

Lactobacillus Genotyping by Fluorophore-Enhanced Repetitive PCR (FERP) and Capillary Electrophoresis

Ania Szary

The use of probiotics has been increasingly investigated, especially in the area of gastrointestinal health. Probiotics are ingested microorganisms that under favorable conditions can prevent and treat specific diseases. Probiotics are also commonly referred to as “healthy” or “good bacteria.” These bacteria have been found to aid in the protection against certain gastrointestinal infections and other common conditions such as vaginal infections and antibiotic-induced diarrhea. There are several mechanisms that are responsible for the beneficial effects of probiotics. For example, the presence of probiotics can cause the modulation and stimulation of immune function, the production of anti-microbial substances that inhibit or kill the pathogenic bacteria, competition for nutrients, and inhibition of mucosal and epithelial adherence. Therefore, the use of probiotics suggests an exciting alternative and advancement in preventive and therapeutic treatment of gastrointestinal diseases.¹

Lactic acid bacteria, or Lactobacilli, are currently the best-studied bacteria used for probiotics. The genus *Lactobacillus* comprises greater than 30 recognized species. They are gram-positive rod-shaped bacteria that commonly colonize the gastrointestinal tract. Additionally, they have never been related to disease and are commonly found in dairy-containing products. They present characteristics that would enable them to be good candidates for probiotic therapy. However, only specific strains of *Lactobacillus* may have probiotic effects. Strain identification is needed for comparative measures during clinical trials of probiotic approaches and for gene therapy. Thus, it is imperative that a rapid,

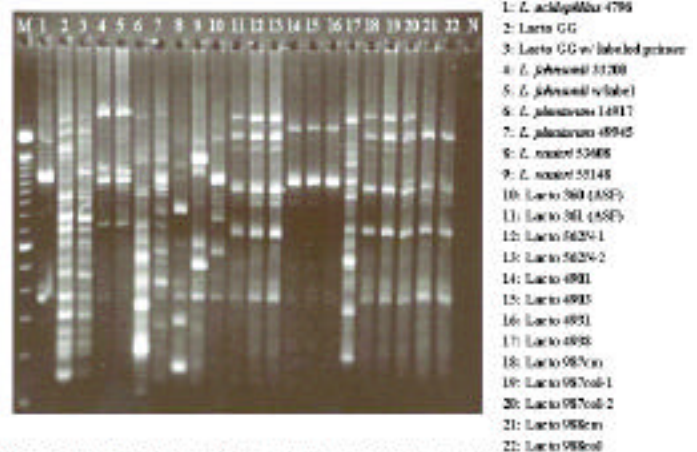


Figure 1: rep-PCR gel of *Lactobacillus* isolates using DCCO ATR primers.

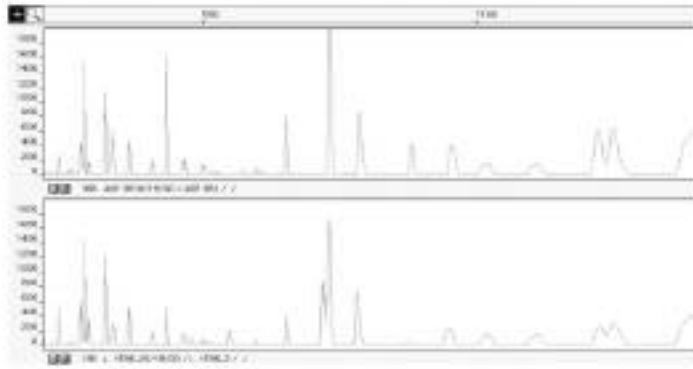


Figure 2: GeneScan® profiles of lactobacilli amplified using Rep-It primers. Electropherogram of marine *Lactobacillus* 1596-2 as compared to *Lactobacillus* ASF 361.

effective, and accurate method can be employed to easily decipher strains of bacteria.

Various approaches have been used to identify strains of *Lactobacillus*. Randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) have been used to distinguish *Lactobacillus* strains with limited success.² Alternative genotyping and sequence-based approaches are necessary to produce an adequate technique for classification and strain tracking.

Versalovic proposed a high-resolution DNA fingerprinting method using fluorophore-enhanced repetitive element PCR (FERP) (see genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction and Automated DNA fingerprinting of pathogenic bacteria by fluorophore-enhanced repetitive sequence-based polymerase chain reaction).³ In 1992, Lupski and Weinstock described the random chromosomal distribution of dispersed repetitive sequences in numerous prokaryotic genomes, which is similar to what is seen in eukaryotic genomes. These sequences differ in size, do not encode proteins, and are located in various parts of the bacterial chromosome. Therefore, it was determined that the DNA sequence arrangement in the bacterial genome may consist of short repeats interspersed with longer single-copy sequences. Based on this information, a technique known as rep-PCR was formed. Rep-PCR is a type of polymerase chain reaction that targets the repetitive sequences in bacterial genomes using specific primers that are designed complementary to bacterial interspersed repetitive sequences.¹ These primers then enable the amplification of differently sized DNA fragments consisting of unique DNA sequences lying between these elements. Electrophoresis is then used to differentiate these differently sized fractions according to their

length on an ethidium bromide stained agarose gel. This provides a complex band, or “fingerprint” pattern, which reflects varying distances between oligonucleotide primer binding sites at repetitive sequence targets and is sometimes referred to as a unique “barcode.” These fingerprint patterns are unique to each bacterial strain, depicting rep-PCR’s success in its ability to differentiate various strains.^{3,4} An example of fingerprints generated from rep-PCR is shown in Figure 1 using BOX A1R primers. These fingerprints are complex, with approximately 10 to 20 bands for each strain. It is also easily seen that the fingerprints for the same strain are similar, indicating the usefulness of rep-PCR. Rep-PCR has multiple applications in molecular epidemiology: in human, animal, and plant diagnosis of infectious pathogens, in quality assurance control for typing strains important for industrial and diagnostic applications, in probiotics, and for the study and monitoring of genetically engineered microorganisms released into the environment. Therefore, rep-PCR shows strong potential not only as a simple generic taxonomic tool but also as a diagnostic method.

However, traditional electrophoresis does not provide optimal fingerprints to easily detect variations between strains. Therefore, a technique utilizing fluorescence detection and capillary gel electrophoresis, which allows for the exact determination of base pairs of a band and the concentrations of amplicons, was studied. Statistical analysis to determine similarity between strains can be used via computer-assisted methods. Thus, one would have the ability to determine similarity and clonal relationships, which would enable the identification of tightly linked genetic “clusters” that colonize multiple animals. One could determine which strains are highly adapted for persistent colonization of the mammalian gastrointestinal tract. Computer-assisted methods, using DNA fingerprints obtained from either gel electrophoresis or capillary gel electrophoresis, can provide statistical analyses that determine the similarity between strain-based on their genotype. Therefore, a computer-assisted method was used to determine the ability of rep-PCR to accurately characterize bacterial strains.

Methodology

To obtain *Lactobacillus* organisms, natural isolates of *Lactobacillus* were obtained from stool, cecum, and colon biopsies from healthy mice. To verify that these bacteria were *Lactobacillus*, gram stain morphology, biochemical testing, and the resistance to vancomycin and penicillin were used. Candidate organisms were

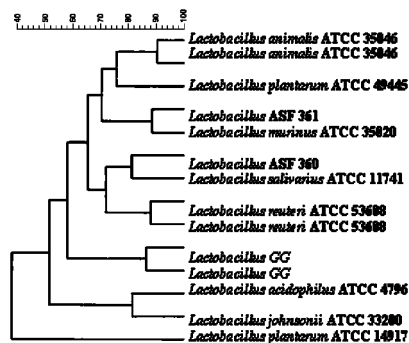


Figure 7: FERP Fingerprint Profile Analyses.

GelComparII-generated dendrograms were created using fingerprints from Genescan®. Strains here represent two separate Genescan runs, with separate rep-PCR reactions. Numerical values (horizontal axis) represent percent similarity.

tion and phylogenetic studies. Therefore, several of the *Lactobacillus* strains were sequenced. Also, GenBank's 16S rDNA database provided some of the reference sequences. *Lactobacillus* similarity and percent identity charts were based on these sequences and constructed with DNASTAR's Lasergene 99 version 4.03. Then, the dendrograms obtained from FERP analysis were compared to those based on the sequence analysis of the various *Lactobacillus* strains to determine whether there was any correlation between the dendrograms, and thus to determine the effectiveness of utilizing FERP as a classification technique.

Results

It was determined that profiles based on Genescan analysis of fluorophore-enhanced rep-PCR effectively distinguish various *Lactobacillus* strain. For example, in Figures 2 and 4, five different strains of *Lactobacillus* are depicted. Each strain has a different profile with varying positions of peaks. The positions of peaks determine the amount of base pairs a fragment has, and the height of the peak determines the quantity of that fragment. However, the quantity of the fragment is based on the amount of DNA, so one can only compare the height of profiles with the same amount of DNA. Also, if the peaks are similar, then the strains are also similar. This similarity can only be determined through statistical analysis (i.e., GelCompar II). Furthermore, it was determined that two fluorophore-enhanced rep-PCRs performed on two different days yielded similar profiles, as seen in Figure 5. Therefore, one can conclude that the results obtained are reproducible. In addition, Genescan provides an electrophoretogram of one strain in

only 40 minutes. The rapid turnaround time and high discriminatory power of Genescan support the use of Genescan as an optimization technique for fluorophore-enhanced rep-PCR.

GelCompar II analysis of FERP showed significant similarities to sequence-based analysis. Figure 6 illustrates GelCompar analysis of an agarose gel, while Figure 7 shows GelCompar analysis of Genescan electrophoretograms. Figure 8 depicts a dendrogram based on sequencing data. It was determined that statistical analyses based on rep-PCR profiles of unknown murine (mouse) *Lactobacilli*, i.e. *Lactobacillus* 1660, grouped most isolates as either *L. animalis/murinus/ASF 361* or *L. reuteri*. Also, fluorophore-enhanced rep-PCR distinguished *L. reuteri*-like isolates, which were grouped together by both rep-PCR and 16S rDNA sequencing. Furthermore, supporting rep-PCR-based identification, phylogenetic analysis of 16S rDNA sequences also grouped most murine *Lactobacillus* isolates into two groups, either *L. reuteri*-like or *L. animalis/murinus/ASF 361*. Therefore, one can conclude that fluorophore-enhanced rep-PCR can be used as a classification technique for varying strains and is useful for genotyping *Lactobacillus*.

Further studies should be performed on a greater variety of *Lactobacillus* species to verify the results of this experiment. Also, it must be determined if FERP would be an appropriate technique for genotyping other bacterial species. If FERP proves to be an effective technique for classification, a classification library for various bacterial species could be created. Furthermore, FERP could provide an alternative for the identification of bacterial species and strains, in which species and strains could be quickly, easily, and effectively identified for various purposes. ☐

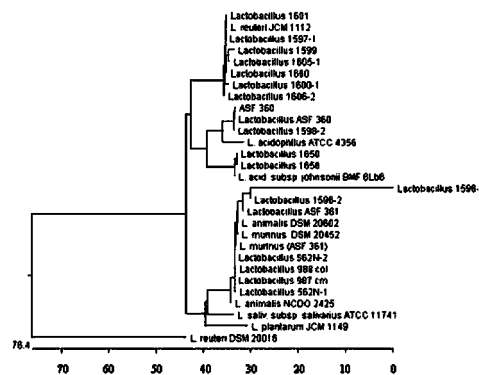


Figure 8: 16S rDNA similarity dendrogram was generated using the Clustal method with a weighted residue table in Megalign (Lasergene 99, DNASTar v 4.03). Two distinct clades were generated: I. *L. reuteri*-like isolates, and II. *L. animalis/murinus*-like.

Acknowledgments

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Recommended Reading

1. Versalovic J, Schneider M, de Bruijn FJ. "Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction." *Meth Mol Cell Bio*, 1994;5:25-40.
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2. Tynkkynen S. "Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains." *Applied and Environmental Microbiology*, 1999;65:3908-3914.
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