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# Rapid discrimination and classification of the *Lactobacillus plantarum* group based on a partial *dnaK* sequence and DNA fingerprinting techniques

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**Abstract**   The *Lactobacillus plantarum* group comprises five very closely related species. Some species of this group are considered to be probiotic and widely applied in the food industry. In this study, we compared the use of two different molecular markers, the 16S rRNA and *dnaK* gene, for discriminating phylogenetic relationships amongst *L. plantarum* strains using sequencing and DNA fingerprinting. The average sequence similarity for the *dnaK* gene (89.2%) among five type strains was significantly less than that for the 16S rRNA (99.4%). This result demonstrates that the *dnaK* gene sequence provided higher resolution than the 16S rRNA and suggests that the *dnaK* could be used as an additional phylogenetic marker for *L. plantarum*. Species-specific profiles of the *Lactobacillus* strains were obtained with RAPD and RFLP methods. Our data indicate that phylogenetic relationships between these strains are easily resolved using sequencing of the *dnaK* gene or DNA fingerprinting assays.

**Keywords**   Phylogenetic marker · *dnaK* gene ·
Sequencing · RAPD · PCR–RFLP · *Lactobacillus plantarum* group

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## Introduction

The current taxonomy of the *L. plantarum* group is comprised of five closely related species: *L. plantarum* subsp. *plantarum, L. plantarum* subsp. *argentoratensis, L. paraplantarum, L. pentosus*, and *L. fabifermentans* (De Bruyne et al. 2009). The *L. plantarum* and *L. pentosus* species have been shown to be probiotic and are widely used in the food and feed industries (Casey et al. 2007; Holzapfel et al. 2001; Merry et al. 1995; Oneca et al. 2003; Ruiz-Barba et al. 1994). Moreover, numerous studies have demonstrated that the *L. plantarum* subsp. *plantarum* strain provides beneficial effects to the immune system (Bujalance et al. 2007; Pathmakanthan et al. 2004), especially in treating inflammatory disease and mitigating pathogenic infection (Connelly 2008). Inconceivably, more than 28% of commercial probiotic products are mislabelled at the genus or species level (Huys et al. 2006). Thus, it is very important to accurately distinguish and identify the correct species designations for this probiotic bacterial species complex.

Traditionally, the methods used for *L. plantarum* group classification were dependent on phenotypic tests such as morphological, physiological and biochemical analyses (Abazinge et al. 1993; Petrovic et al. 2006). Currently, the identification of *Lactobacillus* strains relies mainly on API 50 CH carbohydrate fermentation strips (Boyd et al. 2005; Bringel et al. 2005; De Bruyne et al. 2009). However, this commercial identification kit may not always be adequate in

reliably distinguishing closely related *Lactobacillus* species, especially in the *L. casei* and *L. plantarum* groups (Johansson et al. 1995). Therefore, there has been a shift towards molecular based techniques to improve species identification within the *L. plantarum* group. The 16S ribosome RNA (16S rRNA), an ∼1,500 bp sequence that codes for a portion of the 30S ribosome, and comparative analysis of the sequences is a commonly used molecular method for bacterial identification (Petti 2007). Strains that generally show higher than 97% similarity of the 16S rRNA sequence are considered to be the same species (McCartney 2002; Vandamme et al. 1996). However, it may not always suitable to distinguish closely related species such as those of the *L. plantarum* group because the 16S rRNA sequence similarity of *L. plantarum* group strains reaches 98.9–99.9% (De Bruyne et al. 2009). Thus other phylogenetic markers with higher resolution should be identified.

The *dnaK* gene encodes the 70 kDa heat shock protein (HSP70), which plays a crucial role as a chaperone machine for protein folding and transport and in protecting organisms from heat- or stress-induced damage (Netzer and Hartl 1998). In fact, HSP70 is the most conserved protein found in all biota (Gupta and Golding 1993); thus, the *dnaK* sequence is well suited for examining deep-level phylogenies. In the present study, we analysed partial *dnaK* gene sequences and compared them to the 16S rRNA gene to evaluate the utility of *dnaK* as an alternative phylogenetic marker for *L. plantarum* group strains. In addition, the RAPD (random amplification of polymorphic DNA) and RFLP (restriction fragment length polymorphism) fingerprinting techniques were also evaluated for their ability to discriminate the *L. plantarum* group.

## Materials and methods

### *Lactobacillus* strains

The *Lactobacillus* type strains and isolates used in this study are listed in Table 1. They were obtained from the Bioresource Collection and Research Center (BCRC). All bacterial strains were incubated on Lactobacilli MRS Agar (LMRS agar, Difco) aerobically for 48 h at 37°C.

**Table 1** *Lactobacillus* strains used in this study

| No. | Species | BCRC no. | *dnaK* accession no. |
|---|---|---|---|
| 1 | *Lactobacillus plantarum* subsp. *plantarum* | 10069[T] | GQ998873 |
| 2 | | 10357 | GQ998874 |
| 3 | | 12251 | GQ998876 |
| 4 | | 14059 | GQ998878 |
| 5 | | 17639 | GQ998881 |
| 6 | | 15478 | GQ998885 |
| 7 | *Lactobacillus plantarum* subsp. *argentoratensis* | 17638[T] | GQ998879 |
| 8 | | 17640 | GQ998880 |
| 9 | | 12327 | GQ998877 |
| 10 | *Lactobacillus paraplantarum* | 17178[T] | GQ998882 |
| 11 | | 17970 | GQ998886 |
| 12 | | 17971 | GQ998887 |
| 13 | *Lactobacillus pentosus* | 11053[T] | GQ998883 |
| 14 | | 17972 | GQ998888 |
| 15 | | 17973 | GQ998889 |
| 16 | | 17989 | GQ998890 |
| 17 | | 80018 | GQ998891 |
| 18 | | 12250 | GQ998875 |
| 19 | | 15317 | GQ998884 |
| 20 | | 80017 | GQ998894 |
| 21 | | 12944 | GQ998893 |
| 22 | *Lactobacillus fabifermentans* | 18841[T] | GQ998892 |

BCRC, Bioresource Collection and Research Center at Food Industry Research and Development Institute, Taiwan, ROC

### Genomic DNA preparation

The genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturers' instructions. The DNA concentration and purity was measured with a spectrophotometer and checked by agarose gel electrophoresis. Then, the genomic DNA was used as a template for PCR.

### RAPD–PCR analysis

The RAPD profiles for the test strains were determined as described previously (Huang and Lee 2009). Forty random primers (Operon kits A and T series) were used for RAPD-PCR, and the amplification was carried out in a thermal cycler (Gene-Amp® 2700, Applied Biosystems, CA, USA). The

RAPD profiles were separated according to size by electrophoresis on 2% agarose gels.

## PCR amplification of target gene

The partial *dnaK* fragments were amplified by PCR using primers Lpdnak-500F3 (5′-CCGTTCTTRTCR ATRTCRAA-3′) and Lpdnak-1710R5 (5′-GAAAYY CAAGTYGGHGAAGT-3′), the 16S rRNA were amplified using the MicroSeq Full Gene 16S rRNA Bacterial Identification kit (Applied Biosystems, Foster City, CA, USA). PCR reactions were composed of 81 μL sterile MilliQ water, 10 μL 10× PCR buffer, 1.5 μL dNTPs (10 mM), 2.5 μL forward primer (10 mM), 2.5 μL reverse primer (10 mM), 2.5 U Taq DNA polymerase (DreamTaq, Fermentas) and 3 μL template DNA (100 ng/μL). The thermal protocol using the following conditions: initial strand denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, and a final extension step at 72°C for 7 min. The PCR products were visualized by electrophoresis on a 2% agarose gel with ethidium bromide staining.

## DNA sequencing and RFLP analysis

The PCR products were purified using the QIA quick PCR purification Kit (Qiagen Inc., Valencia, CA, USA) and sequenced with the BigDye Terminator v3.1 cycle-sequencing kit on the 3730 DNA sequencer (Applied Biosystems and Hitachi, Foster City, CA, USA). The sequencing primers of *dnaK* gene were Lpdnak-500F3 and Lpdnak-1710R5, Lplntdnak-340r1 (5′-ATTCCAGCYGTTCAAGAAGC-3′) and Lpd-nak-1340F9 (5′-AAMGTMCCACCACCAAGGTC-3′) designed from the conserved regions of the five type strains of the *L. plantarum* group. The sequencing primers of 16S rRNA gene were the MicroSeq Full Gene 16S rRNA Bacterial Identification kit. Twenty μl of the amplified target DNA of *dnaK* were digested with enzymes *Hae*III, *Alu*I, *Msp*I and *Tsp*509I. The restriction profiles were separated according to size by electrophoresis on 2.5% agarose gels.

## Phylogenetic data analysis

Sequence similarities were calculated using the programs of Wisconsin Package Version 10.1 (Accelrys Inc., San Diego, CA). The *dnaK* and 16S rRNA

sequences were aligned using the Clustal X program, version 1.8 (Thompson et al. 1997). Phylogenetic trees were constructed with the PHYLIP computer program package (Felsenstein 1993) using the neighbor-joining method (Saitou and Nei 1987) with genetic distances computed by using Kimura's 2-parameter model (Kimura 1980). The bootstrap values were based on 1,000 replications.

## Results

### RAPD fingerprinting profiles

Two series of random primers including OPA and OPT (Operon Alameda, CA, USA) were used during the RAPD fingerprinting analysis of strains from within the *L. plantarum* group. Each series consisted of 20 primers of random sequence. Among these 40 random primers, OPT-1 (5′-GGGCCACTC-3′) was the most noteworthy for distinguishing all *Lactobacillus* species within the *L. plantarum* group, producing unique DNA patterns for each species within the group (Fig. 1).

### Target gene amplification and DNA sequencing

Approximately 1,500 and 1,100 bp of the 16S rRNA and *dnaK* genes, respectively, were amplified from all
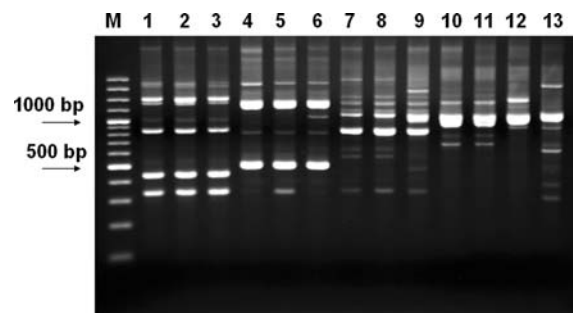


**Fig. 1** RAPD fingerprints of *Lactobacillus plantarum* group strains. Genomic DNA samples were amplified with random primers (OPT-1). M: 100 bp DNA ladder markers. *Lanes*: (*1*) *L. plantarum* subsp. *plantarum* BCRC 10069[T]; (*2*) *L. plantarum* subsp. *plantarum* BCRC 12251; (*3*) *L. plantarum* subsp. *plantarum* BCRC 14059; (*4*) *L. plantarum* subsp. *argentoratensis* BCRC 17638[T]; (*5*) *L. plantarum* subsp. *argentoratensis* BCRC 17640; (*6*) *L. plantarum* subsp. *argentoratensis* BCRC 12327; (*7*) *L. paraplantarum* BCRC 17178[T]; (*8*) *L. paraplantarum* BCRC 17970; (*9*) *L. paraplantarum* BCRC 17971; (*10*) *L. pentosus* BCRC 11053[T]; (*11*) *L. pentosus* BCRC 17972; (*12*) *L. pentosus* BCRC 17973; (*13*) *L. fabifermentans* BCRC 18841[T]

*Lactobacillus* strains. The amplified DNA fragments were used for sequencing and restriction analysis. To obtain the 1,100 bp *dnaK* fragment, sequencing primers Lplntdnak-340r1 and Lpdnak-1340F9 were designed to anneal to the conserved regions. The PCR product sequencing was repeated at least twice to confirm the reading and to resolve any ambiguity. The *dnaK* gene sequences of all *Lactobacillus* strains in this study were submitted to GenBank, and the accession numbers are listed in Table 1.

PCR–RFLP profiles

The *dnaK* amplicons were digested with the enzymes *Hae*III, *Msp*I, *Alu*I and *Tsp*509I, respectively. Among the 22 *Lactobacillus* strains, digestions using *Tsp*509I produced five different restriction profiles and distinguished all *L. plantarum* species simultaneously. Conversely, digestions using the other three enzymes showed that some species produced the same restriction profiles (data not shown).

Phylogenetic tree based on 16S rRNA and *dnaK* gene sequences

Similarities of the *dnaK* sequence ranged from 86.2 to 96.2%, compared to 98.9 to 99.9% for the 16S rRNA (Table 2). The nucleotide sequences of the *dnaK* and 16S rRNA genes from 22 *Lactobacillus* strains were determined, and phylogenetic trees were reconstructed using the neighbor-joining method. The bootstrap values at all nodes of the *dnaK* tree were significantly higher than those of 16S rRNA tree. Further, the topology of the *dnaK* tree showed five clearly separated groups (Fig. 3). In comparison, the 16S rRNA-based tree for all strains only showed two groups (Fig. 4) because the *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. paraplantarum*, and *L. pentosus* strains were indistinguishable from each other.

Discussion

Genomic DNA fingerprinting using the RAPD method has become widely accepted as a valid taxonomic and phylogenetic tool for a large range of organisms such as lactobacilli (Daud Khaled et al. 1997; Du Plessis and Dicks 1995; Huang and Lee 2009). Thus far, the RAPD technique was applied only to *L. plantarum* subsp. *plantarum*, *L. paraplantarum*, and *L. pentosus*; the other two *Lactobacillus* species were not checked (Torriani et al. 2001a). In this study, we used RAPD fingerprinting to classify full *L. plantarum* group strains, and the OPT-1 primer was used to simultaneously discriminate five members within the *L. plantarum* group, each of which formed their own specific-species DNA pattern (Fig. 1). PCR–RFLP technique is a simple, fast and low-cost way to identify bacteria (Olive and Bean 1999). The crucial element of this technique is the selection of the restriction enzymes. In this study, we also used a *dnaK* gene fragment in combination with PCR–RFLP to simultaneously discriminate between

| Table 2 Similarities of *dnaK* and 16S rRNA sequences between the type strains of *Lactobacillus plantarum* group | Strain no. | Strain name | Similarity of *dnaK* nucleotide sequences (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 |
| | 1 | *Lactobacillus plantarum* subsp. *plantarum*<br>BCRC 10069[T] | | 96.2 | 89.8 | 89.2 | 87.4 |
| | 2 | *Lactobacillus plantarum* subsp. *argentoratensis*<br>BCRC 17638[T] | 99.8 | | 90.2 | 89.7 | 87.6 |
| The values on the upper right are the similarities between *dnaK* sequences (1,136 bp), and the values on the lower left are the similarities between 16S rRNA sequences (1,485 bp) | 3 | *Lactobacillus paraplantarum*<br>BCRC 17178[T] | 99.7 | 99.7 | | 89.6 | 86.2 |
| | 4 | *Lactobacillus pentosus*<br>BCRC 11053[T] | 99.9 | 99.9 | 99.7 | | 86.6 |
| | 5 | *Lactobacillus fabifermentans*<br>BCRC 18841[T] | 98.9 | 98.9 | 98.9 | 98.9 | |
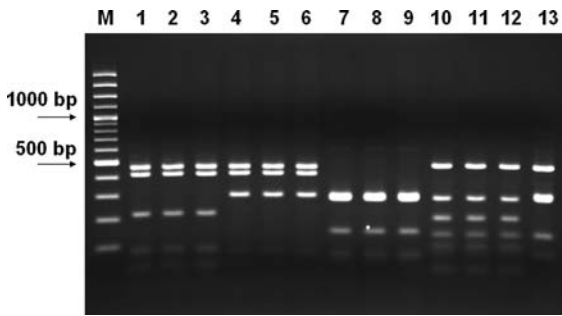
**Fig. 2** *Tsp*509I RFLP profiles of the *dnaK* gene PCR products. M: 100 bp DNA ladder markers. (*1*) *L. plantarum* subsp. *plantarum* BCRC 10069[T]; (*2*) *L. plantarum* subsp. *plantarum* BCRC 12251; (*3*) *L. plantarum* subsp. *plantarum* BCRC 14059; (*4*) *L. plantarum* subsp. *argentoratensis* BCRC 17638[T]; (*5*) *L. plantarum* subsp. *argentoratensis* BCRC 17640; (*6*) *L. plantarum* subsp. *argentoratensis* BCRC 12327; (*7*) *L. paraplantarum* BCRC 17178[T]; (*8*) *L. paraplantarum* BCRC 17970; (*9*) *L. paraplantarum* BCRC 17971; (*10*) *L. pentosus* BCRC 11053[T]; (*11*) *L. pentosus* BCRC 17972; (*12*) *L. pentosus* BCRC 17973; (*13*) *L. fabifermentans* BCRC 18841[T]

members of the *L. plantarum* group. Four restriction enzymes were tested, and the *Tsp*509I PCR–RFLP analysis was found to preliminary give species-specific discrimination (Fig. 2). The RFLP profiles of single species differed by two to three fragments, due to genetic drift. Thus, the other more reliable molecular technique should be identified.

Whole genome DNA–DNA hybridization is a gold standard assay and has long been applied to bacterial species delineation (Stackebrandt et al. 2002; Wayne et al. 1987). However, this technique is time consuming, expensive, not always repeatable and difficult to apply to a large number of bacteria (Mehlen et al. 2004; Pontes et al. 2007). In contrast, DNA sequences of protein-encoding genes seem to be more effective than the 16S rRNA gene and may replace DNA–DNA hybridization for species identification (Stackebrandt et al. 2002; Zeigler 2003). In
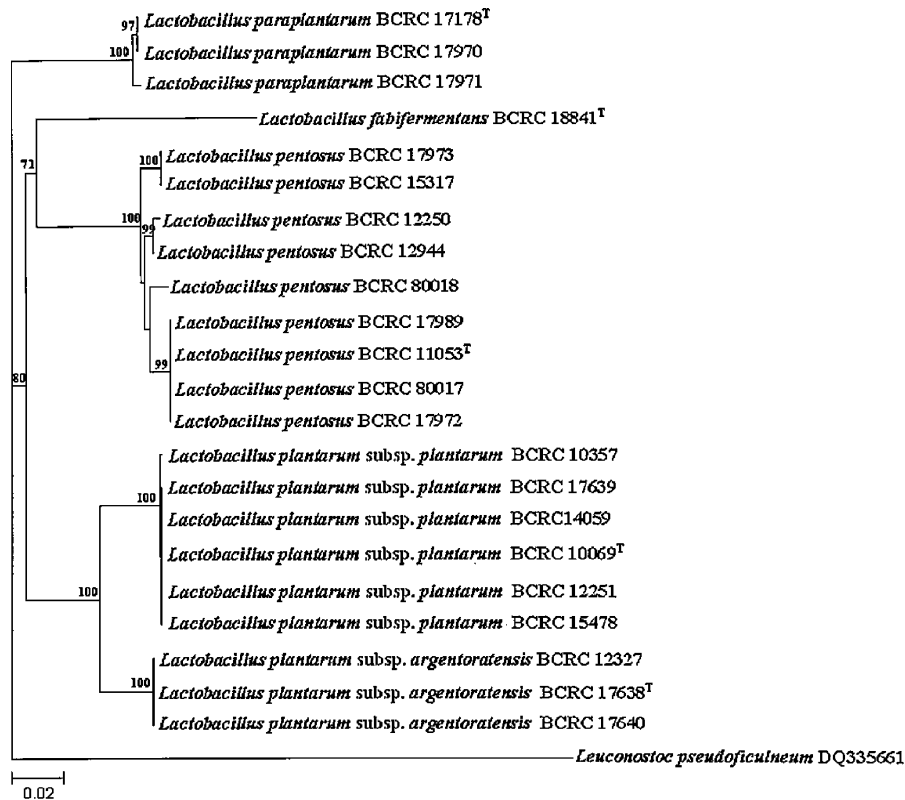


**Fig. 3** Phylogenetic tree of 22 *Lactobacillus* strains based on *dnaK* sequences. The tree was constructed with the neighbour-joining method. Genetic distances were computed by Kimura's two-parameter model. *L. pseudoficulneum* was included as an outgroup. Only bootstrap percentages above 70% are shown (based on 1,000 replications). The *scale bar* represents 0.02% sequence divergence

**Fig. 4** Phylogenetic tree of 22 *Lactobacillus* strains based on 16S rRNA sequences. The tree was constructed with the neighbour-joining method. Genetic distances were computed by Kimura's two-parameter model. *L. pseudoficulneum* was included as an outgroup. Only bootstrap percentages above 70% are shown (based on 1,000 replications). The *scale bar* represents 0.01% sequence divergence



this study, we used the 16S rRNA and partial *dnaK* gene sequences to classify *L. plantarum* group strains. A reliable phylogenetic tree based on *dnaK* clearly showed five species groups. The bootstrap values at all nodes of the *dnaK* tree were significantly higher than those of the 16S rRNA tree. In addition, ten nodes were observed where bootstrap values reached ≥80% (Fig. 3), whereas only one such node existed on the 16S rRNA tree (Fig. 4). The average nucleotide sequence similarity of *dnaK* between the *L. plantarum* group type strains was significantly less than that of 16S rRNA (89.2 and 99.4%, respectively). At present, several phylogenetic targets of protein-encoding genes have been exploited for the differentiation of *L. plantarum* group species (*atpA*, *tuf*, *recA*, *hsp60*, *pheS* and *rpoA*) (Blaiotta et al. 2008; Chavagnat et al. 2002; Naser et al. 2007; Torriani et al. 2001b). However, only two targets (*recA* and *pheS*) showed good resolution at a high discrimination level. In the present study, we found that the phylogenetic information in the *dnaK* gene was compatible with that from other protein-encoding genes in distinguishing phenotypically closely related species *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. paraplantarum*, *L. pentosus* and *L. fabifermentans*.

Our data confirm that the sequence of the *dnaK* gene is significantly more polymorphic than that of the 16S rRNA gene. Thus, we propose that *dnaK* should complement the 16S rRNA for classification of the *L. plantarum* group. Furthermore, the DNA fingerprinting profiles obtained with the RAPD and RFLP methods might also be useful for species discrimination between *L. plantarum* group strains. In conclusion, the *dnaK* target gene appears to be an ideal phylogenetic marker for accurate and rapid discrimination and classification of *L. plantarum* group species, as well as subspecies, using direct sequencing and DNA fingerprinting techniques.

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