Korean vaginal *Lactobacillus* spp.의
특성 규명 및 분자동정

Characterization and molecular identification of Korean vaginal *Lactobacillus* spp.

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Characterization and molecular identification of Korean vaginal *Lactobacillus* spp.
Characterization and molecular identification of Korean vaginal *Lactobacillus* spp.

by

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ABSTRACT

One hundred eight vaginal lactobacilli were isolated from Korean women and characterized in terms of their antibiotics susceptibility, salt susceptibility and PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and SDS-PAGE whole cell protein analysis. The *in vitro* susceptibilities of 108 vaginal isolates to 13 antibiotics were determined by broth dilution method based on NCCLS reference protocol. High rates of resistance were demonstrated for gentamicin, kanamycin, metronidazole, and streptomycin whereas all the isolates were susceptible to erythromycin. The concentrations of gentamicin, kanamycin, metronidazole, streptomycin, and erythromycin at which 90% of the vaginal isolates were inhibited (MIC<sub>90</sub>) were 100 μg/ml, 200 μg/ml, >200 μg/ml, 200 μg/ml and 0.39 μg/ml, respectively. Salt MICs of 108 vaginal isolates which determined by broth microdilution method were varying from 0.4M to 1.7M. Almost half of the isolates were resistant to more than 0.6M salt.

For molecular typing, PCR-RFLP analysis was employed where the 16S rDNA was amplified by PCR and the PCR products were digested with 8 different restriction endonucleases prior to being electrophoresed in agarose gels. Based on PCR-RFLP results, more than 70% of the isolates were identified as *Lactobacillus crispatus*. Several isolates were further identified by DNA sequence analysis of their 16S rDNA. Furthermore, species level identification results of PCR-RFLP were similar to the results of SDS-PAGE whole cell protein analysis. Therefore, both PCR-RFLP and SDS-PAGE whole cell protein analysis can be used for an effective identification of *Lactobacillus* spp. to the species level.
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### I. Introduction

#### 1.1 Lactobacillus spp.

It was first identified in 1894 by the German physician A. Doderlein that *Lactobacillus* sp. was the predominant bacterium in the vaginal microbial flora found in women of reproductive age (Redondo-Lopez et al., 1990). The genus *Lactobacillus* encompasses a diverse assemblage of Gram-positive, catalase-negative, non-spore-forming, rod-shaped organisms.

Lactobacilli are facultative anaerobes that colonize the moist surface of the vaginal epithelium, intestinal tract, and oral cavity of humans and nonhuman animals (Redondo-Lopez et al., 1990; Sharpe, 1981). Lactobacilli play an important role in maintaining healthy vagina and they produce various inhibitory compounds which can prevent the growth of anaerobic pathogenic bacteria. Lactobacilli metabolize glucose to a final end product of lactic acid, which contributes to the maintenance of a low vaginal pH(4.0-4.5) (Sharpe, 1981; Vallor et al., 2001). Many isolates of vaginal lactobacilli produce hydrogen peroxide, a compound having broad antimicrobial activity (Hiller et al., 1991; Klebanoff et al., 1991).
*Lactobacillus crispatus* and *L. jensenii* are the most prevalent species in the vagina, and 94%-95% of these strains produce H$_2$O$_2$ (Antonio, 1999). Women colonized by H$_2$O$_2$-producing lactobacilli have decreased acquisition of bacterial vaginosis (Hawes et al., 1996).

### 1.2 Bacterial Vaginosis (BV)

Bacterial vaginosis is the most frequent cause of vaginal discharge. Symptoms of bacterial vaginosis include vaginal discharge and malodor, although some women are asymptomatic. Clinical signs of bacterial vaginosis include elevated pH (>4.5), homogenous vaginal discharge, a positive whiff test (amine odor produced when 10% potassium hydroxide is added to vaginal fluid), and the presence of clue cells (sloughed vaginal epithelial cells coated with bacteria) (Hiller et al., 1999; Eschenbach et al., 1988). In bacterial vaginosis (BV) patient, the number of lactobacilli decreases while the number of anaerobic bacteria increases for unknown reasons.

To treat BV antimicrobial agents were used to eliminate the pathogenic bacteria but they can also eliminate vaginal lactobacilli. While antimicrobial agents can be effective for eradication of vaginal infections, they are often
unable to cure bacterial vaginosis or prevent complications that arise from it[6]. Furthermore, antimicrobial agents have many side effects such as occurrence of multidrug resistant microorganisms. Also, chemotherapeutic agents ideally should not be used for prophylaxis or health maintenance (Gillian et al., 2002). Therefore, the antimicrobial agent which can selectively inhibit the growth of anaerobic pathogenic bacteria while not inhibiting the growth of lactobacilli is needed for effective treatment of BV.

1.3 Antimicrobial susceptibility

Antimicrobial resistance is an increasing medical problem fueled by the use of antimicrobial agents. In many countries, national surveillance and research programmes have been initiated to monitor resistance in bacteria isolates from human. Antimicrobial resistance genes have been shown to be transferable between bacteria of diverse origin, also under natural conditions (Kruse and Sorum, 1994). Because non-pathogen bacteria may also be a source for resistance genes that can spread to pathogens, surveillance activities should include non-pathogenic as well as pathogen bacteria.
Several methods have been reported for antimicrobial susceptibility testing of lactic acid bacteria isolates, including disc diffusion (Perreten et al., 1998), E-test (Katla et al., 2001), broth dilution (Perreten et al., 1997), and agar dilution.

1.4 Molecular identification

The process of typing is important epidemiologically for recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the infection, recognizing particularly virulent strains of organisms, and monitoring vaccination programs. The typing of lactobacilli has generally been conducted by cell and colony morphology and biochemical tests. However, unambiguous strain identification, based on conventional phenotypic methods such as sugar fermentation profiles or other biochemical/physiological traits, is not always possible. Identification at intraspecies level is also an important issue since it may help to distinguish groups of strains or single strains with peculiar technological properties.

There is sufficient diversity at the species level that organisms isolated from different sources at different times and in different geographical regions may be differentiated or classified into sub-types or strains. Any subtyping method
must have high differentiation power. It must be able to clearly differentiate unrelated strains, such as those that are geographically distinct from the source organism, and at the same time to demonstrate the relationship of all organisms isolated from individuals infected through the same source. Several molecular methods have recently been applied for the identification of Lactic acid bacteria, such as SDS-PAGE of whole cell proteins, restriction fragment length polymorphism analysis of the 16S rDNA gene, hybridization with rRNA probes, species-specific PCR, PCR followed by temperature gradient gel electrophoresis (TGGE) and randomly amplified polymorphic DNA (RAPD)-PCR analysis. Many studies emphasize that the classification of lactobacilli is unsatisfactory and does not reflect the real phylogenetic relatedness of different strains and species (Collins et al., 1991; Pot et al., 1993; Stahl et al., 1990). Molecular methods can differ widely in their ability to differentiate strains. Many of the currently used molecular technique for typing rely on electrophoretic separation of DNA fragments of different molecular lengths. The electrophoretic result is represented by a pattern of bands on a gel. Since these patterns may be extremely complex, the ease with which the patterns are interpreted and related is a factor in evaluating the utility of a particular typing method (Arbeit, 1995). Along with consideration, its ease of use is
also important (Arbeit, 1995). The technical difficulty, cost, and time to obtain a result must also be evaluated in assessing the utility of a particular typing method (TABLE 1).

Several new genetic and chemotaxonomic approaches have been used during the last 14 years with an aim of improving the classification and identification of lactobacilli: for example, analysis of plasmid content (Nes, 1984), sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of whole-cell protein (Pot et al., 1993) and of total soluble cell protein (Collins et al., 1991), sequencing of rRNA (Collins et al., 1991; Pot et al., 1993; Collins et al., 1989) restriction endonuclease fingerprinting (Stahl et al., 1990; Rodtong and Tannock, 1993), and DNA-DNA hybridization (Collins et al., 1991; Pot et al., 1993).

In addition, restriction fragment length polymorphism analysis (RFLP) following 16S rDNA PCR amplification (PCR-RFLP) has been successfully used for the identification of methanogens (Hiraishi et al. 1995).
TABLE 1. Characteristics of molecular identification methods.

<table>
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Estimated costs

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1.5 Whole cell protein analysis

Proteins are amphoteric compounds; their nett charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a nett negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. At a given pH therefore, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of the molecules. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone and SDS binds to proteins fairly specifically in a mass ratio of 1:4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and
uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.

Molecular methods are widely used for identification purposes, with Sodium Dodesyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) whole cell protein pattern analysis offering the advantages of being fairly fast and easy and, when performed under highly standardized conditions, of having a good level of taxonomic resolution at species or subspecies level (Desheemaeker et al., 1994). The SDS-PAGE protein profile analysis has been used successfully to identify lactic acid bacteria isolated from wines and musts, dry salami and Italian ewa’s-milk cheeses and to solve problems in the identification of closely related species in the genera Lactococcus, Vagococcus, Lactobacillus, Leuconostoc, and Streptococcus. In addition, very good correlation between the results of the numeral analysis of protein patterns and DNA/DNA hybridization or 16S rRNA based oligonucleotide probe hybridization has been reported.
II. Objectives

In a previous study, 108 vaginal lactobacilli were isolated from healthy Korean women, and the initial characterization was performed on the 108 vaginal *Lactobacillus* spp. (KLB 1 thru KLB108) in terms of their antimicrobial activity against various pathogenic bacteria and hydrogen peroxide production.

In this study, characterization was performed on the 108 vaginal *Lactobacillus* spp. in terms of their antimicrobial susceptibility and salt susceptibility to select antimicrobial agent for the more effective treatment of BV. The antimicrobial agent which can selectively inhibit the growth of anaerobic pathogenic bacteria while not inhibiting the growth of lactobacilli is needed for effective treatment of BV.

One hundred and eight vaginal isolates were identified by PCR-RFLP and SDS-PAGE whole cell protein analysis to determine the species prevalence of 108 vaginal lactobacilli. Based on the species prevalence of 108 vaginal lactobacilli, the species prevalence of normal human vagina can be expected.
III. Materials and Methods

3.1 Bacterial strains and media

In a previous study, 108 vaginal lactobacilli were isolated from healthy Korean women, and the initial characterization was performed on the 108 vaginal *Lactobacillus* spp. (KLB 1 thru KLB108) in terms of their antimicrobial activity against various pathogenic bacteria and hydrogen peroxide production (Chang et al., 2002). All isolates were presumptively identified as *Lactobacillus* based on their ability to grow on the Rogosa agar, Gram-positive staining, rod cell shape and catalase-negative phenotype. One hundred and eight Korean lactobacilli were characterized, molecular identified and analized by SDS-PAGE whole cell protein profile in this study. Nine *Lactobacillus* reference strains (*L. acidophilus* ATCC 4356, *L. casei* ATCC 393, *L. crispatus* ATCC 33820, *L. delbrueckii* subsp. *lactis* ATCC 15808, *L. fermentum* ATCC 23271, *L. gasseri* ATCC 9857, *L. jensenii* ATCC 25258, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 7469) were obtained from the American Type Culture Collection (ATCC) and one (*L. reuteri* DSM 20016) from Deutsche Sammlung von Mikroorganismen (DSM)
Lactobacillus Rogosa and Man-Rogosa-Sharpe (MRS) agars (Difco) were used for the initial isolation, and MRS broth were used for subsequent culture. Korean vaginal *Lactobacillus* spp. were routinely grown in MRS medium. MRS medium contained 20g of glucose, 10g of peptone No.3, 10g of beef extract, 5g of yeast extract, 5g of sodium acetate, 2g of ammonium citrate, 2g of potassium phosphate dibasic (K₂HPO₄), 1g of tween 80, 35mg of FeSO₄·7H₂O, 575mg of MgSO₄, 120mg of MnSO₄·7H₂O per liter.

### 3.2 Antimicrobial susceptibility test

The antimicrobial susceptibility test of 108 Korean vaginal isolates was performed by broth dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) with slightly modification (National Committee for Clinical Laboratory Standards, 2002).

*Lactobacillus* spp. were cultured overnight anaerobically in 5ml MRS broth. To prepare the culture broth, 10% of overnight cultured cell were transferred to fresh 5ml MRS broth and incubated for 3h up to O.D.600=1. Serially two-fold diluted antibiotics were added to each test tubes containing 5ml MRS broth. Then, the cultures were incubated for 20h
to measure the optical density. MIC (minimal inhibitory concentration)s
were defined as the lowest drug concentration that gave only a slight growth
(corresponding approximately to 10% of the control growth.
The antimicrobial agents tested were ampicillin (Amp), chloramphenicol (Ch),
erthyromycin (Em), gentamicin (Gm), kanamycin (Km), metronidazole (Mtz),
novobiocin (Nov), oxacillin (Oxa), penicillin G (PnG), streptomycin (Sm),
tetracycline (Tc), ofloxacin (OF) and cefotaxime (CF) at twofold
concentrations from 0.1 µg/ml to 200 µg/ml.

3.3 Salt MIC test

Korean vaginal *Lactobacillus* spp. were cultured overnight anaerobically in
5ml MRS broth. To prepare the culture broth, 10% of overnight cultured cell
were transferred to fresh 5ml MRS broth and incubated for 3h up to
O.D.600=2. Concentrations of salt tested were ranged from 0M to 2M every
0.1M. Culture broth was added to each microtubes containing 1ml MRS broth
which contained each concentrations of salt. Then, the cultures were
incubated for 20h to measure the optical density. MICs were defined as the
lowest drug concentration that gave only a slight growth corresponding
approximately to 10% of the control growth.
3.4 Molecular identification (PCR-RFLP)

In order to determine the strain prevalence of Korean vaginal lactobacilli molecular typing was performed on the 108 isolates to the species level using PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism).

3.4.1 Genomic DNA isolation

Isolation of vaginal Lactobacillus spp. chromosomal DNA was performed by the previously reported method (Chang et al., 2002). 5ml of Lactobacillus spp. culture broth which was grown at 37°C to late exponential phase of growth was harvested, washed by TEN buffer (10mM Tris-HCl, 1mM EDTA, 0.1M NaCl, pH8.0), resuspended with TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0). After 40 µl of lysozyme (50mg/ml) was added, the mixture was incubated at 37°C for 1hr and 30 µl of 10% SDS, 60 µl of EDTA (0.5M), and 7 µl of proteinase K (2mg/ml) were added thoroughly into mixed supernatant for cell lysis. After 1hr, the lysate was extracted twice with 600 µl of phenol and once with chloroform. Aqueous phase was separated by centrifugation at 15000rpm for 5min at 4°C and transferred to a
new microtube. DNA was precipitated by adding of 3M sodium-acetate (pH4.8) and 10 µl of RNase (10mg/ml) was added to the dissolved DNA solution. This solution was incubated at 37 °C for 2hr. After RNA and residual protein were removed, DNA solution was treated with phenol/chloroform/isoamylalcohol (25:24:1) and DNA subsequently was precipitated with two volume of absolute ethanol and stored at -20 °C.

DNA pellet was obtained by centrifugation at 15000rpm for 10min at 4 °C followed by washing with 70% ethanol and the DNA pellet was dried and resuspended in 50 µl of TE buffer (FIG. 1).

3.4.2 PCR (Polymerase chain reaction)

PCR was performed with universal primer

rD1(5AGAGTTTGATCTGGCTCAG3) and

rD1(5AAGGAGGTGATCCAGCC3) to amplify 1.6Kb of 16S rDNA (Chang et al., 2002; Weisburg et al., 1991). PCR reaction mixture was as follows;
template DNA 100ng, 10X buffer 5 µl, dNTP 2.5mM, primer 10pM each,
Taq ( TaKaRa Ex Taq™ ) 1.25U, ddH₂O 37.75 µl (TABLE 2). PCR was performed as follows; preheating at 95 °C for 5min, denaturation at 94 °C
for 1min, primer annealing at 60 ℃ for 1min, polymerase extension at 72 ℃ for 2min, final elongation at 72 ℃ for 8min, 35 cycles (TABLE 3).

3.4.3 Restriction enzyme digestion

PCR product was digested with 8 different restriction endonucleases; *Hinfl* I, *Alu I*, *Msp I*, *HaeIII* (TaKaRa, Japan), *Rsa I*, *Taq I*, *Dde I* and *Cfo I* (Promega, U.S.A.) by using the appropriate reaction condition and incubation time according to the supplier's recommendation. Reaction mixture of 8 restriction endonucleases was presented at TABLE 4.

3.4.4 Electrophoresis of DNA

Enzyme digested 16S rDNA was electrophoresed at 100V, 80mA for 2h in 2% agarose gel. Tris-borate-EDTA (TBE) electrophoresis buffer (89mM Tris-barate, 2mM EDTA) was used.
3.4.5 Data analysis

Dendrogram constructed with restriction patterns obtained by PCR-RFLP.

Patterns of vaginal isolates were combined with the NTSYS-pc (numerical taxonomy system of multivariate statistical programs) and grouped with the UPGMA (unweighed pair group method using average linkage cluster analysis) (Chang et al., 2002).
Overnight culture on 5ml MRS broth

↓

Wash with 600 \( \mu l \) of TEN buffer and resuspend in 400 \( \mu l \) of TE buffer

↓

Add 40 \( \mu l \) of lysozyme (50mg/ml)

↓

Add 30 \( \mu l \) of 10% SDS, 60 \( \mu l \) of EDTA (0.5M), and 7 \( \mu l \) of proteinase K (2mg/ml)

↓

1\(^{st}\) phenol extraction;
add equal volume of phenol:chloroform:isoamyl alcohol (25:24:1)

↓

Transfer supernatant to a fresh microtube and add 10\( \mu l \) of RNase (10mg/ml)

↓

2\(^{nd}\) phenol extraction

↓

Transfer supernatant to a fresh microtube and add two volume of 100% ethanol and 3M sodium-acetate

↓

Store at -20 °C

↓

Wash by 1ml of 70% ethanol

↓

Vacuum drying

↓

Resuspend in 50\( \mu l \) of TE buffer


FIG. 1. Preparation procedure for genomic DNA isolation from

*Lactobacillus* spp.
TABLE 2. PCR reaction mixture composition for amplification of 16S rDNA gene. 16S rDNA gene amplification composition.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>2 μl (100ng)</td>
</tr>
<tr>
<td>dNTP</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>10X buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>Primer (rD1, fD1)</td>
<td>10pM each</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1.25U</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>37.75 μl</td>
</tr>
</tbody>
</table>
TABLE 3. PCR procedure for amplification of 16S rDNA gene.

<table>
<thead>
<tr>
<th>PCR procedure</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preheating</td>
<td>95°C</td>
<td>5min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1min</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>60°C</td>
<td>1min</td>
</tr>
<tr>
<td>Polymerase extension</td>
<td>72°C</td>
<td>2min</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>8min</td>
</tr>
</tbody>
</table>

35 cycles
TABLE 4. Restriction endonucleases reaction mixture.

<table>
<thead>
<tr>
<th>Hinf</th>
<th>Alu</th>
<th>HaeIII</th>
<th>Cfo</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>10 μl (1000ng)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme buffer</td>
<td>2 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>2 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td>up to 50 μl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Msp</th>
<th>Rsa</th>
<th>Taq</th>
<th>Dde</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>10 μl (1000ng)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme buffer</td>
<td>2 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>2 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>2 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td>up to 50 μl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 Whole cell protein analysis

3.5.1 Preparation of whole cell protein extracts

Cultures grown on MRS broth for 16-20h were inoculated in 10ml of MRS broth and incubated at 37°C overnight. Stationary phase cell pellets were harvested by centrifugation and washed twice in extraction buffer (200mM Tris-HCl; pH 8.0, 150mM Ammonium sulfate, 10% glycerol, 1mM EDTA, 1mM PMSF, stored at 4°C). For mechanical cell disruption, 1g of glass beads (diameter 2mm) were added to the pellets and vortexing for 10min (1min vortexing/1min in ice) at the maximum setting. Supernatant was transferred to a new microtubes after centrifuging (15000rpm, 10min, 4°C). The supernatant was mixed with 5X sample buffer (60mM Tris-HCl, 25% glycerol, 2% SDS, 14.4% 2-mercaptoethanol, 0.1% bromophenol blue). This was followed by heating at 100°C for 5min, cooling at room temperature and centrifuging (15000rpm, 10min, 4°C). The extracts thus obtained were stored at -20°C.
3.5.2 SDS-PAGE electrophoresis

The polyacrylamide gel consisted of a 4% stacking gel in 0.125M Tris-HCl buffer (pH 6.8) with 0.1% (w/v) SDS and a 12% separating (resolving) gel in 0.375M Tris-HCl buffer (pH 8.8) with 0.1% (w/v) SDS. The electrode buffer was 25mM Tris, 192mM glycine, 0.1% (w/v) SDS (pH 8.3). A volume of sample which was contained 0.05% (w/v) bromophenol blues a visible marker was layered on top of the gel. Electrophoresis was performed using a power supply operated at a constant voltage of 100V through the stacking gel and 150V through the separating gel. The gel was stained by immersing in the stain solution (0.25% (w/v) Coomassie brilliant blue R-250 (Merck, Darmstadt, Germany) in 50% (v/v) methanol and 10% (v/v) acetic acid) overnight with constant shaking on a rocker table. The excess stain was washed out by destaining with a solution of 20% (v/v) methanol, 5% (v/v) acetic acid, and 2.5% (v.v) glycerol.

A standard protein solution (Perfect Protein™ Markers, Novagen, Germany), containing 9 proteins ranging in size from 10 to 225KDa, was used as a molecular weight marker and for the normalization and interpolation of the protein patterns.
IV. Results

4.1 Antimicrobial susceptibility test

Broth dilution method (FIG. 2) was used in this study because in general its results agreed with the ones obtained by the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2002) as tested with several selected strains.

High rates of resistance were demonstrated for gentamicin, kanamycin, metronidazole, and streptomycin whereas all the isolates were susceptible to erythromycin. The MIC ranges, the MICs at which 90% (MIC$_{90}$) of growth was inhibited are shown in TABLE 5. The concentrations of ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, metronidazole, novobiocin, oxacillin, streptomycin, tetracycline, ofloxacin, and cefotaxime, at which 90% of the vaginal isolates were inhibited (MIC$_{90}$) were 3.13 µg/ml, 3.13 µg/ml, 0.2 µg/ml, 100 µg/ml, >200 µg/ml, >200 µg/ml, 0.78 µg/ml, 0.25 µg/ml, 100 µg/ml, 3.13 µg/ml, 200 µg/ml and 1.56 µg/ml, respectively. The concentrations of metronidazole tested in our study ranged
from 0.01 μg/ml to 200 μg/ml and at these concentrations, O.D. of test culture was higher than the growth control. Similar results were previously reported where concentrations between more or equal to 128 μg/ml and less or equal to 256 μg/ml stimulated the growth of Lactobacillus spp. However, high concentrations of metronidazole (i.e. between 1,000 μg/ml and 4,000 μg/ml), partially inhibited the growth while concentrations more or equal to 5,000 μg/ml completely suppressed the growth (Simoes et al, 2001).

4.2 Salt MIC test

NCCLS recommended broth microdilution method was used to determine salt MICs of 108 vaginal isolates. Salt MICs of 108 vaginal isolates were showed in FIG. 3. Salt MICs of vaginal isolates were varying from 0.4M to 1.7M. MICs of 36% of isolates were 0.6M and 23% of isolates were 0.5M. Salt MICs of some isolates were more than 1M, salt MICs of 3% of isolates were even more than 1.5M. Salt MICs of #57-2, #97-2, and #98 were 1.5M, 1.6M, and 1.7M, respectively.
FIG. 2. MIC test by broth dilution method.
TABLE 5. Antimicrobial susceptibility of the 108 Korean vaginal isolates

<table>
<thead>
<tr>
<th>% of isolates</th>
<th>Amp</th>
<th>Ch</th>
<th>Em</th>
<th>Gm</th>
<th>Km</th>
<th>Mtz</th>
<th>Nov</th>
<th>Oxa</th>
<th>PnG</th>
<th>Sm</th>
<th>Tc</th>
<th>OF</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;200(mg/lt)</td>
<td>5.97</td>
<td>84.21</td>
<td>93.97</td>
<td>11.66</td>
<td>24.19</td>
<td>30.5</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200(mg/lt)</td>
<td>25.37</td>
<td>11.84</td>
<td>6.02</td>
<td>6.66</td>
<td>20.96</td>
<td>23.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100(mg/lt)</td>
<td>46.26</td>
<td>3.94</td>
<td>15</td>
<td>46.77</td>
<td>16.94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50(mg/lt)</td>
<td>14.92</td>
<td>1.03</td>
<td>11.66</td>
<td>4.83</td>
<td>13.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(mg/lt)</td>
<td>5.97</td>
<td>4.47</td>
<td>1.03</td>
<td>15</td>
<td>6.77</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5(mg/lt)</td>
<td>1.49</td>
<td>1.49</td>
<td>10.3</td>
<td>18.33</td>
<td>1.61</td>
<td>5.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25(mg/lt)</td>
<td>5.81</td>
<td>46.91</td>
<td>7.46</td>
<td>36.08</td>
<td>13.33</td>
<td>1.61</td>
<td>8.97</td>
<td>3.38</td>
<td>8.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.13(mg/lt)</td>
<td>45.34</td>
<td>53.08</td>
<td>5.97</td>
<td>22.68</td>
<td>5</td>
<td>61.53</td>
<td>39.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.56(mg/lt)</td>
<td>36.04</td>
<td>14.9</td>
<td>14.43</td>
<td>3.33</td>
<td>29.48</td>
<td>45.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.78(mg/lt)</td>
<td>6.97</td>
<td>7.89</td>
<td>31.34</td>
<td>4.12</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.39(mg/lt)</td>
<td>5.81</td>
<td>28.94</td>
<td>29.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20(mg/lt)</td>
<td>53.94</td>
<td>2.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10(mg/lt)</td>
<td>9.21</td>
<td>1.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 3. Salt susceptibility of the 108 vaginal isolates. Percentage of strains which MICs were detected as each salt concentration.
4.3 Molecular identification (PCR-RFLP)

PCR-RFLP analysis was carried out with 108 vaginal isolates of lactobacilli, including 10 reference strains. Genomic DNAs prepared from 10 type strains and 108 vaginal isolates were used to amplify the 16S rDNA with universal primer as described in Materials and Methods. All the strains produced a single band about 1.6Kb long. The amplified 16S rDNAs were digested with 8 restriction endonucleases and the resulting restriction fragments were size-fractionated by 2.0% agarose gel electrophoresis (FIG. 4) (Chang et al., 2002). PCR-RFLP data were analyzed using the program NTSYS-pc that calculated the molecular relativity of the strains tested (FIG. 5).

Based on PCR-RFLP results, we have found that 76% of Korean vaginal isolates belong to either *L. crispatus* or *L. acidophilus* (FIG. 6). Overall species prevalence of Korean vaginal isolates were summarized in FIG. 6. Out of the 108 isolates, 5 randomly selected strains were further identified by 16S rDNA sequencing and their sequences were deposited in GenBank as follows: *Lactobacillus* sp. KLB 46 = *Lactobacillus crispatus* (AF243167) (FIG. 7, 8, 9); *Lactobacillus* sp. KLB 58 = *Lactobacillus paracasei* (AF243168) (FIG. 10, 11, 12); *Lactobacillus* sp. KLB 79 = *Lactobacillus*
crispatus (AF243169)(FIG. 13, 14, 15); Lactobacillus sp. KLB 12 =
Lactobacillus fermentum (AF522394) (FIG. 16, 17, 18); Lactobacillus sp.
KLB39 = Lactobacillus salivarius (AY112743) (FIG. 19, 20, 21). Molecular
identities of these 5 strains based on 16S rDNA sequencing results were the
same as 16S rDNA PCR-RFLP data indicating that PCR-RFLP was as
effective as sequencing for species level identification.
Interestingly, all lactobacilli strains including 108 vaginal isolates and 10 type
strains showed the same PCR-RFLP pattern when they were digested with
Rsa I (FIG. 4-4). To know Rsa I -RFLP pattern of other lactic acid
bacteria (LAB), 7 Leuconostoc strains and 2 Bifidobacterium were tested.
As a result, these 9 LAB strains show the different Rsa I -RFLP pattern from
lactobacilli (FIG. 22). Therefore this uniform RFLP pattern could be used
to distinguish lactobacilli at the genus level from other lactic acid bacteria
such as Bifidobacterium which showed various Rsa I -RFLP pattern (Chang
et al., 2002).
FIG. 4-2. Restriction pattern with Cfo I (B) of PCR-amplified product of 16S rDNA genes of 10 type strains and 4 lab isolates. Lane M: 100bp DNA ladder, lanes 1 to 14: L. acidophilus ATCC 4356, L. casei subsp. casei ATCC 393, L. crispatus ATCC 33820, L. delbrueckii subsp. lactis ATCC 15808, L. fermentum ATCC 23271, L. gasseri ATCC 9857, L. jensenii ATCC 25258, L. plantarum ATCC 14917, L. rhamnosus ATCC 7469, L. reuteri DSM 20016, Lactobacillus sp. KLB 1, Lactobacillus sp. KLB 12, Lactobacillus sp. KLB 39, Lactobacillus sp. KLB 46
FIG. 5-1. Dendrogram, obtained from PCR-RFLP patterns with 8 restriction endonucleases, of Korean *Lactobacillus* spp. and 10 type strains. Data was analyzed by NTSYS-PC. A cluster analysis was conducted with similarity estimates by using UPGMA.
FIG. 5-2. Dendrogram, obtained from PCR-RFLP patterns with 8 restriction endonucleases, of Korean Lactobacillus spp. and 10 type strains. Data was analyzed by NTSYS-PC. A cluster analysis was conducted with similarity estimates by using UPGMA.
FIG. 5-3. Dendrogram, obtained from PCR-RFLP patterns with 8 restriction endonucleases, of Korean *Lactobacillus* spp. and 10 type strains. Data was analyzed by NTSYS-PC. A cluster analysis was conducted with similarity estimates by using UPGMA.
FIG. 6. Species prevalence of vaginal isolates. Pie chart of the percentage distribution of *Lactobacillus* spp. from the vaginas of healthy Korean women.
FIG. 7. 16S rDNA of *Lactobacillus* sp. KLB 46 sequences which were deposited in GenBank.
ORIGIN

1  tgccatctac gcagctcag cgacgccaac taacagattt acctcagtaa tgacgttagg
61  aagcgacgc gcggatgggt gatgaacag tggcacaacct gccccatagctgaggatacc
121  acttgaaac agttgtcaat accggataaa aagccagatg gcgtgatcag ctttttattgga
181  gcggcgttaag ctgctgtatat ggagtgccgg cgccggtcag tagctagttg tgaaggttaaa
241  ggcgtaccaaa gcggtatgtg ctaagcgcag ttgagagact gatcggccac attgggagctg
301  agaacgccgc caaactccta cggagaggcg cagtaggggaactttccacg ggcacaccaag
361  tctgagggag caagccgcccg tgag AGAAGGA aggtttctgg gctgtagggta ctgtgtgggtg
421  gtgaagaaggg atagaggtag taacctggtct tatttgagcg gtaatacacc agaagaactac
481  ggcaacctac gtggccagcg ccggcggtaat acgtagttgg ccaagcgtgt cccgatttta
541  agggcgctaaa ggcagcggcg gcggagagatt aagctgtgatg tggaaacgct ccgcttaacc
601  gaggactgtg aagtgcaacacttttcgtg agtgcagaaag gggaggggtg taaacctggt
661  gtacgcgtgg tggcgttaga tataggggag aacaaccagtg ggcggcggcg ctcggagtgc
721  tgcaacctgac gctggcggtc ggaaagatgg gtagcaacaac ggattgata cctgttggtg
781  ccatcggtg aagctgagt gtaagtttg gggagggttc ccgcctctcg tgcgtggttagt
841  aagacatgaa gcaactcgcgc tcggagcagc gacggccaggg gtaggaactca aaggaattgtga
901  cggggggcgg cacaacgctgt ggcagactgt gtaatctcg aagcacaagcg aagaacctta
961  ccaagtctgt gcacattctg ctcattttag agatcacaag tccctctgg gcagcgtgactaag
1021  acagctggttg onctggctgc gtcaacctgtg tgtaagttagat gttggttaaa ttgccccgacac
1081  gcgcctaaacc cttggtattc gtgccgcatg ttaagtttgc cactcattg agactctccccg
1141  tgcaacaagcc gcaggaaaggg gggatatgct gcaacatcga tegccctattag tggcctgctgtg
1201  acacaagcgcg tacaataagctg agataacaga gagaagcagcc tggcgaagcgc aaccgaatctca
1261  tgaagctgtgt ctactcgagct gacgtagctg tgcaacgctg ctgcaaacag agctgtaggtgc
1321  taggacctgc gggatcggc gcggcggtgg gtagacgtta ccatcggccgcc gggacctggagc
1381  gtcaacacsct gggagtctgc aagccgcaac ccggcttggagctg aacaggctagc
1441  tctagggcc gcgcaagtagc tgaggtggtaa tcgtaacaac tgtcgttagg agaacta

FIG. 8. 16S rDNA of *Lactobacillus* sp. KLB 46 sequences.
*Lactobacillus crispatus* strain KLB79 16S ribosomal RNA gene, partial sequence
Length = 1512
Score = 2872 bits (1449), Expect = 0.0
Identities = 1479/1489 (99%)
Strand = Plus / Plus

*Lactobacillus crispatus* strain TL25a 16S ribosomal RNA gene, partial sequence
Length = 1509
Score = 2870 bits (1448), Expect = 0.0
Identities = 1479/1488 (99%), Gaps = 1/1488 (0%)
Strand = Plus / Plus

*Lactobacillus crispatus* strain TL23a 16S ribosomal RNA gene, partial sequence
Length = 1510
Score = 2863 bits (1444), Expect = 0.0
Identities = 1478/1488 (99%), Gaps = 1/1488 (0%)
Strand = Plus / Plus

*Lactobacillus crispatus* strain KC12b 16S ribosomal RNA gene, partial sequence
Length = 1508
Score = 2855 bits (1440), Expect = 0.0
Identities = 1477/1488 (99%), Gaps = 1/1488 (0%)
Strand = Plus / Plus

*Lactobacillus crispatus* strain TL39b 16S ribosomal RNA gene, partial sequence
Length = 1513
Score = 2853 bits (1439), Expect = 0.0
Identities = 1479/1491 (99%), Gaps = 1/1491 (0%)
Strand = Plus / Plus

*Lactobacillus crispatus* strain ATCC33820 16S ribosomal RNA gene, partial sequence
Length = 1518
Score = 2851 bits (1438), Expect = 0.0
Identities = 1478/1490 (99%), Gaps = 1/1490 (0%)
Strand = Plus / Plus

FIG. 9. Sequence homology of *Lactobacillus* sp. KLB 46.
FIG. 10. 16S rDNA of *Lactobacillus* sp. KLB 58 sequences which were deposited in GenBank.
FIG. 11. 16S rDNA of Lactobacillus sp. KLB 58 sequences.
**Lactobacillus paracasei** subsp. **paracasei** strain F31 16S ribosomal RNA gene partial sequence
Length = 1518
Score = 2926 bits (1476), Expect = 0.0
Identities = 1489/1492 (99%), Gaps = 1/1492 (0%)
Strand = Plus / Plus

**Lactobacillus paracasei** gene for 16S rRNA, partial sequence
Length = 1522
Score = 2918 bits (1472), Expect = 0.0
Identities = 1491/1496 (99%), Gaps = 1/1496 (0%)
Strand = Plus / Plus

**Lactobacillus casei** strain BL23 16S ribosomal RNA gene, partial sequence
Length = 1518
Score = 2912 bits (1469), Expect = 0.0
Identities = 1488/1493 (99%), Gaps = 1/1493 (0%)
Strand = Plus / Plus

**Lactobacillus casei** gene for 16S rRNA
Length = 1520
Score = 2892 bits (1459), Expect = 0.0
Identities = 1488/1496 (99%), Gaps = 2/1496 (0%)
Strand = Plus / Plus

**Lactobacillus casei** gene for 16S rRNA
Length = 1521
Score = 2890 bits (1458), Expect = 0.0
Identities = 1488/1496 (99%), Gaps = 2/1496 (0%)
Strand = Plus / Plus

**Lactobacillus casei** gene for 16S rRNA
Length = 1521
Score = 2884 bits (1455), Expect = 0.0
Identities = 1487/1496 (99%), Gaps = 2/1496 (0%)
Strand = Plus / Plus

FIG. 12. Sequence homology of **Lactobacillus** sp. KLB 58.
**LOCUS**  AF243169  1512 bp  DNA  linear  BCT 07-AUG-2002  
**DEFINITION**  Lactobacillus crispatus strain KLB79 16S ribosomal RNA gene, partial sequence. 
**ACCESSION**  AF243169  
**VERSION**  AF243169.1  GI:7621526  
**KEYWORDS**  .  
**SOURCE**  Lactobacillus crispatus  
**ORGANISM**  Lactobacillus crispatus  
Bacteria; Firmicutes; Lactobacillales; Lactobaccillaceae; Lactobacillus.  
**REFERENCE**  1  (bases 1 to 1512)  
**TITLE**  Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences  
**MEDLINE**  21861876  
**PUBMED**  11872120  
**REFERENCE**  2  (bases 1 to 1512)  
**TITLE**  Direct Submission  
**JOURNAL**  Submitted (08-MAR-2000) College of Dentistry, University of Illinois at Chicago, 801 S. Paulina Street, Chicago, Illinois 60612, USA  
**FEATURES**  Location/Qualifiers  
source  1..1512  
/organism="Lactobacillus crispatus"  
/strain="KLB79"  
/db_xref="taxon:47770"  
rRNA  <1..>1512  
/product="16S ribosomal RNA"  
**BASE COUNT**  398 a  336 c  464 g  314 t  

FIG. 13. 16S rDNA of *Lactobacillus* sp. KLB 79 sequences which were deposited in GenBank.
FIG. 14. 16S rDNA of *Lactobacillus* sp. KLB 79 sequences.
*Lactobacillus crispatus* strain TL23a 16S ribosomal RNA gene, partial sequence  
Length = 1510  
Score = 2964 bits (1495), Expect = 0.0  
Identities = 1508/1511 (99%), Gaps = 1/1511 (0%)  
Strand = Plus / Plus

*Lactobacillus crispatus* strain ATCC33820 16S ribosomal RNA gene, partial sequence  
Length = 1518  
Score = 2942 bits (1484), Expect = 0.0  
Identities = 1503/1508 (99%), Gaps = 1/1508 (0%)  
Strand = Plus / Plus

*Lactobacillus crispatus* strain TL25a 16S ribosomal RNA gene, partial sequence  
Length = 1509  
Score = 2942 bits (1484), Expect = 0.0  
Identities = 1491/1492 (99%), Gaps = 1/1492 (0%)  
Strand = Plus / Plus

*Lactobacillus crispatus* strain KC35b 16S ribosomal RNA gene, partial sequence  
Length = 1510  
Score = 2938 bits (1482), Expect = 0.0  
Identities = 1491/1494 (99%)  
Strand = Plus / Plus

*Lactobacillus crispatus* strain KC12b 16S ribosomal RNA gene, partial sequence  
Length = 1508  
Score = 2936 bits (1481), Expect = 0.0  
Identities = 1487/1489 (99%)  
Strand = Plus / Plus

*Lactobacillus crispatus* 16S rRNA gene  
Length = 1559  
Score = 2934 bits (1480), Expect = 0.0  
Identities = 1501/1507 (99%), Gaps = 1/1507 (0%)  
Strand = Plus / Plus

FIG. 15. Sequence homology of *Lactobacillus* sp. KLB 79.
FIG. 16. 16S rDNA of Lactobacillus sp. KLB 12 sequences which were deposited in GenBank.
ORIGIN

1 gcttagarrr rtagcctggc tcaggatgaa cgcacgcggt gtgcctataa catgcaagtcc
gaaacggcgtt gcacatgaa taatgggctc ctggaacac acgctgaccc gcacagctgg
gtgcccgga ggtgagtaac acctgtaa caaagctggc ggtccccaac acatgttgg
181 aacagagag aatccgggct aacatcgggt gctgcatgaa caaacaagtt aagatggtctc
ccttatgtac ctctctgctg gacgctgggt gcatagctct gtttggtggtg taatggctta
cctaagccgcat gatgctagc gacgagctac gaccaatgg ccacaaatg gaggagacac
361 gcacccatct cctaccgcgg gcagagctag ggaatctcct cacatgggac caagctgtag
421 ggaacacac ccggctgatgt aagaaggttt tggccctggt aagctttgtg gttaaagagg
481 aacaggttgtag agagactatg ttatactgtg gacgtgattt aacacaaagtc tacgctttaa
541 ctacggtgca gcagcgcacgg taactcaggt gttgcaacgc tttcgagat tttatggggc
601 taanaagagct tggccgggatt ttaataagt ctatgtgaa cctccgcgggtaaacgcaagaa
661 gctgactcgg aacactgtaa cttcagctgca ctaagctggta gttggaacctcc atgtgtgagc
721 gttggaagtct tagatataag ggaagaacc acgctcggcag ggcctgtgtcct ggtctgcaac
781 tggctggtag actgctagac atgggttgcgc aacaagattct gataacctgg tagtccacgc
841 cgttaacagc gatgcgtgctgtttggaggg tttccgcccct tcaagtcgaggg atgaacgcatt
901 taagaactcgg cacgtgggag taccacccggc aagttgtaaa cttcaaggaa ttggacgggg
961 cccgacaaag ccgttgagcata tgtgtttaaa tgtgaaggtcc gcaaaaaacc tcaraggttc
1021 tggtcctttc gcggccatcctc tagataggtg gctttccctt ccgggagcctc atsacaggtg
1081 gttcggaggtc gtcctgactgtctggtgata gatgtggtt tagtcggcctt cagcagcgcagn
1141 acccctgtta ctatgtgcca gcattaagtt gcggcacttc gttcagacct gcgtggacaaa
1201 cccgagggag tggcggagcg ctgctagata cttgcccttt tattgacccgt gttacacagct
1261 tggcacatagc acgcgtccagtg cggctgacact cgcctggcag gcaagcaaat ctctaaac
1321 ctgttctgatt cggcactgc ccgccccctg ggtgctgctgtt cagacacagtagcctg
1381 cgcgggagatc gcctgggttc tggatagtgc cccggggttt ctacagcaacgc acgctcagc
1441 ctagaggttt gtaacaccct cagctgggtt gcggtaacct ttagagccga gcgcctcctag
1501 gttgacgaga cttagaggtt gaagttgtaa caagttcgcc gtacggagac ccctgcggcgttg
1561 atccacaccttg ctgcagccgtag tattcact

FIG. 17. 16S rDNA of Lactobacillus sp. KLB 12 sequences.
**Lactobacillus fermentum** 16S ribosomal RNA gene, complete sequence
Length = 1540
Score = 2915 bits (1469), Expect = 0.0
Identities = 1525/1541 (98%), Gaps = 3/1541 (0%)
Strand = Plus / Plus

**Lactobacillus fermentum** strain F53 16S ribosomal RNA gene, partial sequence
Length = 1525
Score = 2857 bits (1440), Expect = 0.0
Identities = 1495/1510 (99%), Gaps = 3/1510 (0%)
Strand = Plus / Plus

**Lactobacillus fermentum** strain KC5b 16S ribosomal RNA gene, partial sequence
Length = 1525
Score = 2849 bits (1436), Expect = 0.0
Identities = 1498/1514 (98%), Gaps = 4/1514 (0%)
Strand = Plus / Plus

**Lactobacillus fermentum** 16S ribosomal RNA gene, complete sequence
Length = 1496
Score = 2784 bits (1403), Expect = 0.0
Identities = 1476/1497 (98%), Gaps = 3/1497 (0%)
Strand = Plus / Plus

**Lactobacillus fermentum** 16S ribosomal RNA
Length = 1580
Score = 2780 bits (1401), Expect = 0.0
Identities = 1488/1517 (98%), Gaps = 3/1517 (0%)
Strand = Plus / Plus

**Lactobacillus fermentum** strain PL9006 16S ribosomal RNA gene, complete sequence
Length = 1486
Score = 2750 bits (1386), Expect = 0.0
Identities = 1466/1484 (98%), Gaps = 7/1484 (0%)
Strand = Plus / Plus

FIG. 18. Sequence homology of *Lactobacillus* sp. KLB 12.
FIG. 19. 16S rDNA of Lactobacillus sp. KLB 39 sequences which were deposited in GenBank.
ORIGIN

1 gcttagagtt tgatcctggcc tcaggacgaa cgtcggcggc tgccttataa catgcaa gtgc  
61 gaacgaaact ttcttacacc gaaatgtttgc attcagcga agaaggtgga tggccgacg g  
121 gtagataa ccctggttaa atcgctaaaa gaaagggata acacttggac acaaggtgctta  
181 atacgctgta ctcttaaga gattgatgtaa cattgtaga acaggtgctta  
241 tgtaagggcg cccgagcgtta attaactggtg gttcggttaa ggcctaca cagaatgatg  
301 aecgtggcgca ccctgaggggg atcggcaca aatgagcagcattaga ccagaacggc  
361 acgggaggca gacgttagggga atcttccaca atggacgcaaa tgcctagtgga gcaacgcgc  
421 gtgatgtgaa aagggcttcct gatcggtaaa ctctttggtt agagaaggaac cagagttagttg  
481 gtaactgttc attacgtagac ggtaacttac cagcaagtcagcgtaa cggctcaactgcgtggcagca  
541 gcgcggtttaa taagcgtggt ggacggttgc tcggcgattta tggccgcttta agggaacgc  
601 gcgcggttcttt taagtctgatt gtagaaacct tgtgcttac ccggagtattg cattgaaact  
661 ggaagaacttg agtgcagaaag aggaagttgg cagacacatgt gtagcgggtga aatcctgtgag  
721 tatatggaag aacacccagtgc ggaacacgcct ctcttgggtc tgaatacgact cggagtattg  
781 gagaagctgg tgaagacaac agattagata ccttggttatt ccaacggcgta aacgattagaaat  
841 gtaggtgtt gcgagcggcc aacccctcag tgccgcaagct aacgcaataag cattccggcc  
901 tgggagatgc gcggcgaagg tgtgaactca aaggaatttga cgccgccc ccacagcggt  
961 ggacgcatgag gttataatcc aagcaacgcg aagaacactta ccaagatcttg acatccttgg  
1021 accacctaaag aggattagct ttcttcggcg gacaaagtgga caggtggtggc atggctgtcg  
1081 tcaagctgttg tgtgagatgc tggggttaag tccgcaagcgc gcggcgtacc ggctttgcc  
1141 tgtgcagcgtca ttaaggggtga aactcggccga gacgtcgggt tgcgaaacggcaggaggtg  
1201 gcggagccgta aaggtcatat gccctttagt acctggggtta ccaacgtggc aacagatgctgg  
1261 gtaacagcg tcgaggagcc gcggggttta gcaatctcttat aagggctttcg ctacgttcg  
1321 atgtgaaggc tcaacagcgc tacaataggtcg ccgaatctgct atgaatgctgg aatcagcatgtt  
1381 tgcgctggta acatcctcgg ggctttgtaact cagcggccgg tgcagcgtcagc tgcagcgttcg  
1441 acacaacaag cccgtggggcg cagcgaacgg aaccaagcgct ctaaggggtg acagaggtattt  
1501 ggagatcgtaa ctaacagcggg tgcgggttagc agaaggtggc tgggtgtagcactccttg  
1561 cgctgaactcg ttaagttggcg agggtggttgc gcgtggcgtgc gtcggtggc 

FIG. 20. 16S rDNA of Lactobacillus sp. KLB 39 sequences.
**Lactobacillus salivarius subsp. salivarius** 16S ribosomal RNA gene, complete sequence
Length = 1587
Score = 2912 bits (1469), Expect = 0.0
Identities = 1538/1555 (98%), Gaps = 5/1555 (0%)
Strand = Plus / Plus

**Lactobacillus salivarius** 16S ribosomal RNA gene, partial sequence
Length = 1515
Score = 2886 bits (1456), Expect = 0.0
Identities = 1505/1516 (99%), Gaps = 4/1516 (0%)
Strand = Plus / Plus

**Lactobacillus salivarius subsp. salicinii** strain JCM 1044 16S ribosomal RNA gene. partial sequence
Length = 1519
Score = 2880 bits (1453), Expect = 0.0
Identities = 1502/1513 (99%), Gaps = 4/1513 (0%)
Strand = Plus / Plus

**Lactobacillus salivarius subsp. salicinii** strain JCM 1042 16S ribosomal RNA gene partial sequence
Length = 1519
Score = 2880 bits (1453), Expect = 0.0
Identities = 1502/1513 (99%), Gaps = 4/1513 (0%)
Strand = Plus / Plus

**Lactobacillus salivarius subsp. salivarius** 16S ribosomal RNA gene, complete sequence
Length = 1517
Score = 2880 bits (1453), Expect = 0.0
Identities = 1502/1513 (99%), Gaps = 4/1513 (0%)
Strand = Plus / Plus

**Lactobacillus salivarius subsp. salicinii** strain JCM 1047 16S ribosomal RNA gene partial sequence
Length = 1519
Score = 2872 bits (1449), Expect = 0.0
Identities = 1501/1513 (99%), Gaps = 4/1513 (0%)
Strand = Plus / Plus

FIG. 21. Sequence homology of *Lactobacillus* sp. KLB 39 sequences.
4.4 Whole cell protein analysis

The reproducibility of SDS-PAGE was estimated by duplicate loading of independent, duplicate whole cell protein extracts from 108 vaginal isolates on gels. SDS-PAGE results showed various whole cell protein patterns (FIG 23). The similarity correlation between SDS-PAGE of whole cell protein pattern and PCR-RFLP was 76%.
V. Discussion

A study of the surveys of antimicrobial agent resistance in lactobacilli showed that a variety of methods had been used (Etest: Charteris et al., 2001; agar dilution: Goldstein et al., 2000; disk diffusion: Charteris et al., 1998; and microbroth: Klein et al., 2000). In order to determine MICs of vaginal isolates, broth dilution method was performed by the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) with slightly modification (National Committee for Clinical Laboratory Standards, 2002). The MICs at which 90% of growth are inhibites for metronidazole is for all lactobacilli (more than 200 μg/mL) (TABLE 5). The concentrations of metronidazole tested in our study ranged from 0.01 μg/mL to 200 μg/mL and at these concentrations, O.D. of test culture was higher than the growth control. Similar results were previously reported where concentrations between more or equal to 128 μg/mL and less or equal to 256 μg/mL stimulated the growth of Lactobacillus spp. However, high concentrations of metronidazole (i.e. between 1,000 μg/mL and 4,000 μg/mL), partially inhibited the growth while concentrations more or equal to 5,000 μg/mL completely suppressed the growth (Simoes et al, 2001). Resistance of lactobacilli to
metronidazole might be because of the absence of hydrogenase activity (Church et al., 1996). Thus, these results might help in the determination of the antimicrobial susceptibilities of the *Lactobacillus* species.

In this study, PCR-RFLP was used to identify the vaginal lactobacilli. PCR-RFLP is rapid and cheaper than conventional phenotypic identification methods. When PCR amplified 16S rDNAs of vaginal isolates and 10 reference *Lactobacillus* spp. were digested by *Rsa I* they showed same electrophoresis band pattern (FIG. 4). It was not effective for identification of vaginal lactobacilli but it could used to distinguish lactobacilli at the species level from other lactic acid bacteria such as *Bifidobacterium* and *Leuconostoc* which showed various *Rsa I*-RFLP pattern (FIG. 22).

Based on PCR-RFLP results, most of vaginal isolates belonged to two species *L. crispatus*, *L. acidophilus*. It is noteworthy that the predominant lactobacilli in Japanese women were found to be *L. crispatus* (52.7%) and *L. gasseri* (20.8%) by DNA-DNA hybridization analysis (Song et al., 1999). More recently, most vaginal *Lactobacillus* strains from women of geographically separated countries have been found to belong to three species, *L. crispatus*, *L. gasseri* and *L. jensenii* (Pavlova et al., 2002). According to the previous report by Pavlova et al. (2002), most vaginal *Lactobacillus* strains from women of multiple geographically-separated countries belonged
to three species: *L. crispatus*, *L. gasseri* and *L. jensenii*. The high degree of species consistency in the vaginal indigenous lactobacilli among several different women’s populations is significant for the future development of bacterial replacement therapy. The present study with 16S rDNA sequence analysis confirms the DNA homology studies of Giorgi et al. (1987), Antonio et al. (1999) and Song et al. (1999), who found that the most prevalent species of vaginal lactobacilli in women from Italy, the United States and Japan, respectively, were homologous to the type strains of *L. crispatus*, *L. gasseri* and/or *L. jensenii*.

SDS-PAGE whole cell analysis was used for another method for species level identification. SDS-PAGE electrophoresis remains the standard method of species determination (Weinrichter et al., 2001). Whole cell protein of vaginal isolates which analyzed by SDS-PAGE showed various pattern (FIG. 23).

Both PCR-RFLP and SDS-PAGE whole cell protein analysis were reliable for the identification to the species level of all the isolates.
VI. References


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Manachini, P. L., and C. Parini. 1983. DNA restriction endonuclease cleavage patterns, DNA sequence similarity and phenotypical characteristics in some strains of Lactobacillus helveticus and Lactobacillus jugurti. Ant. Leeuwenhoek. 49:143-152


감사의 글

2000년 8월 대학원이라는 곳에 처음 설을 달고 한학기 동안 실험실 생활을 경험했을 때마다, 교수님께서 한학기 동안 생활해 본 느낌을 물으셨던 일이 있었었습니다. 그 때 이미 생각했던 것보다 시간이 무척 빨리 지나가고 2년이란 시간이 그리 질지향을 것임은 예상하고 있었지만 어느덧 그 2년이 흐르고 이제 대학원 생활을 뒤돌아보고 있습니다. 돌아보면 후회 되는 일도 많지만 대학원이라는 곳을 경험할 수 있었음을 후회할 적은 없으며 그런 기회가 나에게 주어졌던 것을 항상 감사하고 있습니다.

세심한 배려와 관심으로 꾸준히 지켜 waktu 주시며 격려를 아끼지 않으신 소재성 지도 교수님께 감사의 마음을 전합니다. 또한 실험실과 허병기 교수님, 허태현 교수님, 구윤호 교수님, 김은기 교수님, 김동일 교수님, 윤현식 교수님, 이철균 교수님 그리고 김영수 교수님께도 감사의 말씀 드립니다.

대학원 생활에서 얻은 지식 못지않게 깊은 것이 선후배와의 인연이라고 할 수 있습니다. 유전자공학 실험실에서의 처음 대학원 생활에 잘 적응할 수 있도록 도와주신 현구오빠, 경민오빠, 항상 우리를 위해 이벤트를 준비해 주시던 지철오빠, 세 명의 부사수들을 이끄느라 마음고생이 많았던 석용오빠, 말없이 저커와 주고 먹으러 할 때 위로의 말을 아끼지 않던 제상선배에게 감사의 말씀을 전하고 싶습니다. 방방으로 말없이 많은 일을 이끌어 나가던 은택오빠, 그 누구보다도 오랜 시간을 함께하며 많은 도움을 주었던 다연이, 말없이 어느 곳에서도 빛이 되는 주현이, 가장 많은 시간을 같이 보내 동기 한명 한명에게도 감사하다는 말을 전하고 싶습니다. 같이 지낸 시간은 적었지만 풍부한 경험으로 많은 도움을 주셨던 흥운표 박사님께도 감사드립니다. 동기가 없어 의롭지만 꾸준히 열심히 하는 민지 그리고 올해 신입생이 될 영준선배, 윤도, 정민이, 미경이도 실험실 생활 열심히 하려리 믿으며 계획한 일 모두 이루길 바랍니다.

다른 방에 있지만 항상 도움을 주신 경은언니에게 너무 감사하다는 말씀 전하고 싶고, 트리오에게 많이 관심 가져 주시던 상욱오빠께도 감사하다는 말씀 전합니다.
그리고 환경방 백석오빠, 세포방 상윤오빠, 규화오빠, 지숙언니, 식품방 수임언니, 반응방 세경신범, 강모오후에도 김사의 말씀 전하며 그 외 생물공학과 대학원 선배님들께도 김사의 말씀 드립니다. 또한 2 년을 인하대학교 생물공학과에서 함께한 동기들께도 고마운 말씀 전하고 싶습니다.

비록 같은 길을 걷는 못하였지만 그들로 많이 도와준 경민오빠, 후상선배, 원식이, 홍재에게도 감사한다는 말씀 전하고 싶습니다.

대학 4 년 동안 항상 함께 있어 힘을 되었던 신희, 수영이, 혜이, 광신오빠, 진수오빠 그리고 그 외 친구들, 어울림 때 힘을 되어주었던 정훈오빠, 기덕오빠, 기섭오빠, 선진이, 규복이, 성현이, 그 외 동문 선배들 동기들에게 감사하다는 말씀 전하고 싶습니다.

고등학교시절 저의 미래를 결정하는데 많은 도움을 주신 권성일 선생님, 정삼훈 선생님, 임용은 선생님께 감사한다는 말씀을 전합니다.

10 여년을 한결같이 함께 있어주신 미연이, 주영이, 경숙이, 대영이, 영애, 경화, 의성이, 동복이에게도 감사의 말씀 전합니다.

20 세기의 마지막 해를 캐나다에서 같이 보내며 미련 추억을 간직할 수 있게 도와준 혜진언니, 성은언니, 장준오빠, 성희오빠, 윤혁오빠, 해준이에게 감사의 말씀 전하며 풋보이트 마사코, 가나미에게도 감사의 마음을 전하고 싶습니다.

그 누구보다도 저를 아끼주시고 사랑해주시며 저의 발전을 위해 지원을 아끼지 않으셨으며 이만큼 잘 키우시느라 너무나 고생하신 부모님께 가슴 깊이 감사의 말씀 드리며 사랑의 마음을 전하고 싶습니다. 절없는 손녀를 따뜻하게 지켜봐 주시는 할머니, 외할머니, 외할머니, 딸씨 지켜봐 주시는 고모, 고모부님, 이모, 이모부님, 삼촌께도 감사의 말씀 전합니다. 항상 누나를 위해주는 하나뿐인 동생에게도 감사한다라는 말씀 전하고 싶습니다.

결에서 항상 아껴주고 힘을 되어주며 변함없이 밀어주고 지켜주고 주고 있는 그 사람에게 깊은 감사의 말씀 전합니다.

이제 새로운 곳, 사회로 나가게 됩니다. 관심과 사랑으로 여기까지 올 수 있게 도와주신 부모님과 교수님의 기대에 어긋나지 않도록 바르게 그리고 모든 일에 더 열심히 하는 데, 제자가 되도록 노력하겠습니다. 마지막으로 제 걸에 있는 모든 분들이 건강하시고 행복하시길 바라며 이 놀문을 바칩니다.