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Differentiation of Three *Lactobacillus rhamnosus* Strains (E/N, Oxy, and Pen) by SDS–PAGE and Two-Dimensional Electrophoresis of Surface-Associated Proteins

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SDS–PAGE of extracted surface-associated proteins of *Lactobacillus rhamnosus* strains E/N, Oxy, and Pen, was performed. The obtained protein patterns allowed differentiation of the examined strains, which was not accomplished by the commonly used RAPD genotypic method. The differentiation by the SDS–PAGE method proved to be a useful tool for strain-specific identification, which was further confirmed by 2DE analysis. Therefore, it can be used as an alternative or complementary method for both conventional and genotypic identification procedures, especially when closely related lactobacilli isolates are identified.

Keywords: *Lactobacillus*, polyacrylamide gel electrophoresis, random amplification of polymorphic DNA, surface-associated proteins

Genus *Lactobacillus* is a group of Gram-positive, non-sporing, catalase-negative microorganisms that are associated with habitats that are rich in nutrients, such as plant materials, dairy products, and meat. They are also common inhabitants of the human gastrointestinal tract and the vagina, contributing to the stabilization of the microflora and maintaining the colonization resistance against pathogens [2, 19]. Many studies have shown that lactobacilli may also decrease lactose intolerance, adverse effects of inflammatory bowel disease, diarrhea, constipation, food allergy, and even colon cancer [12, 15]. Moreover, evidence exists on the positive effects of probiotics on mucosal immunity and blood cholesterol level [9, 10]. Because of their beneficial influence on human health, many of them are used as food additives and probiotic pharmaceuticals, for instance *L. rhamnosus* strains E/N, Oxy, and Pen, which are an ingredient of Lakcid, medicine commonly used for the prevention of antibiotic-associated diarrhea in children in Poland [5, 15, 23]. According to results obtained previously using classical microbiological methods, it was confirmed that Lakcid contains three different *L. rhamnosus* strains that differ from one another in carbohydrate utilization patterns, colony and cell morphology, and in antibiotic sensitivity (J. Bardowski *et al.* 2005, 3rd Probiotics, Prebiotics and New Foods. ATTI Abstracts, Rome: University Urbaniana).

Owing to enormous demands on probiotic bacteria, manufacture of high-quality pharmaceuticals requires rapid and accurate identification of lactobacilli at the strain level. *Lactobacillus* species commonly are identified using variety of genomic methods, for example, randomly amplified polymorphic DNA (RAPD) [21] analysis, pulsed-field gel electrophoresis (PFGE) [17], the analysis of rRNA sequences [18], and ribotyping [14]. Some of them are very laborious, time-consuming, or relatively expensive, and in most cases, these methods could not distinguish two isolates because of their low discriminative power at the strain level. Therefore, an approach based on SDS–PAGE of surface-associated or whole-cell proteins seems to be a more rapid and simple alternative for genetic methods in lactobacilli strains differentiation [4, 13].

The objective of this study was to compare the discriminatory power of two rapid techniques (RAPD and SDS-PAGE) of surface-associated proteins for the identification of closely related lactobacilli species and strains. Additionally, we used SDS–PAGE and two-dimensional (2D) electrophoresis analysis of surface-associated proteins for the differentiation of *L. rhamnosus* strains E/N, Oxy, and Pen.

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MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *L. rhamnosus* strains used in this study were obtained from Biomed Serum and Vaccine Production Plant Ltd. in Lublin, Poland. These strains are also available in the Institute of Biochemstry and Biophysics, The Polish Academy of Sciences, under the numbers 2593 (*L. rhamnosus* Pen), 2594 (*L. rhamnosus* E/N), and 2595 (*L. rhamnosus* Oxy). Other microorganisms, *Lactobacillus acidophilus* K1, *Lactobacillus casei* LBY, and *Lactobacillus plantarum* Ib, originate from the Division of Food Science Institute of Animal Reproduction and Food Research of The Polish Academy of Sciences, Olsztyn, Poland. All strains were routinely cultured in Man–Rogosa–Sharpe broth (BTL, Poland) at 37°C.

DNA Isolation

Total bacterial DNA was extracted according to the method described by Varmanen [22]. Briefly, the mid-log-phase cells in 3 ml of MRS were harvested, washed twice with 6.7% (w/v) sucrose–50 mM Tris–1 mM EDTA (pH 8.0), and finally resuspended in 380 μl of the same buffer. One hundred μl of a 50 mg/l lysozyme solution (MP Biomedicals) and 100 U of mutanolysin (Sigma) were added, and the cells were incubated at 37°C for 1 h. Next, 50 μl of 0.25 M EDTA–50 mM Tris (pH 8.0) was added, and the cells were then treated with 30 μl of 20% (w/v) sodium dodecyl sulfate–50 mM Tris–20 mM EDTA (pH 8.0). The proteins were digested by adding 20 μl of proteinase K (20 mg/ml) (Fermentas) and samples were incubated for 1 h at 50°C. DNA was isolated from the cell debris by a phenol–chloroform extraction method.

Polymerase Chain Reaction

The PCR-specific reaction described by Alander [1] was used to identify *L. rhamnosus* strains. Reaction mixtures (20 μl) consisted of 0.25 U of *Taq* DNA polymerase (Fermentas), 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer (5'-CTTGCAT CTTGATTTAATTTTG-3', forward; 5'-CCGTCAATTCCTTTGAGTTT-3', reverse), 50 ng of bacterial DNA, and PCR buffer (Fermentas). Amplifications were performed with template denaturation at 94°C for 5 min; followed by 30 cycles of annealing for 30 s at 62°C in cycles 1 to 10, at 60°C in cycles 11 to 20, and at 58°C in cycles 21 to 30, with extension for 1 min at 72°C and denaturation for 40 s at 94°C; and a final extension for 10 min at 72°C. RAPD analysis was performed with several different random primers (RB, 5'-AGTCAG CCAC-3' [21]; OPL-05, 5'-ACGAGGCAC-3'; PL1, 5'-ACGCGCCCT-3' [20]; OPG25, 5'-GGAAGTCCTG-3'; OPG28, 5'-AGGCATCGTG-3'; P4, 5'-GTGTGCCCCA-3'; P7, 5'-CTACGCTCAC-3' [6]), using only one primer and two primers in the multiplex RAPD reaction. Twenty μl of reaction mixtures consisted of 0.25 U of *Taq* DNA polymerase (Fermentas), 200 μM of each deoxynucleoside triphosphate (Fermentas), 0.4 μM of random primer, 100 ng of template, and PCR buffer (Fermentas). The amplification profile was as follows: 5 cycles of 1 min at 94°C, 2 min at 27°C, 2 min at 72°C; 45 cycles of 1 min at 94°C, 2 min at 32°C, 2 min at 72°C; and 1 cycle of 15 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis with 1.2% (w/v) agarose in 0.5× Tris–borate–EDTA buffer (10×89 mM Tris, 89 mM boric acid, and 25 mM EDTA, pH 8.0) (TBE). Gels were stained with ethidium bromide (0.5 μg/ml) and visualized under UV light.

Preparation of Surface-Associated Proteins

The surface-associated proteins were extracted from the bacterial strains with 8 M urea. Cells from 100 ml of 24-h culture were harvested by centrifugation, washed twice in PBS, pH 7.4, and then incubated for 1 h at 37°C in 10 ml of 8 M urea. The suspensions were centrifuged to remove bacterial biomass and the supernatants were filtered through a 0.2-μm filter and then dialyzed against 10 mM Tris-HCl, pH 7.4, at 4°C for 48 h. The obtained extracts were lyophilized and resuspended in 2 ml of water. Protein concentration was determined by the method of Bradford.

SDS–PAGE and 2D Electrophoresis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli and stained with Coomassie Brilliant Blue R-250. Resolving and stacking gels were of 10% and 4% acrylamide, respectively. The gel was 20 cm long and 1.0 mm thick. Protein extract samples were resuspended in two volumes of the sample buffer [62.5 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) Bromophenol Blue, 5% (v/v) β-mercaptoethanol]. For denaturation, samples were heated for 5 min at 95°C. Electrophoresis was performed using Protean II xi Cell (Biorad) according to the manufacturer's procedures.

For two-dimensional electrophoresis analysis, 50-μg protein samples were prepared with a 2D Clean-Up kit (Amersham Biosciences) and resuspended in rehydration buffer (Biorad) containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (v/v) Bio-Lyte 3/10 ampholyte, and 0.001% (w/v) Bromophenol Blue (Biorad). Proteins were subjected to active rehydration (12 h; 50 V; 20°C) on 7-cm, pH 3–10, nonlinear IPG strips and then to isoelectric focusing by using Protean IEF (Biorad) for a total of 14 kVh at 20°C under mineral oil to prevent evaporation. IPG strips were equilibrated in 6 M urea, 2% (v/v) glycerol, 0.375 M Tris-HCl (pH 8.8), reduced with 2% (w/v) DTT, and alkylated with 135 mM iodoacetamide. After focusing, the IEF strips were applied to 10% polyacrylamide gels, sealed with 0.5% (w/v) low melting point agarose containing Bromophenol Blue in 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, and then electrophoresis was carried out at 200 V for 40 min using a Mini Protean (Biorad). Proteins were visualized by staining with Coomassie Brilliant Blue R-250. All experiments were performed twice.

RESULTS AND DISCUSSION

PCR Screening for the *L. rhamnosus* Strains

For the identification and detection of the *L. rhamnosus* strains, species-specific primers designed into the 16S rRNA gene have been used. Fig. 1 shows the electrophoresis patterns of the PCR products obtained for 6 lactobacilli when the specific primers were used. All of the *L. rhamnosus* strains (E/N, Oxy, and Pen) produced the same size of amplicon, whereas the other *Lactobacillus* strains, including *L. acidophilus* K1, *L. casei* LBY, and *L. plantarum* Ib, were negative in the PCR screening with the primer set used in this study. The specificity of the primer pair was determined by the forward primer, and the expected size of

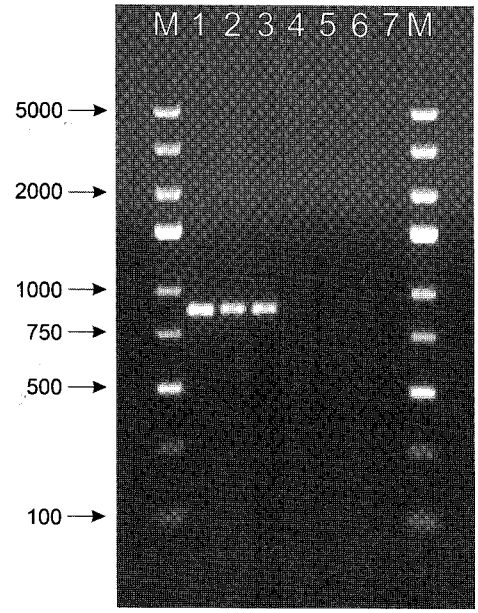

Fig. 1. PCR reaction with species-specific primers.
Lanes: M, DNA molecular weight marker (in base pairs); 1, *L. rhamnosus* E/N; 2, *L. rhamnosus* Oxy; 3, *L. rhamnosus* Pen; 4, *L. acidophilus* K1; 5, *L. casei* LBY; 6, *L. plantarum* Ib; 7, negative control (no DNA added).

the PCR product was 863 bp, which was in good agreement with the sizes reported earlier [1, 21].

RAPD Analysis

Several RAPD primers and primer combinations were used to evaluate the discriminative power of this method in differentiation of lactobacilli strains [6, 20, 21]. We have also tested different reaction conditions and reaction mixture compositions (data not shown). Our results showed that distinct fingerprint patterns were revealed only between different bacterial species (at the species level) in both reactions with one random primer (Fig. 2A) and multiplex RAPD reactions (Fig. 2B). Earlier studies have shown the application of RAPD to discriminate between different species [11], subspecies [8], and even strains [20] of *Lactobacillus*. In this work, it was shown that the RAPD method is not capable of distinguishing between different strains of the same species. However, the procedure turned out to be a useful genetic tool in differentiation of lactobacilli at the species level.

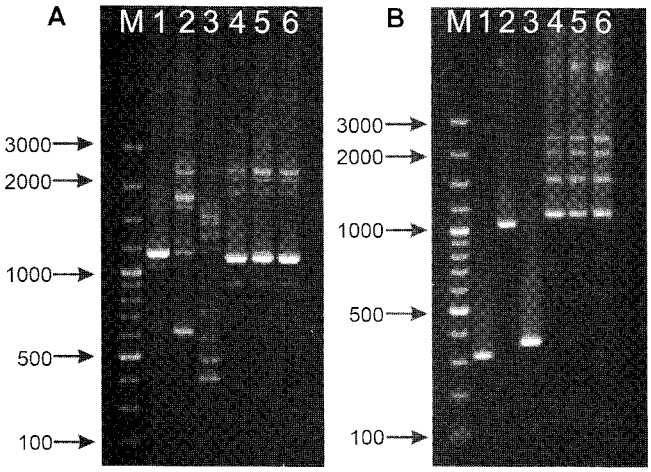

Fig. 2. Band patterns obtained after RAPD amplifications of *Lactobacillus* strains with (**A**) primer Rb, and (**B**) two different primers, P7 and PL1.
Lanes: M, molecular weight marker (in base pairs); 1, *L. acidophilus* K1; 2, *L. casei* LBY; 3, *L. plantarum* Ib; 4, *L. rhamnosus* E/N; 5, *L. rhamnosus* Oxy; 6, *L. rhamnosus* Pen.

Differentiation of Strains by SDS–PAGE

Numerous extraction methods for the removal of proteins from the surface of bacterial cells exist [16, 24]. In our study, different procedures to prepare the cell-wall extract were analyzed, including the use of 1 M LiCl, 2 M guanidine hydrochloride, 8 M urea, and 0.2 M glycine, pH 2.2 (data not shown). The use of urea releases large quantities of the surface-associated proteins, which after SDS–PAGE analysis allow us to obtain well-defined and reproducible patterns. Moreover, the urea extraction did not generate cellular lysis during the process of surface-associated proteins removal, which was confirmed by the obtained protein profiles from cells before and after the extraction experiment. The electrophoretic profiles of the examined strains showed the presence of numerous bands

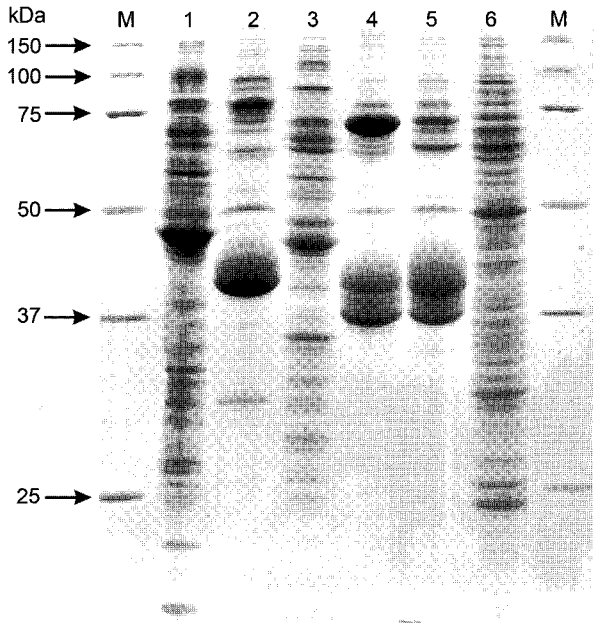

Fig. 3. SDS–PAGE fingerprinting of surface-associated proteins of *Lactobacillus* strains.
Lanes: M, molecular weight marker; 1, *L. acidophilus* K1; 2, *L. casei* LBY; 3, *L. plantarum* Ib; 4, *L. rhamnosus* E/N; 5, *L. rhamnosus* Oxy; 6, *L. rhamnosus* Pen.

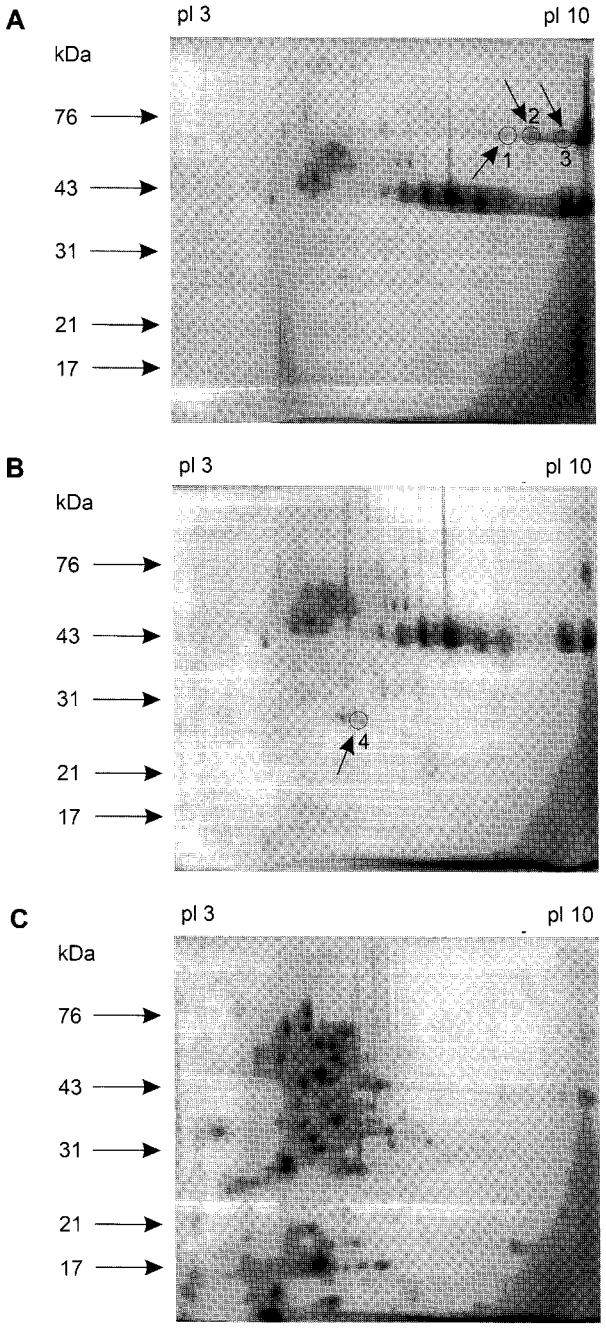

Fig. 4. Two-dimensional protein profiles of surface-associated proteins obtained for (**A**) *L. rhamnosus* E/N, (**B**) *L. rhamnosus* Oxy, and (**C**) *L. rhamnosus* Pen.
The proteins (about 50 µg) were first sparated in a nonlinear pH gradient of 3–10, followed by separation in an SDS–PAGE (10%) and Coomassie Brilliant Blue R-250 staining. Different protein spots between *L. rhamnosus* E/N and Oxy are marked by spot number plus an open circle.

of different molecular masses between 20 and 150 kDa (Fig. 3). The analysis of different protein bands obtained after electrophoresis confirmed the effectiveness of this method for *Lactobacillus* species differentiation [3, 7]. Additionally, a clear overall visual difference in the surface-associated protein patterns among *L. rhamnosus* strains was detected (Fig. 3, lanes 5–7). *L. rhamnosus* strains E/N and Oxy showed a high similarity of protein patterns in comparison with *L. rhamnosus* strain Pen, which gave a large number of protein bands not detected for E/N and Oxy. However, there were some minor differences in SDS–PAGE patterns within the areas of 50–75 kDa between the E/N and Oxy strains. Therefore, this method is working well for identification of *L. rhamnosus* strains used in our study and appears to be an efficient taxonomic tool for rapid differentiation of other closely related lactobacilli strains [4]. To confirm the reliability of SDS–PAGE of surface-associated proteins as a differentiation method for *L. rhamnosus* strains, protein samples were subjected to two-dimensional electrophoresis analysis (Fig. 4A–4C). After electrophoretic separation, the 2D maps were analyzed by PDQuest (Biorad). The approximate number of spots detected was 105±8 for *L. rhamnosus* Pen, 44±2 for *L. rhamnosus* E/N, and 41±2 for *L. rhamnosus* Oxy. The obtained results confirm significant differences of surface-associated protein bands between *L. rhamnosus* Pen and the other examined strains, Oxy and E/N. The 2D maps for *L. rhamnosus* E/N and *L. rhamnosus* Oxy were characterized by high similarity, but clear differences between these two strains were detected, as was for SDS–PAGE analysis.

The application of genetic and proteomic tools revealed different discriminating powers for *Lactobacillus* taxonomy. RAPD analysis has the potential to give species-specific information but failed to distinguish among strains of *L. rhamnosus* tested in this investigation. Differentiation of *L. rhamnosus* strains E/N, Oxy, and Pen was only possible using SDS–PAGE of extracted surface-associated proteins under the conditions described in this paper. This method turned out to be a reliable and specific procedure, allowing rapid differentiation of *Lactobacillus* strains. Moreover, easy gel interpretation is advantageous in comparison with fingerprinting of whole-cell extracts. In order to obtain rapid and accurate results, traditional methods need to be combined with molecular typing methods like SDS–PAGE of extracted surface-associated proteins, irrespective of the fact that microbiological and biochemical methods are routinely used for the identification of industrial lactic acid bacteria. SDS–PAGE fingerprinting gave a substantial power for strain-specific identification. Therefore, it can be used as an alternative or complementary method for both conventional and genotypic identification procedures.

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