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RFLP of 16S-ITS rDNA region to differentiate Lactobacilli at species level

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Summary

The 16S-ITS (internal transcribed spacer) region of the *rrn* operon was amplified by polymerase chain reaction (PCR). The amplification products were analysed by restriction fragment length polymorphism (RFLP) using a set of restriction enzymes, *Alu*I, *Hae*III, and *Taq*I. Restriction pattern analyses revealed that *Taq*I restriction enzyme could clearly differentiate the nine reference strains of *Lactobacillus* used in the study.

Introduction

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S–23S–5S) and they are transcribed as a single polycistronic RNA molecule (Luz *et al.* 1998). The 16S rRNA gene sequence has been used for assessing bacterial phylogenies at genus level (Abd-El-Haleem *et al.* 2002; Shaver *et al.* 2002). Below the genus level, additional rDNA sequences have been necessary. The intergenic/internal transcribed spacer (ITS), also known as the intergenic spacer region (ISR) (Jensen *et al.* 1993; Fisher & Triplett 1999; Daffonchio *et al.* 2000; Toth *et al.* 2001; Abd-El-Haleem *et al.* 2002; Shaver *et al.* 2002) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (*boxA*) (Abd-El Haleem *et al.* 2002). ITS is generally found in multiple copies in most bacterial genomes. Since ITS are hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio *et al.* 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez *et al.* 1999). Amplification size polymorphisms of the ITS alone have been used for identification at the species level (Jensen *et al.* 1993; Cho & Tiedje 2000; Abd-El Haleem *et al.* 2002).

In the present work, the 16S rDNA and ITS region were amplified as a single amplicon. The amplification products were then digested with *Taq*I restriction enzyme. Restriction patterns were analyzed in a gel documentation system and the similarity between the strains was determined automatically. Results obtained from nine strains of *Lactobacillus* indicated that 16S-ITS rDNA RFLP could differentiate these bacteria at species level.

Materials and methods

Strains and growth conditions

Reference strains *Lactobacillus brevis* (NRRL B-4527), *Lactobacillus coryniformis* ssp. *coryniformis* (NRRL B-4391), *Lactobacillus delbrueckii* ssp. *bulgaricus* (NRRL B-548), *Lactobacillus delbrueckii* ssp. *delbrueckii* (NRRL B-443), *Lactobacillus coryniformis* ssp. *torquens* (NRRL B-4390), *Lactobacillus curvatus* (NRRL B-8768), *Lactobacillus farciminis* (NRRL B-4566), *Lactobacillus fermentum* (NRRL B-4524), and *Lactobacillus pentosus* (NRRL B-227) were obtained from Agricultural Research Service Culture Collection (ARS/NRRL; Peoria, IL., USA) and were kept at −80 °C in 20% glycerol. The strains were grown in MRS agar, (peptone, 10 g/l; meat extract, 10 g/l; yeast extract, 5 g/l; D(+)-glucose, 20 g/l; 0,1% (vol/vol) Tween 80; K_2HPO_4, 2 g/l; Na-acetate, 5 g/l; tri-ammonium citrate, 2 g/l; $MgSO_4 \cdot 7H_2O$ 0.2 g/l; $MnSO_4 \cdot 4H_2O$, 0.05 g/l; 15 g/l agar, pH 6.6) for 24 h at 30 °C.

Preparation of genomic DNA

Two methods (Ausubel *et al.* 1994; Cardinal *et al.* 1997) were combined. Briefly, 5 ml of overnight cultures were harvested and cells were suspended in 200 μl sucrose solution (25% sucrose, 30 mg lysozyme/ml in TE). Samples were then incubated for 1 h at 37 °C. After the cell lysis, 370 μl proteinase K solution (1 mg proteinase K/ml in TE) and 30 μl SDS solution (10%)

were added and the samples were further incubated for 1 h at 37 °C. Following deproteinization, 100 μl NaCl (5M) and 80 μl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7M NaCl) were added and the samples were then incubated for 10 min at 65 °C. Chloroform extraction was performed twice using one equal volume of chloroform (chloroform/isoamyl alcohol, 24:1). DNA wool was obtained by the addition of isopropanol (one equal volume) and was then washed in 500 μl ethanol (70%). DNA was pelleted, dried, and dissolved in 100 μl RNase solution (100 μg/ml RNase in TE). After incubation for 1 h at 37 °C, the sample volumes were adjusted to 400 μl with TE and DNA was solubilized by alternating heat shocks, for 10 min at 80 °C, and for 20 min at −20 °C. Phenol/chloroform extraction was performed and DNA precipitated with 0.5M NaCl (final concentration) and two volumes of ethanol (99%). Pellets were washed in 500 μl ethanol (70%). Finally DNA was solubilized as above in 50–250 μl TE, depending on the solubility of the DNA pellet. Samples were stored at −20 °C.

Amplification of the 16S rDNA – ITS region

Polymerase chain reactions were performed in a volume of 50 μl containing approximately 500 ng of genomic DNA as the template, 0.2 mM dNTPs, 1.5 mM MgCl, 10 pmol of each of the DNA primers, 1 α × PCR buffer (Fermentas), and 1.25 u *Taq* DNA polymerase (Fermentas). PCR conditions were as follows: an initial denaturation step for 5 min at 94 °C; 40 cycles of amplification including 1 min denaturation at 94 °C, 1 min annealing at 42 °C, and 1 min elongation at 72 °C, steps. The reactions were terminated with a final extension step for 10 min at 72 °C. Amplifications were performed in a Mini Cycler System (MJ Research INC, USA). DNA primers used in the experiments were, forward, 5′-AGAGTTTGATCCTGGCTCAG-3′ (Mora *et al.* 1998) and reverse, 5′-CAAGGCATCCACCGT-3′ (Jensen *et al.* 1993). The forward primer is complementary to the upstream of 16S rDNA of *E. coli* K12 strain (Accession number: AE000452, nucleotides from 1 to 20), and the reverse is complementary to the upstream sequences of 23S rDNA of *E. coli* 278710 (Accession number: AJ278710, nucleotides from 18 to 32).

Restriction fragment length polymorphism (RFLP)

One fourth of the amplification products, approximately 700 ng, were digested with 10 units of *Taq*I (Fermentas) restriction enzyme. Digestion samples were overlaid with mineral oil and carried out overnight in a water bath at 65 °C. Before and after the digestion, DNA was extracted twice with chloroform and ethanol precipitated (Sambrook *et al.* 1989). Half of the restriction products were resolved in 2.5% agarose (Applichem, low EEO) by gel electrophoresis for 2.5 h at 60 A in 1x TAE (40 mM Tris-acetate and 1 mM EDTA pH 8.0), and stained with ethidium bromide (1 μg/ml, final concentration). Image of the gel was recorded for further analysis in a gel documentation system (Vilber Lourmat, France).

RFLP pattern analysis

RFLP patterns were stored into and refined by Adobe Photoshop 7.0 and were then analyzed by using BIO-1D++ software (Vilber-Lourmat, France). The similarity between strains was determined automatically by specifying the formula of Nei and Li (Vilber-Lourmat). Strain clustering was performed by the unweighted pair group method with arithmetic averages, UPMGA, (BIO-1D++, Vilber-Lourmat).

Results and discussion

The length of 16S rDNA-ITS amplification products varied from 1500 to 2000 bp. These fragments were purified and digested with three frequent cutter restriction enzymes: *Alu*I, *Hae*III, and *Taq*I. After chloroform extraction, digestion products were separated by agarose gel electrophoresis. In the report, only the results of *Taq*I restriction enzyme digestion were included because this enzyme was found to be the most discriminative among the three restriction enzymes used.

RFLP with TaqI restriction enzyme

RFLP with *Taq*I restriction enzyme could differentiate the nine *Lactobacillus* strains into three major groups (Figure 1 A–C). Group A contained five strains (lanes 1–5). Consistent with the expectations, two subspecies of *Lb. coryniformis* (lanes 1 and 2) produced identical restriction patterns, thus the similarity between them was 100%. The *Lb. pentosus* restriction pattern (lane 3) appeared to be the closest to those of the two *Lb. coryniformis* strains, with approximately 67% similarity. *Lb. brevis* and *Lb. fermentum* strains, on the other hand, yielded quite dissimilar digestion profiles within themselves (lanes 4 and 5, respectively), and they both were equidistant to the other members of the group. Two members of group B (lanes 6 and 7) were the two subspecies strains of *Lb. delbrueckii* and these also produced identical digestion profiles. Although included within the same group, *Lb. farciminis* restriction profile (lane 8) showed only 50% similarity to those of *Lb. delbrueckii* ssp. strains. The most outstanding feature of the pattern analysis was that the restriction profile of *Lb. curvatus* formed a group of its own (group C), and it appeared to be the most distant strain in the whole dendrogram.

Initially 150 local isolates of lactic acid bacteria (LAB), including both cocci and bacilli, were analyzed by 16S rDNA RFLP, and it was not possible to unambiguously differentiate each species of LAB genera (data not shown). This problem prompted us to extend

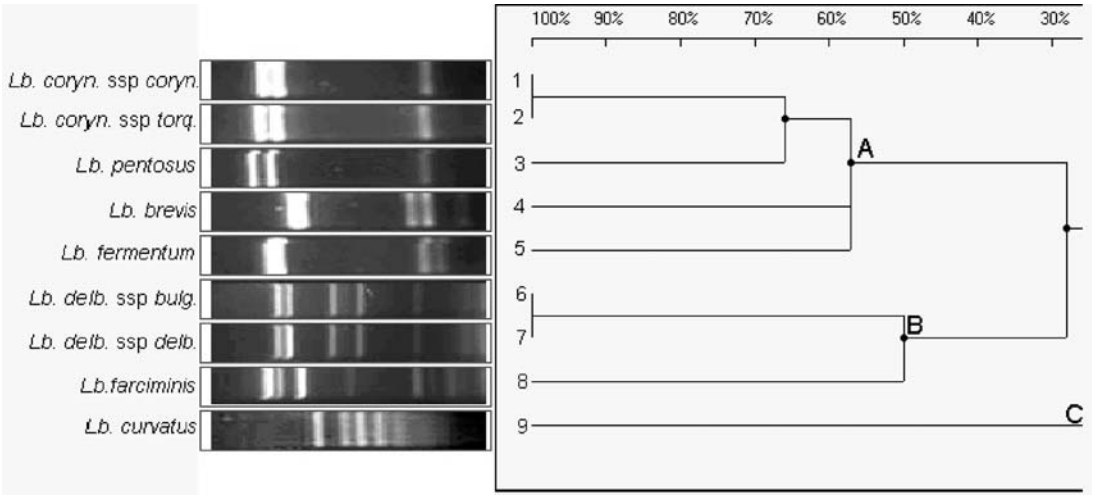

Figure 1. Cluster analysis of 16S rDNA–ITS RFLP profiles: The restriction profiles were produced with *Taq*I restriction enzyme. Strain names (left) were given with respective RFLP profiles (gel strips) and with the dendrogram. Lane 1, *Lactobacillus coryniformis* ssp. *coryniformis* (NRRL B-4391). Lane 2, *Lactobacillus coryniformis* ssp. *torquens* (NRRL B-4390). Lane 3, *Lactobacillus pentosus* (NRRL B-227). Lane 4, *Lactobacillus brevis* (NRRL B-4527). Lane 5, *Lactobacillus fermentum* (NRRL B-4524). Lane 6, *Lactobacillus delbrueckii* ssp. *bulgaricus* (NRRL B-548). Lane 7, *Lactobacillus delbrueckii* ssp. *delbrueckii* (NRRL B-443). Lane 8, *Lactobacillus farciminis* (NRRL B-4566) and lane 9, *Lactobacillus curvatus* (NRRL B-8768). Before the dendrogram, the bands within the each of the restriction profiles were normalised against a DNA size marker (1 kb DNA ladder, Fermentas).

the 16S rDNA sequence down to the 5′-end of 23S rDNA sequences, covering the whole ITS region. Results demonstrated that by using 16S rDNA-ITS RFLP it would be possible to discriminate species of Lactobacilli. Discriminative power of the approach should of course be revealed by further applications. The results also indicated that among the three frequent cutter restriction enzymes used, *Taq*I was the most discriminative enzyme.

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