# Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA* Gene Sequence Analysis and Multiplex PCR Assay with *recA* Gene-Derived Primers

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In this study, we succeeded in differentiating *Lactobacillus plantarum, Lactobacillus pentosus,* and *Lactobacillus paraplantarum* by means of *recA* gene sequence comparison. Short homologous regions of about 360 bp were amplified by PCR with degenerate consensus primers, sequenced, and analyzed, and 322 bp were considered for the inference of phylogenetic trees. Phylograms, obtained by parsimony, maximum likelihood, and analysis of data matrices with the neighbor-joining model, were coherent and clearly separated the three species. The validity of the *recA* gene and RecA protein as phylogenetic markers is discussed. Based on the same sequences, species-specific primers were designed, and a multiplex PCR protocol for the simultaneous distinction of these bacteria was optimized. The sizes of the amplicons were 318 bp for *L. plantarum*, 218 bp for *L. pentosus,* and 107 bp for *L. paraplantarum*. This strategy permitted the unambiguous identification of strains belonging to *L. plantarum, L. pentosus,* and *L. paraplantarum* in a single reaction, indicating its applicability to the speciation of isolates of the *L. plantarum* group.

The species *Lactobacillus plantarum, Lactobacillus pentosus,* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. The genetic heterogeneity of the *L. plantarum* group has been demonstrated by Dellaglio et al. (8) on the basis of DNA-DNA hybridization data: three groups were identified which were later classified as *L. plantarum* sensu stricto (2), *L. pentosus* (28), and *L. paraplantarum* (7).

Despite the importance of these species for fermented foods of plant, animal, and fish origin, their correct identification is complicated by the ambiguous response of traditional physiological tests and molecular methods: Fourier transform infrared spectroscopy of lactobacilli from breweries was not able to differentiate spectra from *L. plantarum* and *L. pentosus* (6). Randomly amplified polymorphic DNA-PCR and related numerical analysis gave satisfying results, but the methods were applied only to *L. plantarum* and *L. pentosus* (27). Finally, two papers reported the selection of species-specific PCR primers for the *L. plantarum* group; however, in one case specificity towards *L. paraplantarum* was not checked (21), and in the other the primers did not guarantee sufficient specificity (3). Satisfying results have been obtained by Southern-type hybridization with a *pyrDFE* probe (4), randomly amplified polymorphic DNA-PCR, and AFLP (Keygene NV, Wageningen, The Netherlands) (S. Torriani, F. Clementi, F. Dellaglio, B. Hoste, M. Vancanneyt, and J. J. Swings, Proc. Sixth Symp. Lactic Acid Bacteria, poster B2, 1999), but such methods are not suitable for routine identification requirements. The difficulty of correct identification of these species and the increasing interest in some of their properties, e.g., probiotic activities (13) and tannin degradation (19), indicates the need for a simple and reliable molecular method for the definite differentiation of *L. plantarum, L. paraplantarum,* and *L. pentosus*.

PCR using species-specific oligonucleotides designed based on phylogenetic molecular markers could be a useful approach, since these molecules are ubiquitous and relatively highly conserved. For this purpose, 16S ribosomal DNA sequences are not suitable because of the high identity value (>99%) shared by *L. plantarum* and *L. pentosus* (5, 21). Consequently, the definition of phylogenetic distances is also not feasible by such a classical approach for *L. plantarum* group species. It has been proposed that the *recA* gene could be used as a phylogenetic marker (11, 17), and it has already given satisfying results for many bacterial genera, including bifidobacteria (15).

RecA is a small protein (352 amino acids in *Escherichia coli*) implicated in homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis (11). This panoply of functions implies multiple biochemical activities, including DNA binding (double and single stranded), pairing and exchange of homologous DNA, ATP hydrolysis, and coproteolitic cleavage of the LexA, λcI, and UmuD proteins (11). Due to its fundamental role, the *recA* gene is ubiquitous, and its gene product has been proposed as a phylogenetic marker for distantly related species (11, 17).

In this study, short *recA* gene sequences were obtained and analyzed in order to infer a phylogenetic classification scheme, still lacking for the three species of the *L. plantarum* group. Moreover, species-specific primers were designed based on the obtained sequences and were used in a multiplex PCR assay.

## MATERIALS AND METHODS

**Bacterial strains and cultivation.** The bacterial strains tested for inferring phylogeny were *L. paraplantarum* LMG 16673[T], *L. paraplantarum* LMG 18402,

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*L. paraplantarum* NIRD P2, *L. pentosus* LMG 10755[T], *L. pentosus* LMG 18401, *L. plantarum* ATCC 14917[T], *L. plantarum* B41, and *L. plantarum* NCFB 340.

The strains used as positive or negative controls in the multiplex PCR assay were *L. plantarum* strain A, *L. pentosus* strain 3 and strain 6 from our collection, *Acetobacter aceti* LMG 1261[T], *Bifidobacterium breve* LMG 11042[T], *Enterococcus faecium* LMG 8147[T], *Lactobacillus casei* ATCC 393[T], *L. casei* ATCC 334, *Lactobacillus paracasei* subsp. *paracasei* NCFB 151[T], *Lactobacillus rhamnosus* LMG 6400[T], *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842[T], *Lactobacillus helveticus* ATCC 15009[T], *Lactococcus lactis* subsp. *lactis* LMG 6890[T], and *Streptococcus thermophilus* strain S203 (our collection).

*E. coli* strain XL1-Blue, used for cloning procedures, was grown in Luria-Bertani broth (0.1% tryptone, 0.1% sodium chloride, 0.05% yeast extract, pH 7.0) for 16 h at 37°C. Transformed *E. coli* XL1-Blue was grown on Luria-Bertani agar plates plus 100 μg of ampicillin/ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside, and 80 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml.

Lactobacilli and *E. faecium* were grown in MRS broth (Oxoid) for 16 h at 37°C, *B. breve* was grown in MRS broth with 0.05% cysteine added for 48 h at 37°C in anaerobiosis obtained with Anaerocult A (Merck, Darmstadt, Germany), *A. aceti* was grown in YPM broth (0.5% yeast extract, 0.3% peptone, 2.5% mannitol; pH not adjusted) for 3 days at 30°C, and *L. lactis* and *S. thermophilus* were grown in M17 broth (Oxoid) for 24 h at 37°C.

**DNA preparation.** Genomic DNA of lactic acid bacteria was extracted as described by Marmur (18). In genomic DNA extraction from *A. aceti* the method was modified by the addition of 2× hexadecyltrimethylammonium bromide solution (2% hexadecyltrimethylammonium bromide, 100 mM Tris [pH 8], 20 mM EDTA [pH 8], 1.4 mM NaCl, and 1% polyvinylpyrrolidone), with incubation at 65°C for 10 min.

***recA* gene fragment amplifications.** For the amplification of *recA* gene regions, 300 μl of chromosomal DNA from each *Lactobacillus* strain was added to a 50-μl PCR mixture (2.5 U of Sigma-Aldrich RED*Taq* DNA polymerase in 1× RED-*Taq* reaction buffer, 50 μM each primer, 100 μM deoxynucleotide triphosphates) and submitted to initial denaturation of 1 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min and 30 s at 55°C, and 1 min at 72°C in a GeneAmp PCR System 2400 (Perkin-Elmer). The primers used for the amplification were 5′-T T(C,T) AT(A,T,C) GA(C,T) GC(A,T,C,G) GA(A,G) CA(C,T) GC-3′ (forward primer, corresponding to amino acid region 92 to 98 of the *E. coli* RecA protein [10]) and 5′-CC(A,T) CC(A,T) G(T,G)(A,T) GT(A,T,C) GT(C,T) TC(A,T,C ,G) GG-3′(reverse primer, corresponding to amino acid region 206 to 211 of the *E. coli* RecA protein [9]). The expected size of the amplicons was about 360 bp.

Amplification products were purified from 2% agarose gel by the QIAEX II Gel Extraction System (Qiagen GmbH, Hilden, Germany).

**Cloning of PCR products.** Three microliters of a suspension containing 30 ng of purified amplicons of *recA* gene fragments (360 bp) was added to a ligation mixture containing 50 ng of pGEM-T vector (Promega Corp., Madison, Wis.) and 3 U of T4 DNA ligase (Promega) in 1× ligation buffer and incubated overnight at 4°C. Two microliters of the ligation mixture was used to transform *E. coli* XL1-Blue by electroporation in an *E. coli* Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Recombinant clones carrying the insert were selected by blue-white screening. Plasmids containing DNA fragments were extracted with the Quiaprep Spin Miniprep kit (Qiagen). The presence of the insert was verified by restriction analysis with enzyme *Bgl*II (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan). Restriction was carried out for 1 h at 37°C in 1× reaction buffer with 10 U of enzyme in a final volume of 20 μl.

**Sequencing.** Sequencing reactions were performed at Centro Genoma Vegetale-ENEA CR Casaccia, Rome, Italy, on purified plasmids with primers T7 and SP6 (both strands for each sequence).

**Analysis of sequence data and construction of phylogenetic trees.** The identities of sequences obtained were verified by a BLASTX (1) search against major molecular databases. Putative amino acid sequences were deduced by the GeneDoc program version 2.5.000 (K. B. Nicholas and H. B. Nicholas, unpublished data).

The nucleotide and putative amino acid sequences of the *recA* gene fragments were aligned with the CLUSTAL W program (26). Phylogenetic trees were constructed using programs in the PHYLIP software package version 3.5c. Calculation of distance matrices was carried out by DNADIST and PROTDIST programs on nucleotide and putative amino acid sequences, respectively, using the default models. Trees were inferred using the NEIGHBOR program, which implements the neighbor-joining method of Saitou and Nei (22). Phylogenetic trees from gene sequences were also constructed with the DNAML (maximum likelihood) and DNAPARS (parsimony) programs. The "jumble" option in program menus was always selected to test different sequence orders in inferring phylogenies to minimize the influence of sequence order on the tree topology (16). A bootstrap confidence analysis was performed by generating 1,000 repli-

cated data sets with the SEQBOOT program, constructing trees with DNADIST and NEIGHBOR, and generating the consensus tree with the CONSENSE program (all in the PHYLIP software package version 3.5c).

**Reference sequences used in phylogenetic analysis.** The following bacterial *recA* gene sequences were tested as outgroups in phylogenetic analysis: accession number M81465 (*Acholeplasma laidlawii*), AJ006705 (*Acidothermus cellulolyticus*), M29680 (*Anabaena variabilis*), AF214780 (*Arthrobacter globiformis*), X52132 (*Bacillus subtilis*), AF094756 (*B.breve*), U61497 (*Clostridium perfringens*), AJ006707 (*Frankia alni*), AJ006706 (*Geodermatophilus obscurus*), AJ286124 (*L. paracasei* subsp. *paracasei* NCFB 151[T]), AJ286116 (*Lactobacillus zeae* LMG 17315[T]), M88106 (*L. lactis*), L22073 (*Mycoplasma mycoides*), U30293 (*Ruminococcus albus*), U33924 (*Spirulina platensis*), L25893 (*Staphylococcus aureus*), U21934 (*Streptococcus pyogenes*), and M29495 (*Synechococcus* sp.).

**Multiplex PCR assay.** A multiplex PCR assay (20 μl) was performed with the *recA* gene-based primers paraF (5′-GTC ACA GGC ATT ACG AAA AC-3′), pentF (5′-CAG TGG CGC GGT TGA TAT C-3′), planF (5′-CCG TTT ATG CGG AAC ACC TA-3′), and pREV (5′-TCG GGA TTA CCA AAC ATC AC-3′). The PCR mixture was composed of 1.5 mM MgCl$_2$, the primers paraF, pentF, and pREV (0.25 μM each), 0.12 μM primer planF, 12 μM deoxynucleotide triphosphates (3 μM each), 0.025 U of *Taq* (Polymed, Florence, Italy)/μl, 1× PCR buffer (Polymed), and 5 ng of DNA/μl. PCRs were performed on a Perkin-Elmer GeneAmp PCR System 2400 with initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C (30 s), annealing at 56°C (10 s), and elongation at 72°C (30 s), and final extension at 72°C for 5 min. The PCR products were visualized on a 2% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) buffer. Purified amplicons were sequenced using the designed multiplex species-specific primers to confirm their identities.

**Nucleotide sequence accession numbers.** The EMBL-GenBank-DDBJ accession numbers assigned to the *recA* internal region for the lactic acid bacterial strains investigated in this study are AJ286120 (*L. paraplantarum* LMG 16673[T]), AJ271963 (*L. paraplantarum* LMG 18402), AJ271964 (*L. paraplantarum* NIRD P2), AJ286118 (*L. pentosus* LMG 10755[T]), AJ292254 (*L. pentosus* LMG 18401), AJ286119 (*L. plantarum* ATCC 14917[T]), AJ271962 (*L. plantarum* B41), and AJ292255 (*L. plantarum* NCFB 340).

## RESULTS

**Identification and alignment of *recA* sequences.** Sequence identities were verified by a BLASTX (1) search against available molecular databases. Moreover, the presence of Asp-100, Tyr-103, Asp-144, and Ser-145 (*E. coli* numbering) in the putative translation fragments further confirms their identity, since those residues are considered functionally important (25).

In the phylogenetic analysis, sequence fragments of 322 bp were considered, excluding regions of primer annealing. In these regions, point mutations, likely due to primer degeneration, were indeed observed.

Sequence alignment did not introduce any gaps, and there were no ambiguous positions that could bias the inference of phylogeny. This was not surprising, since the sequences were derived from closely related species.

**Phylogenetic analysis.** Different factors known to influence the phylogenetic analysis, i.e., the choice of outgroup sequences (24) and bioinformatic methods (14), were considered. *recA* gene sequences of several reference organisms, which are available in major databases, were selected as outgroups to evaluate the resulting classification scheme. Congruent results were obtained with all reference sequences tested (data not shown). An example of a phylogenetic tree obtained with *B. subtilis* as the outgroup and *L. paracasei* subsp. *paracasei* NCFB 151[T] and *L. zeae* LMG 17315[T] as reference sequences is shown in Fig. 1.

Three different methods (parsimony, maximum likelihood, and distance matrix data with neighbor-joining) were applied for the inference of the tree. The phylogenetic trees obtained
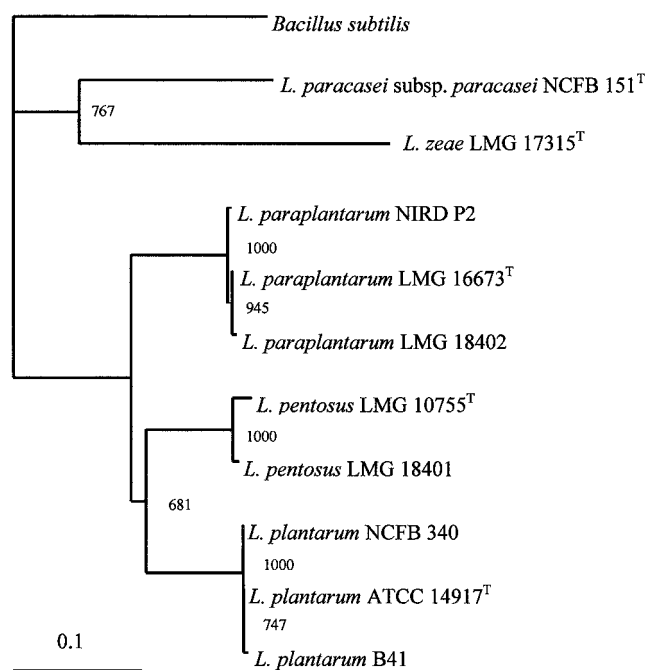
FIG. 1. Phylogenetic tree showing the relative positions of *L. plantarum, L. paraplantarum,* and *L. pentosus* as inferred by the neighbor-joining method with the *recA* gene from *B. subtilis* as the outgroup sequence. Bootstrap values for a total of 1,000 replicates are given. Trees generated by the maximum likelihood and parsimony methods have the same topology. The bar indicates 10% sequence divergence. In this tree topology, the phylogenetic distance between organisms is the sum of the horizontal segments.

had the same topology (Fig. 1), proving the accuracy of the classification scheme. The three species under study were unambiguously differentiated by the comparative sequence analysis of the short fragment of the *recA* gene, as indicated by the bootstrap values in Fig. 1.

Since analysis of RecA protein sequences has been proposed for reconstructing bacterial phylogenetic trees (11), a comparison between the resolution powers of gene sequences and

those of the corresponding putative amino acid sequences was carried out. The putative amino acid sequences share very high identity, so the lengths of branches in the amino acid tree are practically null (data not shown). Percentage identity values for the nucleotide (upper right) and amino acid (lower left) sequences are given in Table 1.

**Primer design and multiplex PCR assay.** The 322-bp *recA* fragment sequences were aligned, and four regions were selected for the design of species-specific primers. A single reverse primer (pREV) and three forward primers (paraF, pentF, and planF) were used in the PCR tests. The designed pREV primer partially overlapped (5 bases; replacing an N with a G) the degenerate primer used by Duwat et al. (9). Numbering the fragment sequences from 1 to 322, the forward specific primers were located as follows: planF, the specific primer for *L. plantarum,* nucleotide positions 9 to 28; pentF, the specific primer for *L. pentosus,* nucleotide positions 109 to 127; and paraF, the specific primer for *L. paraplantarum,* nucleotide positions 220 to 239. Consequently, the expected sizes of the amplicons were 318 bp for *L. plantarum,* 218 bp for *L. pentosus,* and 107 bp for *L. paraplantarum.* To avoid the presence of aspecific bands, the multiplex PCR protocol was optimized in relation to the temperature and time of annealing and the relative concentrations of the primers. The amplification of DNA from all of the *L. plantarum, L. pentosus,* and *L. paraplantarum* strains produced only the expected product, while none of the negative controls produced any amplicon. The identities of the three fragments were confirmed by sequencing. An example of the results is shown in Fig. 2.

## DISCUSSION

Phylogenetic analysis of partial *recA* gene sequences permitted a clear distinction among *L. plantarum, L. pentosus,* and *L. paraplantarum.* This assignment relies only on sequence properties, since results produced by several outgroups and different methods prove the robustness of *recA* phylogenetic analysis. The high bootstrap values obtained are much more significant considering that the *recA* sequences of two faculta-

TABLE 1. Identity values calculated for 322-bp nucleotide sequences of *recA* gene fragments and putative 107-residue amino acid sequences

| Strain | Identity (%)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *L. paraplantarum* LMG 16673[T] | *L. paraplantarum* LMG 18402 | *L. paraplantarum* NIRD P2 | *L. pentosus* LMG 10755[T] | *L. pentosus* LMG 18401 | *L. plantarum* ATCC 14917[T] | *L. plantarum* B41 | *L. plantarum* NCFB 340 |
| *L. paraplantarum* LMG 16673[T] | | **99.7** | **99.4** | **83.9** | **84.8** | **85.4** | **85.1** | **85.1** |
| *L. paraplantarum* LMG 18402 | 99.1 | | **99.1** | **83.5** | **84.5** | **85.1** | **84.8** | **84.8** |
| *L. paraplantarum* NIRD P2 | 100 | 99.1 | | **84.2** | **85.1** | **85.4** | **85.1** | **85.1** |
| *L. pentosus* LMG 10755[T] | 97.2 | 98.1 | 99.1 | | **100** | **86** | **85.7** | **85.7** |
| *L. pentosus* LMG 18401 | 97.2 | 98.1 | 99.1 | 100 | | **86.7** | **86.3** | **86.3** |
| *L. plantarum* ATCC 14917[T] | 98.1 | 98.1 | 99.1 | 100 | 100 | | **99.7** | **99.7** |
| *L. plantarum* B41 | 97.2 | 97.2 | 98.1 | 99.1 | 99.1 | 99.1 | | **99.7** |
| *L. plantarum* NCFB 340 | 98.1 | 98.1 | 99.1 | 100 | 100 | 100 | 99.1 | |

[a] Identity values are shown for *recA* gene fragments (bold face) and amino acid sequences.

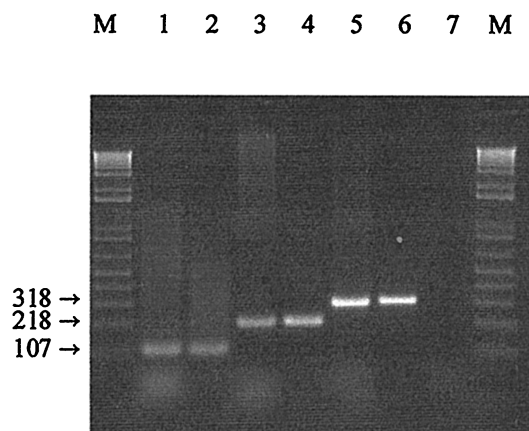M 　1　 2　 3　 4　 5　 6　 7　 M



318 →
218 →
107 →

FIG. 2. Amplification products obtained from the *recA* multiplex assay. Lane M contained a 1-kb PLUS DNA ladder (Life Technology Inc., Gaithersburg, Md.). Lanes 1 and 2, PCR amplification products from *L. paraplantarum* LMG 16673[T] and NIRD P2, respectively; lanes 3 and 4, PCR amplification products from *L. pentosus* LMG 10755[T] and LMG 18401, respectively; lanes 5 and 6, PCR amplification products from *L. plantarum* ATCC 14917[T] and NCFB 340, respectively; lane 7, *L. casei* ATCC 334 (negative control).

tively heterofermentative lactobacilli belonging to the same 16S rRNA phylogenetic group were included in the analysis.

From the comparative analysis of gene sequences and those of the corresponding amino acids, some particularities emerged concerning nucleotide substitutions and identity values. Nearly all nucleotide mutations are synonymous substitutions; consequently, identity values for gene sequences are variable and intraspecific groups are clearly distinguishable, while for amino acid sequences there is little variability. A maximum of three nonsynonymous nucleotide substitutions occurred, which produced the following amino acid replacements: isoleucine (*L. paraplantarum* strains) instead of valine at amino acid position 4, isoleucine (*L. paraplantarum* LMG 18402) instead of threonine at position 23, and alanine (*L. plantarum* B41) instead of valine at position 66. The sole nonconservative substitution was the introduction of isoleucine with a nonpolar side chain instead of threonine with a polar one. The remaining substitutions involved exchanges between nonpolar amino acids.

From a taxonomic point of view, our results confirm that short *recA* gene sequences have high discriminating power for species difficult to differentiate, as already demonstrated for *Bifidobacterium infantis* and *Bifidobacterium longum* (15). At present, the most reliable method for the definition of a species is DNA-DNA hybridization, and it has also been stated that there is a correspondence between these data and the degree of identity of 16S rRNA sequences (23). It is generally accepted that species having 70% or greater DNA similarity usually have more than 97% 16S rRNA sequence identity (23). On the other hand, the identity of 16S rRNA sequences may not be sufficient to guarantee that two taxa belong to the same species (12). This suggests that 16S ribosomal DNA sequence analysis is not the appropriate method to replace DNA reassociation to delineate species and measure intraspecies relationships (23). In the present study, the species rank was indicated by a range of *recA* identity values of 83.5 to 86.7%, which corresponds to DNA hybridization homologies of 15 to 56%

(7). In light of our results and those of previous studies (e.g., reference 15), the *recA* gene can be proposed as a new method for inferring relationships among very closely related species. *recA* gene analysis can have a double potential as a phylogenetic marker: its amino acid sequence can be used to infer phylogenies between distantly related taxa, thus avoiding the disadvantages of rRNAs, e.g., overestimation of the relatedness of species with similar nucleotide frequencies, nonindependence of substitution patterns at different sites, and bias derived from different GC contents of the organisms (11). Furthermore, the nucleotide sequence can be used to evaluate phylogenetic relationships and relative taxonomic positions of recently diverged species with similar GC contents and nucleotide frequencies. In fact, at the nucleotide level, *recA* can tolerate mutations which do not or only slightly alter its product. These mutations give us information about recent evolutionary history, which is too recent to be fixed in slowly diverging sequences like 16S rRNAs (20).

By exploiting the variable nucleotide regions of *recA* gene fragments, we have also succeeded in designing a set of species-specific PCR primers. These can be used separately as primer pairs in different species-specific PCRs but also in a multiplex assay. The optimization of this multiplex PCR makes it possible to assign one isolate to a definite species of the *L. plantarum* group in a single step rather than with three distinct reactions. This assay is rapid (less than 2 h), reliable for its high-specificity reaction conditions, and complete, since all three species of the group are investigated.

The clear distinction obtained with short gene sequences validates the possibility of using the *recA* gene as a phylogenetic-taxonomic marker for closely related species and opens new possibilities for a rapid and reliable identification of lactic acid bacteria of importance for food.

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