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# Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species

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*Teanpaisan R, Dahlén G. Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral* Lactobacillus *species*.
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**Background/aims:** The genus *Lactobacillus* has been associated with dental caries in humans, although it is seldom speciated due to lack of simple and nonlaborious identification methods. A considerable heterogeneity among *Lactobacillus* species has been demonstrated. The purpose of this study was to develop simple methods combining restriction fragment length polymorphism analysis of polymerase chain reaction (PCR)-amplified 16S rRNA (16S rRNA gene PCR-RFLP) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the identification of 13 reference strains of *Lactobacillus*.
**Methods:** The 16S rRNA gene sequences were amplified by PCR using universal primers and digestion of PCR products with the restriction endonucleases, *Hpa*II and *Hae*III. The 16S rRNA gene PCR-RFLP is reproducible and has been proved to be useful for differentiating *Lactobacillus* strains to species level. Seventy-seven *Lactobacillus* isolates from a Thai population were used to show the applicability of the identification test.
**Results:** PCR-RFLP alone had limitations, because the RFLP patterns of *Lactobacillus casei* and *Lactobacillus rhamnosus* and of *Lactobacillus acidophilus* and *Lactobacillus crispatus* showed similar patterns; however, these could be differentiated by SDS-PAGE. Of the 77 isolates, 38 were identified as *Lactobacillus fermentum*, 25 as *L. rhamnosus,* 5 as *Lactobacillus salivarius,* 5 as *L. casei,* 3 as *L. acidophilus* and 1 as *Lactobacillus plantarum*.
**Conclusion:** 16S rRNA gene PCR-RFLP, using *Hpa*II and *Hae*III, together with SDS-PAGE protein profiles could be an alternative method for the identification of oral *Lactobacillus* strains to species level, and may be applicable for large-scale studies on the association of *Lactobacillus* to dental caries.

Key words: *Lactobacillus*; polymerase chain reaction-restriction fragment length polymorphism analysis; 16S rRNA genes; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; protein profiles

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The genus *Lactobacillus* contains a diverse assembly of gram-positive, catalase-negative, nonsporulating, rod-shaped organisms and includes more than 25 species (5, 8). They inhabit a wide variety of habitats, including the oral cavity. Frequently isolated from carious dentin lesions, *Lactobacillus* have been associated with the development of dental caries due to their aciduric characteristics (1, 2, 10). These bacteria have been identified by conventional methods such as morphological and

phage typing, which are sometimes inconsistent. Other methods for identification previously tested showed variable results. Commercial kits based on biochemical, enzymatic and fatty acid analysis were found to be unstable, perhaps due to their susceptibility to different bacterial physiological conditions. Consequently, in most of the dental literature they are not specified and are referred to only as lactobacilli.

Several molecular methods have been utilized during the past few years to differentiate *Lactobacillus* strains to the species level (3, 6), including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is an accepted technique used for species identification of many organisms and is also described as a simple and convenient method for the identification of *Lactobacillus* strains to species level (4).

The usefulness of the restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal RNA genes (16S rRNA) genes (PCR-RFLP) for the identification of streptococci was recently demonstrated (13). The method was shown to be simple and convenient, giving reproducible results.

In this study, we demonstrate that application of SDS-PAGE in combination with 16S rRNA gene PCR-RFLP is useful for identification of *Lactobacillus* strains to species level.

## Material and methods

### Bacterial and culture conditions

Thirteen reference strains of *Lactobacillus*, each representing one species, were studied: *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus casei* (CCUG 31610), *Lactobacillus crispatus* (ATCC 33820), *Lactobacillus curvatus* (ATCC 25601), *Lactobacillus delbrueckii* (ATCC 9649), *Lactobacillus fermentum* (ATCC 14931), *Lactobacillus gasserie* (ATCC 33323), *Lactobacillus paracasei* (CCUG 32212), *Lactobacillus plantarum* (ATCC 14917), *Lactobacillus reuteri* (CCUG 33624), *L. rhamnosus* (ATCC 7469), *Lactobacillus salivarius* (ATCC 11741), and *Olsenella (*formerly *Lactobacillus) uli* (CCUG 31166). Cultures were incubated in 5% $CO_2$ or in 10% $H_2$, 10% $CO_2$ and 80% $N_2$ for 2–3 days.

Seventy-seven clinical isolates were recovered from saliva samples collected by the spatula method (9) from 58 children and adults living in Southern Thailand. The spatula was placed directly onto selective Rogosa medium and incubated anaerobically. The colonies were tentatively identified as *Lactobacillus* based on their growth on Rogosa agar, colonial morphology, gram staining, being catalase negative and by the protein profiles of whole cell total protein using SDS-PAGE (4, see below). After pure culture, the 77 isolates were kept at −80°C until used. Ten isolates representing the identified species were used for identification by fermentation using the API System (API 50 CH, bioMerieux, Marcy-l'Etoile, France).

### DNA extraction

DNA samples were prepared using the modified method of Gevers et al. (7). Cells from one plate were harvested into 1 ml of sterile distilled water, centrifuged and frozen for at least 1 h at −20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspended in 300 µl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA). Lysis buffer 75 µl (TES containing 1330 U/ml mutanolysin and 40 mg/ml lysozyme) was added and the suspension was incubated at 37°C. After addition of 40 µl preheated (37°C) 20% SDS in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. TE buffer 100 µl was added and the lysate was extracted with 1 vol. phenol/chloroform/isoamylalcohol. The phases were separated by centrifugation (12,000 × *g*, 10 min). The aqueous phase was carefully mixed with 70 µl 5 M NaCl and 1 ml isopropanol, and DNA was precipitated on ice for at least 15 min. DNA was collected by centrifugation (12,000 × *g*, 10 min) and the pellet washed in cold 70% ethanol. The pellet was re-suspended in 250 µl of distilled water, 250 µl of 1.4 M sodium chloride and 50 µl of 10% CTAB and incubated 60°C for 30 min. The mixture was then extracted with 1 vol. phenol/chloroform/isoamylalcohol. After centrifugation, the DNA from the aqueous phase was precipitated with 1 ml cold absolute ethanol and washed in cold 70% ethanol. The DNA pellet was finally dissolved in distilled water and stored at −20°C.

### 16S rRNA genes PCR-RFLP

The 16S rRNA gene sequences were amplified by PCR using the universal primer 8UA and 1492 R (13). The primer sequences were: 8UA, 5′-AGA GTT TGA TCC TGG CTC AG-3′; and 1492R, 5′-TAC GGG TAC CTT GTT ACG ACT T-3′. The 50-µl reaction mixture contained 100 ng template DNA, 5 µl 10 × buffer, 1.0 unit *Taq* DNA polymerase, 0.2 mM of dNTP, 1.0 µM of each primer and 2.0 mM $MgCl_2$. Amplification was done using a PCR System 2400 (Applied Biosystems, Foster City, CA) programmed as follows: 15 min at 95°C for initial heat activation and 35 cycles of 1 min at 94°C for denaturation, 1 min at 60°C for annealing and 1.5 min at 72°C for extension and 10 min at 72°C for a final extension. The PCR products of 16S rRNA genes were individually digested with *Hpa*II or *Hae*III (New England Biolab, Ipswich, MA) according to the manufacturer's instructions. Digestion products were run on 7.5% polyacrylamide gel, stained with silver staining kit (Amersham, Little Chalfont, Bucks, UK). A DNA ladder of EZ load 100 bp Rulers (Bio-Rad, Hercules, CA) was used as a size marker.

### SDS-PAGE

Organisms were grown for 2–3 days on Rogosa plates, harvested, washed, and resuspended in distilled water. The cells were sonicated for 20 s with a cell disrupter to lyse the bacterial cell wall. For SDS-PAGE, equal volumes of sonicated cells and SDS sample buffer (0.125 M Tris buffer pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue) were mixed and boiled for 5 min. The samples were electrophoresed on 12% polyacrylamide gels, and stained in Coomassie blue.

## Results

The RFLP profiles generated by *Hae*III and *Hpa*II digestion are shown in Fig. 1A and Fig. 1B, respectively. *Hae*III produced different RFLP patterns for seven reference species: *L. curvatus* (ATCC 25601), *L. delbrueckii* (ATCC 9649), *L. fermentum* (ATCC 14931), *L. gasserie* (ATCC 33323), *L. reuteri* (CCUG 33624), *L. salivarius* (ATCC 11741), and *O. uli* (CCUG 31166). The patterns for *L. casei* (CCUG 31610), *L. paracasei* (CCUG 32212), *L. rhamnosus* (ATCC 7469) and *L. plantarum* (ATCC 14917) (Fig. 1A, lanes 2, 3, 4, and 6) were similar. The patterns of *L. acidophilus* (ATCC 4356) and *L. crispatus* (ATCC 33820) were the same (Fig. 1A, lanes 8 and 13).

In contrast, PCR-RFLP using *Hpa*II could clearly distinguish most of the 13 strains (Fig. 1B), except for *L. casei*

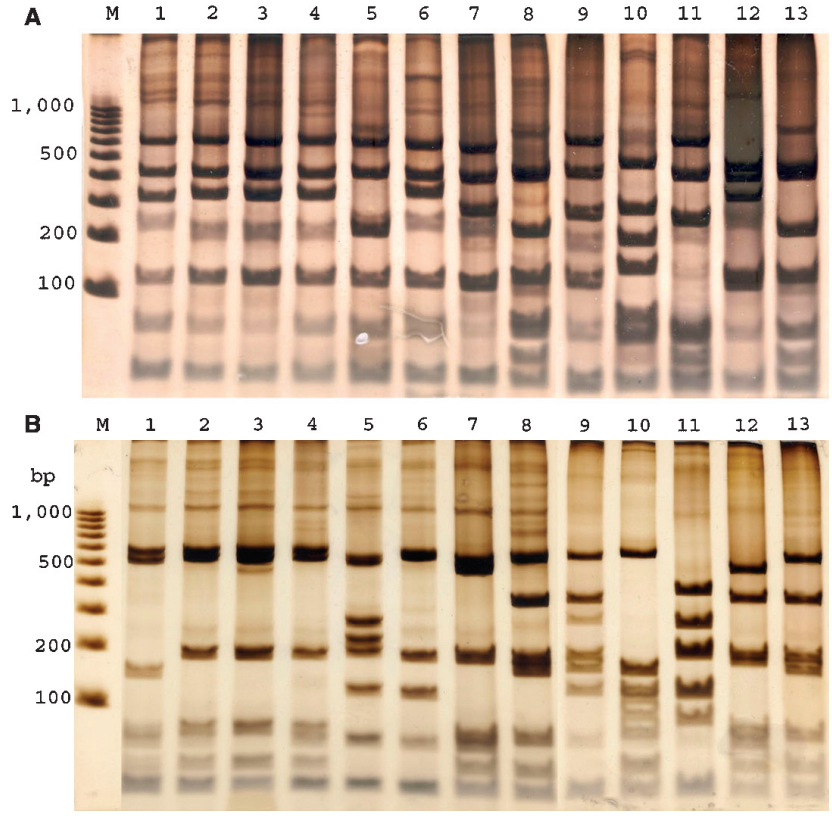


*Fig. 1.* PCR-amplified 16S rRNA genes of 13 reference strains of *Lactobacillus* digested with *Hae*III (panel A) and *Hpa*II (panel B). Lanes: 1, *L. curvatus* (ATCC 25601); 2, *L. casei* (CCUG 31610); 3, *L. paracasei* (CCUG 32212); 4, *L. rhamnosus* (ATCC 7469); 5, *L. fermentum* (ATCC 14931); 6, *L. plantarum* (ATCC 14917); 7, *L. salivarius* (ATCC 11741); 8, *L. acidophilus* (ATCC 4356); 9, *L. delbrueckii* (ATCC 9649); 10, *O. uli* (CCUG 31166); 11, *L. reuteri* (CCUG 33624); 12, *L. gasserie* (ATCC 33323); 13, *L. crispatus* (ATCC 33820). M, Molecular size markers (100 bp DNA Ladder, Bio-Rad).

(CCUG 31610) and *L. rhamnosus* (ATCC 7469) (Fig. 1B, lanes 2 and 4), and *L. acidophilus* (ATCC 4356) and *L. crispatus* (ATCC 33820) (Fig. 1B, lanes 8 and 13). It was noticed that using *Hpa*II, the pattern of *L. salivarius* (ATCC 11741) looked quite similar to that of *L. casei* and *L. rhamnosus* (Fig. 1B, lanes 7, 2 and 4, respectively); however, the two bands at about 500 bp of *L. salivarius* were clearly lower than the bands of *L. casei* and *L. rhamnosus*. *L. salivarius* could be easily distinguished from *L. casei* and *L. rhamnosus* using *Hae*III (Fig. 1A, lanes 7, 2 and 4). A reproducible minor band could be seen below the 500 bp in the PCR-RFLP of *L. paracasei* (CCUG 32212) using *Hpa*II, which gave a unique pattern different from that of *L. casei* and *L. rhamnosus*.

The whole cell protein profiles using SDS-PAGE of 13 *Lactobacillus* reference strains are shown in Fig. 2. Clear and reproducible differences in the profiles of 13 species could be demonstrated. Interestingly, the RFLP patterns of *L. casei* and *L. rhamnosus* generated either by *Hpa*II or *Hae*III, which were identical, showed different protein profiles using SDS-PAGE (Fig. 2, lanes 2 and 5). The protein profiles obtained for *L. casei* were very close that of *L. paracasei* with the exception of a heavy band between 14.4 and 21.5 kDa (Fig. 2, lane 3). The protein profiles of *L. acidophilus* and *L. crispatus* were also different (Fig. 2, lanes 7 and 8). Thus, combining the results of 16S rRNA gene PCR-RFLP using *Hpa*II, *Hae*III, and SDS-PAGE allowed 13 species of lactobacilli to be distinguished from one another. The scheme for differentiating between the various species of *Lactobacillus* spp. using PCR-RFLP and SDS-PAGE is shown in Table 1.

The 77 clinical isolates tentatively identified as *Lactobacillus* were examined using SDS-PAGE and PCR-RFLP using *Hpa*II and *Hae*III digestion. All 77 isolates were identified to species level. The most common species of *Lactobacillus* recovered from saliva of this Thai population were *L. fermentum* (38/77), followed by *L. rhamnosus* (25/77), *L. salivarius* (5/77), *L. casei* (5/77), *L. acidophilus* (3/77) and *L. plantarum* (1/77) (Table 2). Ten isolates were further identified using the API fermentation test. Only five of these isolates corresponded sufficiently with the PCR-RFLP and SDS-PAGE identification (Table 3).

## Discussion

*Lactobacillus* spp. are acidophilic gram-positive rods, commonly recognized by their growth on Rogosa agar plates (pH 5.2). The mode of fermentation divides this genus into three groups – two anaerobic groups of homolactic fermenters, which producing only lactic acid, and heterolactic fermenters, which produce both lactic and acetic acids, and the facultative group of heterofermenters (10). All three groups differ enzymatically; however, biochemical tests often give imprecise specifications. Due to complicated and uncertain identification, *Lactobacillus* spp. is usually referred only as

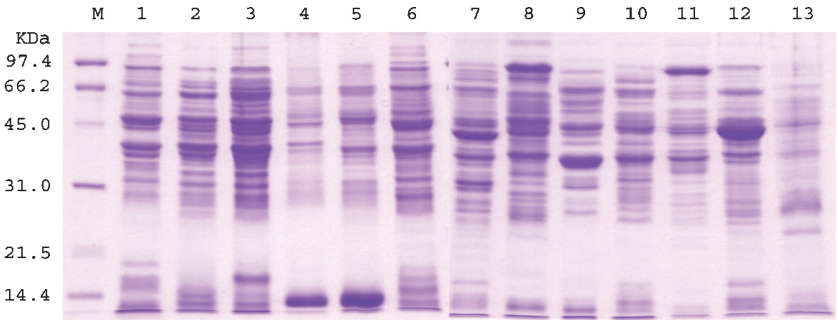


*Fig. 2.* SDS-PAGE protein profiles of 13 reference strains of *Lactobacillus*. Lanes: 1, *L. curvatus* (ATCC 25601); 2, *L. casei* (CCUG 31610); 3, *L. paracasei* (CCUG 32212); 4, *L. plantarum* (ATCC 14917); 5, *L. rhamnosus* (ATCC 7469); 6, *L. salivarius* (ATCC 11741); 7, *L. acidophilus* (ATCC 4356); 8, *L. crispatus* (ATCC 33820); 9, *L. delbrueckii* (ATCC 9649); 10, *L. gasserie* (ATCC 33323); 11, *L. fermentum* (ATCC 14931); 12, *L. reuteri* (CCUG 33624); 13, *O. uli* (CCUG 31166). M, Molecular size markers (Bio-Rad).

*Table 1.* Scheme for differentiating between the various strains of *Lactobacillus* spp.

| Strains | PCR-RFLP | | SDS-PAGE |
|---|---|---|---|
| | *Hpa*II | *Hae*III | |
| *L. curvatus* (ATCC 25601) | √ | – | – |
| *L. delbrueckii* (ATCC 9649) | √ | – | – |
| *L. fermentum* (ATCC 14931) | √ | – | – |
| *L. gasserie* (ATCC 33323) | √ | – | – |
| *L. reuteri* (CCUG 33624) | √ | – | – |
| *L. paracasei* (CCUG 32212) | √ | – | – |
| *L. plantarum* (ATCC 14917) | √ | – | – |
| *O. uli** (CCUG 31166) | √ | – | – |
| *L. salivarius* (ATCC 11741) | √ | (√) | – |
| *L. casei* (CCUG 31610) | √ | – | √ |
| *L. rhamnosus* (ATCC 7469) | √ | – | √ |
| *L. acidophilus* (ATCC 4356) | √ | – | √ |
| *L. crispatus* (ATCC 33820) | √ | – | √ |

( ), confirmation required.
*Formerly *L. uli.*

*Table 2.* Specification of 77 clinical isolates of *Lactobacillus* as identified by PCR-RFLP and SDS-PAGE

| Species | No. of isolates |
|---|---|
| *L. fermentum* | 38 |
| *L. rhamnosus* | 25 |
| *L. salivarius* | 5 |
| *L. casei* | 5 |
| *L. acidophilus* | 3 |
| *L. plantarum* | 1 |
| Total | 77 |

*Table 3.* Correlations between 10 *Lactobacillus* isolates identified with PCR-RFLP/SDS-PAGE and API fermentation test

| Isolates | PCR-RFLP/ SDS-PAGE | API |
|---|---|---|
| 24/4 | *L. salivarius* | *L. fermentum* |
| 36/1 | *L. acidophilus* | *L. rhamnosus* |
| 36/2 | *L. acidophilus* | *L. fermentum* |
| 44/3 | *L. fermentum* | *L. rhamnosus* |
| 51/1 | *L. rhamnosus* | *L. rhamnosus* |
| 79/1 | *L. salivarius* | *L. salivarius* |
| 105/4 | *L. casei* | *L. paracasei/ L. casei** |
| 145/4 | *L. plantarum* | *L. plantarum* |
| 326/2 | *L. fermentum* | *L. fermentum* |
| 343/2 | *L. rhamnosus* | *L. paracasei* |

*Doubtful distinction.
PCR-RFLP/SDS-PAGE, polymerase chain reaction-restriction fragment length polymorphism/ sodium dodecyl sulfate-polyacrylamide gel electrophoresis. API, API 50 CH (bioMerieux).

'lactobacilli' in most clinical studies on dental caries. Efforts have been made to develop simple, fast and discriminating methods for identification of *Lactobacillus* species. SDS-PAGE is a technique available in most laboratories and has been previously used to characterize numerous oral species including *Lactobacillus* species (4). It has been accepted to be a useful method for many bacterial species because it offers a means of characterizing species and of identifying individual strains of closely related bacteria (16). In this study, SDS-PAGE gave useful information for the identification of lactobacilli. SDS-PAGE was particularly valuable in the discrimination between *L. casei* and *L. rhamnosus* and between *L. acidophilus* and *L. crispatus*, which could not be differentiated using PCR-RFLP. Moreover, this methodology is inexpensive should many strains have to be identified. The technique does suffer from the production of sometimes complex banding patterns, and from the problem that differences are small, hampering comparison. Although, SDS-PAGE was successfully used for the specification of most *Lactobacillus* spp., the difficulties we found led us to initiate the speciation by 16S rRNA gene PCR-RFLP analysis.

In this study, 16S rRNA gene PCR-RFLP analysis was successfully used to differentiate most of 13 reference species. This method requires only universal primers for 16S rRNA genes that are widely used in studies of bacterial taxonomy (14). The advantage of PCR is that it greatly reduces the number of bands and more readily facilitates comparisons between strains. The results suggested that *Hpa*II and *Hae*III are complementary, thus both enzymes could be used when one of them alone did not distinguish the strains. However, PCR-RFLP is less discriminatory, and it is a relatively expensive and time-consuming method compared to SDS-PAGE.

A combination of PCR-RFLP and SDS-PAGE has been shown to be a practical method to use for identification of *Lactobacillus* to species level, and to be applicable for a wide range of clinical isolates. Although it was reported that 16S rRNA sequence comparison could be used to identify the clinical isolates, the identification by sequencing is sometimes difficult in large-scale studies and for small laboratories because it is time-consuming and expensive.

Both streptococci and lactobacilli have been strongly associated with caries (11, 12). While streptococci are usually speciated to mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*), lactobacilli are not commonly examined to species level. Consequently, we have little knowledge as to the prevalence of various *Lactobacillus* species in the human oral cavity in general or in association with caries development and carious lesions in particular. In this study *L. fermentum* and *L. rhamnosus* were the two most frequently isolated species, both of which are well known inhabitants of the human oral microflora (15). The correlation between the PCR-RFLP identification and a phenotypic characterization using biochemical tests was not overwhelming, mainly because of the shortcomings of the API fermentation method described above. It was not the aim of this study to do a prevalence study on various *Lactobacillus* species in the oral cavity and in relation to caries frequency. This will be a matter for a separate study.

In conclusion, our results show that a combination of PCR-RFLP and SDS-PAGE techniques provides a rapid, easy-to-perform, and reproducible tool for differentiation of *Lactobacillus* at the species level. The discriminating power of the methods is well illustrated by the analysis of the clinical isolates, which showed clear differentiation of each strain.

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