A, B, C, D, E, H, I, J, K, L – strains of *Lactococcus* sp., N, O, P, R – strains of *Leuconostoc* sp. identified by API 50CHL biochemical tests. F, G – type strains of *Lactococcus* sp., M – type strain of *Leuconostoc* sp. MM – size marker (BTL, Poland).

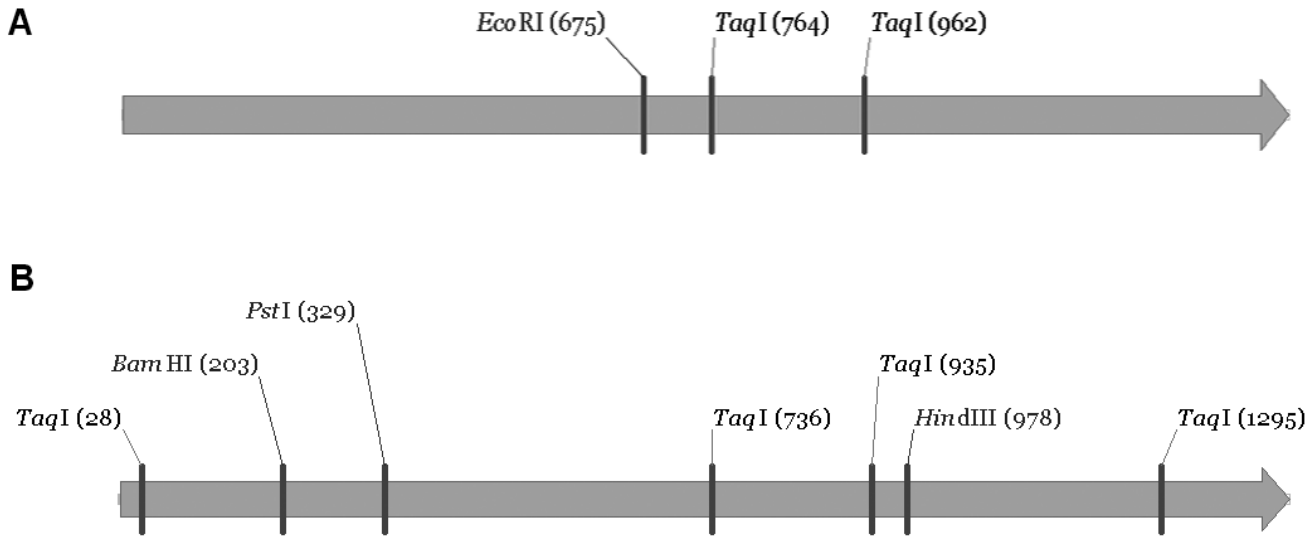

FIGURE 3. Localization of *Taq*I, *Eco*RI, *Bam*HI and *Hin*dIII restriction sites in the 16S rRNA gene for *Lactococcus* sp. (A) and *Leuconostoc* sp. (B).

showed a pattern typical of *Lactococcus* sp. Strains K, J and N classified by conventional testing to *Lactococcus lactis* ssp. *lactis var. diacetylactis* or *Lactococcus lactis* exhibited a pattern characteristic for *Leuconostoc* sp. Nine of the 14 lactic acid bacteria strains were identified as *Lactococcus* sp. and the remaining 5 as *Leuconostoc* sp.

DISCUSSION

The identification of lactic acid bacteria strains has previously been based on colony morphology, Gram-stain reaction, sugar-fermentation profiles and enzymatic activities. The API 50CHL system, which utilizes the characteristics of bacterial sugar fermentation and enzymatic activities, has been commonly used for the identification of lactic acid bacteria [Le Jeune & Lonvaud-Funel, 1994; Dickson *et al*., 2005]. However, these conventional methods often lead to ambiguous results and even misidentifications. It is known that some properties of LAB have a tendency to vary as a result of changes in growth conditions, growth phase, environmental conditions and spontaneous mutations due to the differences in gene expression level especially in strains containing plasmids that encode industrial phenotypes (lactose utilization, proteinase activity, phage resistance) [Deveau & Moineau, 2003]. This may result in the non-reproducibility of the tests or difficulties in interpretation and therefore limits the use of traditional methods. A comparison of the results obtained in biochemical tests with RFLP analysis of 16S rRNA gene showed that only 64% of the examined strains were correctly identified by the API 50 CHL system. Therefore, the application of molecular methods is more accurate than that of the conventional phenotypic methods. Jang *et al*. [2003] and Kim *et al*. [2003] used RFLP analysis of 16S rRNA gene for the identification of *Leucobnostoc* sp. isolated from kimchi. Although Deveau & Moineau [2003] proposed RFLP analysis using *Hin*dII and *Acy*I enzymes for strain differentiating of *Lactococcus* able to produce of extracellular exopolysaccharides.

In our study, we demonstrated that the RFLP analysis of 16S rRNA with the use of four enzymes (*Eco*RI, *Bam*HI, *Hin*dIII and *Taq*I) could be a good method for taxonomic differentiation of LAB and it has been successfully used for at least the identification to the genera level of *Lactococcus* sp. or *Leuconostoc* sp. Our results show that the PCR-RFLP method is more reliable than the biochemical identification for the lactic acid bacteria examined. We suppose that it is also faster and simpler than other molecular techniques that require large quantities of cells and involve complex and time-consuming steps. In addition, it is more economic because it does not require numerous specific primers and expensive equipment. Usefulness of the RFLP analysis of 16S rRNA gene has been proved by Yu *et al*. [2009], who characterised 171 strains of lactic acid bacteria, isolated from home-made fermented milk in Tibet.

CONCLUSIONS

In conclusion, the PCR-RFLP analysis of 16S rRNA gene sequences allows for genus identification and achieves a good level of species differentiation with only one set of primers and few restriction enzymes [Gonzalez *et al.*, 2006]. This technique is a fast, simple, and suitable alternative method to conventional identification procedures for reliable characterization of *Lactococcus* and *Leuconostoc* species.

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