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Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*

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Conventional phenotypic methods lead to misidentification of the lactic acid bacteria *Lactobacillus hilgardii* and *Lactobacillus brevis*. Random amplified polymorphic DNA (RAPD) and repetitive element PCR (REP-PCR) techniques were developed for a molecular study of these two species. The taxonomic relationships were confirmed by analysis of the ribosomal operon. Amplified DNA fragments were chosen to isolate *L. hilgardii*-specific probes. In addition to rapid molecular methods for identification of *L. hilgardii*, these results convincingly proved that some strains first identified as *L. brevis* must be reclassified as *L. hilgardii*. The data clearly showed that these molecular methods are more efficient than phenotypic or biochemical studies for bacterial identification at the species level.

Keywords: *Lactobacillus hilgardii*, *Lactobacillus brevis*, RAPD, REP-PCR

INTRODUCTION

Lactic acid bacteria are responsible for malolactic fermentation, an important step in winemaking (Lafon-Lafourcade *et al.*, 1983; Renault *et al.*, 1988). However, some of them induce spoilage (Lonvaud-Funel & Joyeux, 1982; Lonvaud-Funel *et al.*, 1990). Therefore, specific detection and identification are useful for quality control. Precision and reliability of the phenotypical descriptions are not sufficient enough, and some misidentifications occur (Kandler & Weiss, 1986; Lafon-Lafourcade & Joyeux, 1979). Molecular approaches offer new opportunities to characterize micro-organisms. Lonvaud-Funel *et al.* (1990, 1991) describe the identification of lactic acid bacteria during vinification and wine storage by DNA–DNA hybridization. Genomic DNA of the strain to be identified is hybridized with total genomic DNA probes extracted from reference strains. However a problem arose because of cross-hybridization between some *L. brevis* strains isolated from wines, *Lactobacillus brevis* ATCC 11577 and *Lactobacillus hilgardii* ATCC 8290^T (Vescovo *et al.*, 1979; Lonvaud-Funel *et al.*, 1991). Although these two species are phenotypically close (Kandler & Weiss, 1986), they differ by their ability to ferment arabinose: *L. brevis* can use this carbohydrate while *L. hilgardii* cannot.

In the present study, we intended to discriminate *L. hilgardii* and *L. brevis* by using molecular methods based on DNA amplification. Genomic fingerprinting of both species using random amplified polymorphic DNA (RAPD) amplification on the one hand, and repetitive element PCR (REP-PCR) on the other, was assayed. Single primers corresponding to an arbitrary sequence can be used to amplify genomic DNA sequences in order to generate genomic patterns (William *et al.*, 1990). Depending on the type of primer used and on the conditions of the reaction, random amplification of bacterial genomes generate suitable fingerprints for characterization at the genus, species, or particular strain level (Fani *et al.*, 1993). The REP-PCR developed by Versalovic *et al.* (1991) is currently used to estimate relative degrees of similarity between different isolates and to generate species-specific fingerprints (Gilson *et al.*, 1991; Lupski & Weinsthock, 1992; Rodriguez-Barradas, 1995). This method uses a short, highly conserved extragenic repetitive sequence named the Repetitive Extragenic Palindromic (REP) consensus sequence. This sequence is present throughout the eubacterial kingdom, and it has been demonstrated by Gilson *et al.* (1991) that its location is conserved in a given bacterial species. Thus the distribution of these dispersed repetitive DNA

Abbreviations: RAPD, random amplified polymorphic DNA; REP-PCR, repetitive element PCR.

The GenBank accession number for the 1.Rep probe sequence reported in this paper is AJ006299.

sequences is supposed to be different through *L. hilgardii* and *L. brevis* genomes, in order to generate species-specific patterns.

Molecular hybridization methods using specific DNA probes are also useful for the detection and identification of micro-organisms. They are particulary efficient when used in colony hybridization to study mixed populations. According to the strategy developed by Fani *et al.* (1993), the amplification products specific to *L. hilgardii* were purified and used as species-specific DNA probes.

The rRNA-based studies represent an alternative to establish or to confirm relationships between strains and species (Delong *et al.*, 1989; Barry *et al.*, 1990; Amann *et al.*, 1996). Such investigations were performed by comparing the 16S rDNA genes of representatives of the two species.

METHODS

Bacterial strains and growth conditions. The strains used in this study were obtained from ATCC (American Type Culture Collection) and the IOEB (Institut d'Œnologie de Bordeaux) collection (Table 1). They were cultured at 25 °C in MRS medium (supplier: Prolabo except where indicated): 4 g yeast extract l^{-1} (Difco); 8 g beef extract l^{-1} (Difco); 10 g bactopeptone l^{-1} (Difco); 20 g glucose l^{-1}; 2 g Tris sodium citrate l^{-1}; 5 g sodium acetate l^{-1}; 2 g KH_2PO_4 l^{-1}; 0·2 g $MgSO_4.7H_2O$ l^{-1}; 0·05 g $MnSO_4.H_2O$ l^{-1}; Tween 80, 1 ml; malic acid to adjust pH to 5. Carbohydrate assimilations were established by API 50 test (bioMérieux) for each strain at the initiation and during the work to avoid any doubt due to possible contamination.

Total genomic DNA extraction. DNA was extracted according to a slightly modified version of the method described by Gasson & Davies (1980). Cells from 10 ml cultures were collected, washed and treated with lysozyme (5 mg ml^{-1} final concentration) for 15 min at 37 °C. The protoplasts were lysed with 20% SDS for 15 min. After addition of NaCl (1 M final concentration), the cell debris were precipitated for 1 h at 4 °C and eliminated by centrifugation for 20 min at 10000 g. The sample was emulsified with an equal volume phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and centrifuged for 15 min at 10000 ***g***. The upper phase was emulsified with a volume of chloroform/isoamyl alcohol (24:1, v/v). The DNA was then precipitated, dried and suspended in sterile water with RNase (10 mg ml^{-1}).

In order to evaluate DNA concentration, an aliquot of the sample was run in an agarose gel electrophoresis with ethidium bromide. The fluorescence was then compared with amounts of standard DNA.

Primers and DNA amplification conditions. Single-stranded oligonucleotides were synthesized by Genset (Paris, France) (Table 2). Amplification reactions were performed in a 50 µl volume containing 50 ng total genomic DNA, 10^{-10} M each primer, 0·2 mM dNTPs, 1·25 U *Taq* polymerase (Appligene Oncor) with 5 µl 10× PCR reaction buffer. The amplification reactions were performed in a MiniCycler DNA thermal cycler (MJ Research). After a primary denaturation at 95 °C for 12 min, *Taq* polymerase was added. Each amplification consisted of 30 cycles of 30 s denaturation at 95 °C, 30 s at the annealing temperature of the primers and an extension time of 2 min at 72 °C. Repeated experiments were done for each strain in order to evaluate the reproducibility of the results. Blanks without genomic DNA were used as control for each primer.

Table 1. Bacterial strains used

ATCC, American Type Culture Collection; IOEB, Institut d'Œnologie de Bordeaux; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; [T], Type strain.

Species and strain	Source
Lactobacillus hilgardii	
ATCC 8290[T]	Grape
IOEB 7701	Grape
IOEB 7902	Fermenting white grape must
IOEB 720	Wine
IOEB 8408	Sweet white wine
IOEB 8510	Red wine
IOEB 9101	Muscat
IOEB 9102	Maury
IOEB 9202	Port
IOEB 9515	Rivesaltes
IOEB 9518	Floc de Gascogne
IOEB 9519	Muscat
IOEB 9644	Sweet white wine
IOEB 9648	Sweet white wine
Lactobacillus brevis	
ATCC 14869[T]	Human faeces
ATCC 11577	Cheese contamination
ATCC 367	
DSM 20556	Green olives
IOEB 7702	Grape
IOEB 7903	Fermenting white grape must
IOEB 8404	White wine
IOEB 8407	Sweet wine
IOEB 8511	Red wine
IOEB 8907	Red wine
IOEB 9112	Fermenting white grape must
IOEB 9647	Fermenting white grape must
IOEB 9649	Fermenting white grape must

Isolation, cloning and sequencing of amplified DNAs. Ten microlitres of each amplification mixture was loaded on a 1% (w/v) agarose (Eurobio) gel with TBE electrophoresis buffer (89 mM boric acid, 89 mM Tris, 2 mM EDTA) pH 8·0) containing 0·2 mg ethidium bromide ml^{-1} and electrophoresed at 100 V for 30 min. Gels were visualized at 312 nm with a UV transilluminator. DNA fragments were extracted from agarose gels using the GenElute Supelco kit (Boehringer Mannheim).

Amplified DNA fragments from the *L. hilgardii* type strain were cloned in pBluescript II KS (+/−) vector (Stratagene). The DNA fragments were extracted from agarose gels and inserted into *Eco*RV site of the plasmid. *Escherichia coli* XL-1 Blue (Stratagene) was transformed by electroporation. Plasmid DNA from *E. coli* was extracted according to the method of Birnboim & Doly (1979)

Amplicons or cloned DNAs were sequenced by ACT Gene (Evry, France). Searches for DNA homologies were performed via GenBank, EMBL and Ribosomal Database

Table 2. The different primers used in this study

Amplification	Primer	Sequence
RAPD	9898	5′ GCAGCCGG 3′
REP-PCR	REP1R-I	5′ IIIICGICGICATCIGGC 3′
	REP2-I	5′ ICGICTTATCIGGCCTAC 3′
Specific amplification of ribosomal operon	H1	5′ TCTTGGTCAATGAAGT 3′
	H2	5′ ACTNATTTGACATTAAGA 3′
	B1	5′ GAGCTTCCGTTGAAT 3′
	B2	5′ CTNATTTCAACAATGAAG 3′
	8623	5′ CTGGTTCACTATCGGTCTC 3′
Universal primers for 16S rDNA	fD1	5′ ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG 3′
	rD1	5′ cccgggatccaagcttAAGGAGGTGATCCAGCC 3′

Project databases. Sequences were aligned by using CLUSTAL program.

Probe labelling and DNA dot-blot hybridization. This was performed according to the method developed by Lonvaud-Funel *et al.* (1991). About 50 ng to 1 μg DNA were recovered from each amplified band and labelled with digoxigenin-11-dUTP using the random-primed method (DIG DNA Labelling and Detection Kit; Boehringer Mannheim).

DNAs were denatured and fixed to a nylon membrane with 0·4 M NaOH. Membranes were rinsed in 5 × SSC (1 × SSC: 0·1 M NaCl, 0·015 M trisodium citrate, pH 7·0). Prehybridization was performed at 65 °C in a hybridization solution containing 5 × SSC, 1% blocking reagent (Boehringer Mannheim), 0·02% SDS, 0·1% lauroyl sarcosine. Hybridization was performed overnight at 65 °C in the hybridization solution containing 10 ng ml^{-1} digoxigenin-labelled probe. After incubation, membranes were washed twice at room temperature for 5 min with 2 × SSC, 0·1% SDS and twice at 65 °C for 15 min in 0·2 × SSC, 0·1% SDS. The chemiluminescence detection was carried according to the instructions of the supplier.

RESULTS

RAPD and REP-PCR genomic patterns

Amplification methods using short arbitrarily chosen primers were performed. Best results were obtained using a single 8-mer primer (Table 2) with a G+C content of 87·9 mol% and a T_m of 28 °C. An annealing temperature of 33 °C allowed amplification of a few fragments while still providing good discrimination. The genomic fingerprints of all *L. hilgardii* strains tested were similar, the amplified bands ranged from 1500 to 200 bp and shared four fragments of 700, 600, 400 and 350 bp (Fig. 1). The genomic patterns of the *L. brevis* strains were different from each other and from those of *L. hilgardii*. However, similarities could be found. Interestingly, the *L. brevis* strains IOEB 7903, IOEB 9647 and IOEB 9649 presented patterns very similar to those obtained for *L. hilgardii* (Fig. 1).

These results were confirmed by REP-PCR analysis. Two oligonucleotides (REP1R-I and REP2-I) were

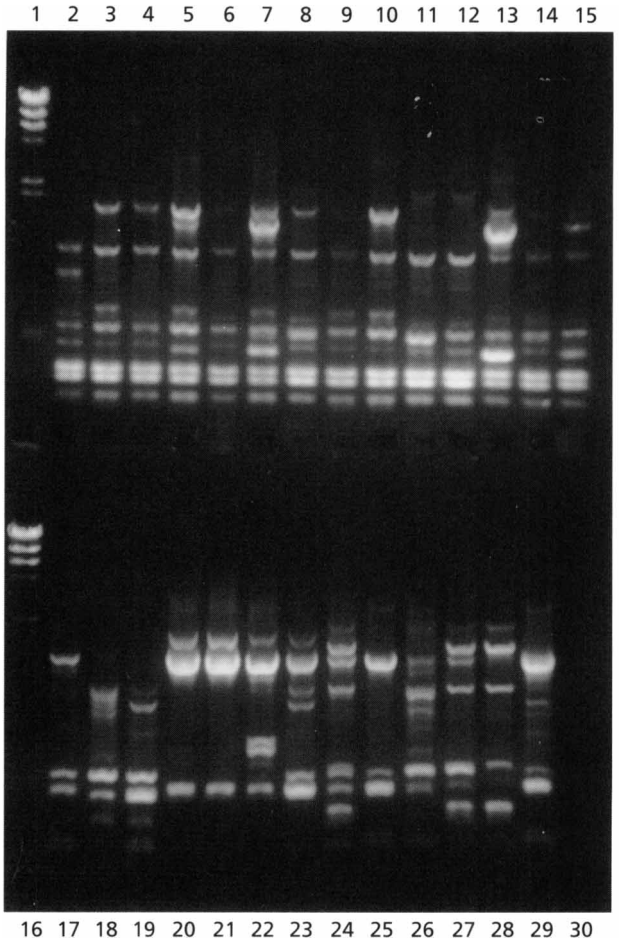

Fig. 1. RAPD genomic patterns of the *L. hilgardii* and *L. brevis* strains. Lane 1, molecular mass marker XIV (2642, 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300 bp). Lanes 2–15, amplification profile of the *L. hilgardii* strains ATCC 8290^T and IOEB 9515, 7701, 9518, 8510, 9102, 8408, 9202, 9519, 9648, 720, 9101, 7902 and 9644. Lane 16, molecular mass marker XIV. Lanes 17–29, amplification profile of the *L. brevis* strains ATCC 14869^T, ATCC 11577 and IOEB 8407, 8907, 8511, 20556, 367, 7903, 8404, 7702, 9647, 9649 and 9112. Lane 30, no template DNA was added to the negative control PCR.

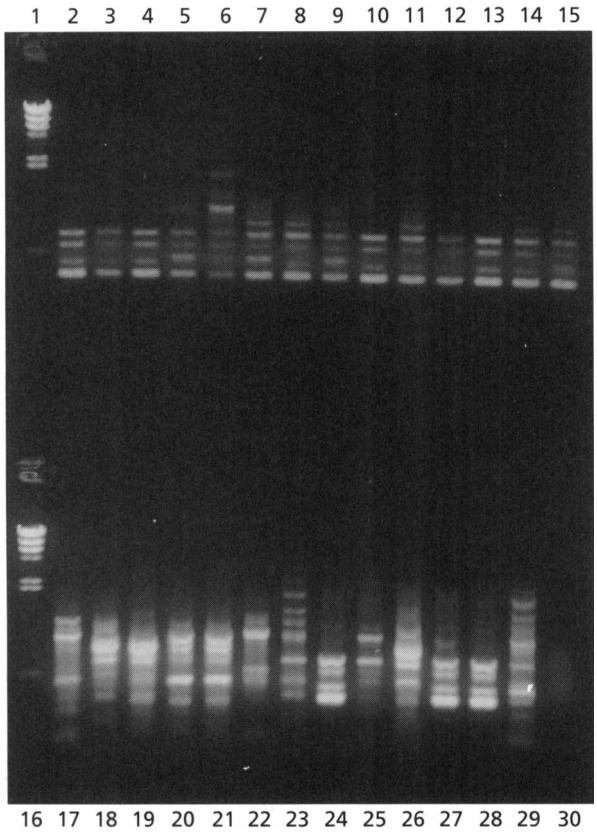

Fig. 2. REP-PCR genomic patterns of the *L. hilgardii* and *L. brevis* strains. Lanes as in Fig. 1.

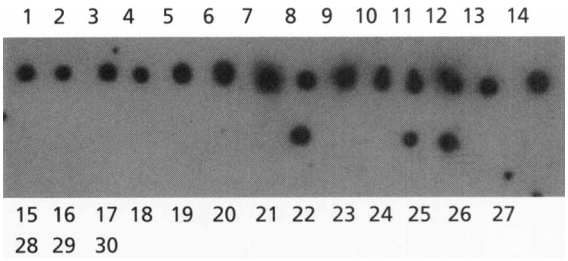

Fig. 3. DNA–DNA hybridization (dot-blot) using the 1.Rep probe. Dots 1–14, *L. hilgardii* strains ATCC 8290^T and IOEB 9515, 7701, 9518, 8510, 9102, 8408, 9202, 9519, 9648, 720, 9101, 7902 and 9644. Dots 15–27, *L. brevis* strains ATCC 14869^T, ATCC 11577 and IOEB 8407, 8907, 8511, 20556, 367, 7903, 8404, 7702, 9647, 9649 and 9112. Dot 28, *L. collinoides* ATCC 27612. Dot 29, *O. oeni* ATCC 23277. Dot 30, *L. plantarum* ATCC 8014.

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ggggcggcggcatcgggcggggaatcatcacgattatgttggcactaaaacgatgtttgca
 G  R  R  H  R  A  G  N  H  H  D  Y  V  G  T  K  T  M  F  A
aaagcagccggcgtcacttcgatcatttttgatgaggttgatacgggagtttcaggccga
K  A  A  G  V  T  S  I  I  F  D  E  V  D  T  G  V  S  G  R
gtagcccaagcaatgggaaataaaatttatacaatttcaacgaagtcccaggtcttatgt
V  A  Q  A  M  G  N  K  I  Y  T  I  S  T  K  S  Q  V  L  C
attactcacttaccacaggttgcggcaatgagtgaccatcactactttattcaaaagcag
I  T  H  L  P  Q  V  A  A  M  S  D  H  H  Y  F  I  Q  K  Q
atacatgacggtcgaacaaccactaccatcacggaactgaacaagcaggatagcgttaac
I  H  D  G  R  T  T  T  T  I  T  E  L  N  K  Q  D  S  V  N
gaaatttctagaatgctttcaggaacgacggtgaccaaattaactaaagaactcgcgagt
E  I  S  R  M  L  S  G  T  T  V  T  K  L  T  K  E  L  A  S
gaactcataacaatggccgatgccgccgcctttgtaaagggttctttctcaaacttgaga
E  L  I  T  M  A  D  A  A  A  F  V  K  G  S  F  S  N  L  R
agggactcttttgcatccgcaccaccccggtattcgcctatttcgccgttagaccgaatg
R  D  S  F  A  S  A  P  P  R  Y  S  P  I  S  P  L  D  R  M
acacgatgacaggggatgacaattaagaatggatttttggcaacggcactggcaaccgct
T  R  stop
Cggatcgcccttggcctaccaattgtttcggcctattgcttat
```

Fig. 4. Nucleotide sequence of the *L. hilgardii* species-specific 1.Rep probe. The amino acid sequence of the deduced protein is reported beneath the nucleotide sequence by using the standard single-letter code placed under the first appropriate codon. The region of the ORF which possesses strong similarity with RecN proteins is underlined.

chosen in opposite orientation corresponding to half of the imperfect palindrome (Table 2). Total degeneracy was represented by inosine placed at specific positions. The annealing to the matrix proceeded at 45 °C. The amplification patterns generated by the REP2-I primer do not present enough complexity to be exploited (data not shown). Primer REP1R-I seemed the most appropriate for this study, since the genomic fingerprint presented enough bands without being too complex (Fig. 2). Similar patterns were obtained with all *L. hilgardii* strains tested with bands ranging from 4300 to 300 bp and all shared four common fragments (700, 600, 450 and 350 bp). With either combination of primers, *L. brevis* patterns did not show any species homogeneity. However, some strains could be grouped according to the fingerprint obtained with REP1R-I alone. Moreover, three *L. brevis* strains, IOEB 7903, IOEB 9647 and IOEB 9649, shared common patterns with the *L. hilgardii* species, as with the previous RAPD method.

Isolation and characterization of a *L. hilgardii*-specific probe

According to the genomic patterns, *L. hilgardii* strains showed an homogeneous genomic entity. Therefore, the ability of RAPD and REP-PCR to generate species-specific markers was exploited. We assumed that the fragments common to the patterns could be specific. For both RAPD and REP amplifications, the four common fragments were extracted from the agarose gel and labelled. The eight probes were tested on dot-blots with spotted genomic DNA of *L. hilgardii* and *L. brevis* strains, as well as DNA of *Lactobacillus collinoides*, *Oenococcus oeni* and *Lactobacillus plantarum*. To demonstrate that all DNAs were correctly spotted, a duplicate set of dot-blots was hybridized with an ubiquitary probe corresponding to a part of the 16S rDNA of *Oenococcus oeni* which included positions 408–1205. Five probes were rejected since they hybridized with the total DNA of several *L. brevis* strains as well as with the total DNA of all *L. hilgardii* strains tested. Three probes, named 1.Rep, 1.9898 and 3.9898, only hybridized with the total DNA of *L. hilgardii* and with the total DNA of the *L. brevis* strains IOEB 7903, IOEB 9649 and IOEB 9649. This reproducibility suggested the use of these probes as *L. hilgardii*-specific markers; Fig. 3 shows the result

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(a)  16 KTMFAKAAGVTSIIFDEVDTGVSGRVAQAMGNKIYTISTKSQVLCITHLPQVAAMSDHHY 75
        *++*+    ********************  **+ +*  ****************+* •
(b) 114 KSIFSSQQDVTSIIFDEVDTGVSGRVAQAIAEKIHKVSIGSQVLCITHLPQVAAMADTHL 173

(a)  76 FIQKQIHDGRTTTTITELNKQDSVNEISRMLSGTTVTKLTKELASELITMAD 127
        +* *++ ****** +  *+**+ * ** **++*  ** ***  * **+  **
(b) 174 YIAKELKDGRTTTRVKPLSKQEKVAEIGRMIAGVEVTDLTKRHAKELLKQAD 225
```

Identities = 69/112 (61%), Similarities = 85/112 (75%).

Fig. 5. Alignments of the ORF deduced from (a) the 1.Rep nucleotide sequence with (b) the C-terminal end of the RecN DNA-repair protein of *Bacillus subtilis*. Amino acid identity (*) and similarity (+) are shown.

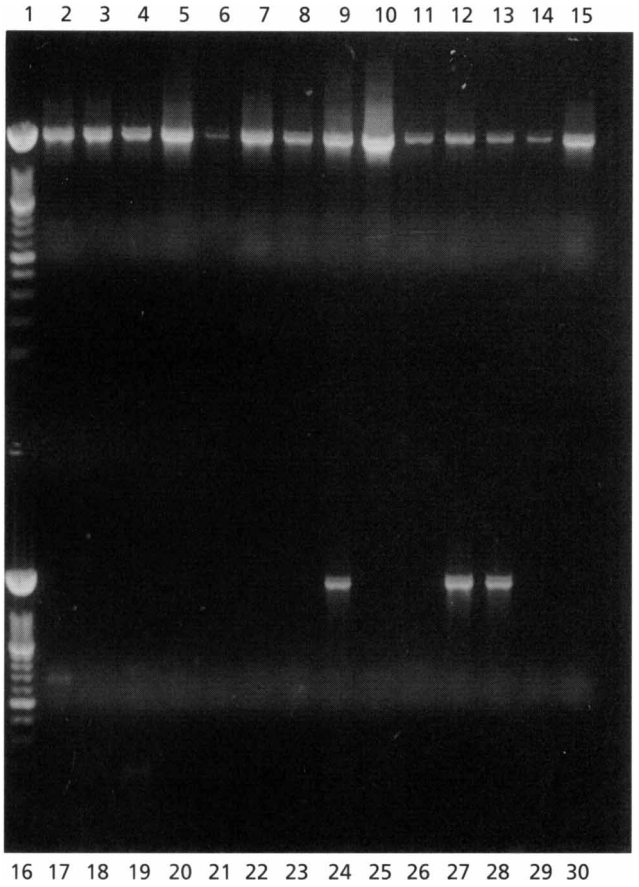

Fig. 6. Specific amplification of the *L. hilgardii* ribosomal operon. Lanes as in Fig. 1.

with the 1.Rep probe. However, the specificity of these probes would be supported if strains IOEB 7903, IOEB 9647 and IOEB 9649 deserve to be reclassified as *L. hilgardii*.

The amplified fragment corresponding to the 1.Rep probe was cloned and sequenced. Analysis of this 584 bp sequence showed that it contained one putative ORF (Fig. 4) which may encode a RecN DNA-repair protein, but no specific signature. A part of the deduced protein sequence exhibited the highest identity and similarity, respectively 61 and 75%, with the C-terminal end of the RecN DNA-repair protein of *Bacillus subtilis* (Figs 4 and 5); but, such values were also obtained with other bacteria such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Vibrio cholerae*, *Bordetella pertussis*, *Escherichia coli* and *Haemophilus influenzae*. Although this protein is ubiquitous, the nucleic acid sequences differences between both species, *L. hilgardii* and *L. brevis*, and the hybridization conditions may allow the probe to target only the *L. hilgardii* genome.

Analysis of the ribosomal operons

To verify the previous results, the 16S rDNA of the *L. hilgardii* and *L. brevis* strains were analysed. Comparative analysis of the 16S rDNA sequences of *L. hilgardii* and *L. brevis* from gene databases showed that several differences could be used to design species-specific primers (Table 2). Four oligonucleotides (H1 and H2 from the 16S rDNA sequence of strain *L. hilgardii* DSM 20176, B1 and B2 from the 16S rDNA sequence of strain *L. brevis* NCDO 1749) were tested together with a second primer named 8623, from a highly conserved region in the 23S rRNA gene. The 8623 sequence is located next to the 16S–23S ribosomal spacer. A specific amplification of *L. hilgardii* ribosomal operon was performed at an annealing temperature of 36 °C using the primers H2 and 8623. PCR yielded two bands for each *L. hilgardii* strain tested (Fig. 6) corresponding to the two rRNA operons reported in lactic acid bacteria (Lamoureux *et al.*, 1993). Using the same set of primers, identical amplification patterns were obtained for strains IOEB 7903, IOEB 9647 and IOEB 9649 which are classified as *L. brevis* according to their capacity to ferment arabinose. No amplification product was obtained for other *L. brevis* strains. The other selected primers did not yield any interesting results.

To confirm that strains IOEB 7903, IOEB 9647 and IOEB 9649 belonged to *L. hilgardii*, we amplified the 16S rRNA gene by using universal primers fD1 and rD1 (Weisburg *et al.*, 1991) at an annealing temperature of 42 °C (Table 2). PCR reactions were performed on isolated colonies of these different strains. The capacity of these clones to ferment arabinose was verified by API CH50 gallery and their *L. hilgardii* species-specific fingerprinting was confirmed by REP-PCR. The 16S rDNA amplicons of each strains were about 1500 bp (Fig. 7). A 500 bp region of the 5′ end of the 16S rRNA gene of the three strains was sequenced and compared with the 16S

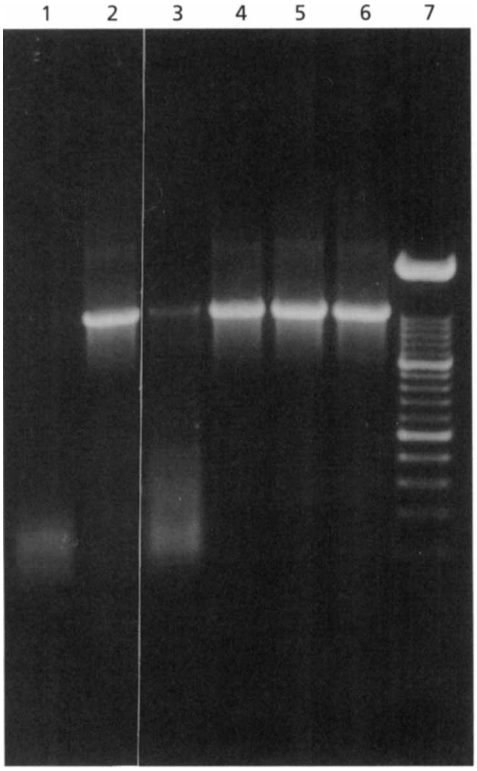

Fig. 7. 16S rDNA amplification patterns using the universal primers fD1 and rD1 (24). Lane 1, no template DNA was added to the negative control PCR. Lanes 2–6, amplification profile of strains IOEB 720, IOEB 7903, IOEB 9647, IOEB 9649 and IOEB 9112. Lane 7, molecular mass marker XIV (see Fig. 1 legend).

Strains		16s rDNA sequences	
(a)	36bp	GTGCCTAATN-CATGCAAGTCGAACGCGTCTTGGTCAATGAAGTTGAGTG	
(b)		GTGCCTAATAACATGCGAGTCGAACGCGTCTTGGTCAATGAAGTTGAGTG	
(c)		GTGCCTAATA-CATGCAAGTCGAACGCGTCTTGGTCAATGAAGTTGAGTG	
(d)		GATCAGACTTAC---CGTGGCTGGATCACCTN---C--TTAAGCTTGGAT	
(e)		GATCANNCTTAC---CGTGGCTGGATCACCTA---C--TTAAGCTGGGNN	
(f)		ATGCCTAATA-CATGCAAGTCGAACGAG-CT---TCCGTTGAATGACGTG	
(g)	36bp	ATGCCTAATA-CNTGCAAGTCGAACGAG-CT---TCCGTTGAATGACGTG	
(a)		CTTGCATTTAACTNATTT-GACATTAAGACGAGTGGCGAACTGGTGAGTN	
(b)		CTTGCATTTAACTGATTT-GACATTAAGACGAGTGGCGAACTGGTGAGTA	
(c)		CTTGCATTTAACTGATTT-GACATTAAGACGAGTGGCGAACTGGTGAGTA	
(d)		CCCGGGTTTAACTGATTT-GACATTAAGACGAGTGGCGAACTGGTGAGTA	
(e)		CCCGGGTTTAACTGATTT-GACATTAAGACGAGTGGCGAACTGGTGAGTA	
(f)		CTTGCA-----CTGATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTA	
(g)		CTTGCA-----CTNATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTA	
(a)		ACNCGTGGGTAACTTGCCCCGAAGCGGGGGATAACATTTGGAAACAGGTG	184bp
(b)		ACACGTGGGTAACTTGCCCCGAAGCGGGGGATAACATTTGGAAACAGGTG	
(c)		ACACGTGGGTAACTTGCCCCGAAGCGGGGGATAACATTTGGAAACAGGTG	
(d)		ACACGTGGGTAACTTGCCCCGAAGCGGGGGATAACATTTGGAAACAGGTG	
(e)		ACACGTGGGTAACTTGCCCCGAAGCGGGGGATAACATTTGGAAACAGGTG	
(f)		ACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTG	
(g)		ACNCGTGGGAAATCTGCCCAGAAGCNGGGGATNACNCTTGGAAACAGGTG	176bp

Fig. 8. Alignment of the 5′ end of the 16S rRNA genes of strains *L. hilgardii* DSM 20176 (a) accession number M58821, *L. hilgardii* IOEB 720 (b), IOEB 9649 (c), IOEB 7903 (d), IOEB 9647 (e), *L. brevis* IOEB 9112 (f), *L. brevis* NCDO 1749 (g) accession number X61134 (Collins *et al.*, 1991). The H2 oligonucleotide is underlined in the sequences where it appeared.

rDNA from the libraries. The best identities were obtained with the *L. hilgardii* species, in particular they all contained the H2 oligonucleotide (Fig. 8).

DISCUSSION

L. hilgardii and *L. brevis* were first differentiated by their carbohydrate-assimilation capacities. According to Kandler & Weiss (1986), the profiles are similar for the two species except concerning the arabinose fermentation. All strains used in this study were classified *L. brevis* or *L. hilgardii* according to their phenotype. However, there is evidence suggesting that assimilative properties may not be adequate enough for the delineation of both taxa as inter-strain variability may occur. Even if culture conditions are standardized, the control of all parameters appears difficult, since adaptation and growth in synthetic culture medium might differ according to the strain. Molecular approaches based on total genomic DNA hybridization cannot clear up the problem, owing to the fact that some cross-hybridization between *L. brevis* and *L. hilgardii* is observed (Vescovo *et al.*, 1979; Pidoux *et al.*, 1990; Lonvaud-Funel *et al.*, 1991). Therefore, PCR methods were developed for a better understanding of the taxonomic relationships within these two lactic acid bacteria species.

Three types of primers were chosen to achieve RAPD, REP-PCR and rRNA operon-specific amplification. Our analyses revealed that both fingerprinting methods (RAPD and REP-PCR) were equally suited to revealing species-specific genetic profiles. Moreover the classification of strains according to the specific rRNA operon amplification patterns gave the same result. The RAPD and REP-PCR fingerprintings showed a great amplification polymorphism among the *L. brevis* strains, contrary to the *L. hilgardii* strains. The strains could be separated into two genetically distinct clusters: the first group contained all the *L. hilgardii* (arabinose-negative) strains plus the IOEB 7903, IOEB 9647 and IOEB 9649 *L. brevis* (arabinose-positive) strains, while the second group contained all the other *L. brevis* strains tested in this study. These results did not correlate with those based on arabinose assimilation. However, in the DNA–DNA hybridization method (Lonvaud-Funel *et al.*, 1991), strains IOEB 7903, IOEB 9647 and IOEB 9649 hybridized not only with the different *L. brevis* reference probes, but also with the *L. hilgardii* reference probe. As an ultimate result, the 5′ end of their 16S rDNA was found to correspond to that of *L. hilgardii* one. This clearly confirms that these strains genotypically belong to the *L. hilgardii* in spite of their ability to ferment arabinose. Herewith, it proves the validity of the different molecular methods developed in this study to characterize *L. hilgardii*.

According to DNA–DNA homology, only some strains identified as *L. brevis* are known to form a homogeneous cluster (Vescovo *et al.*, 1979). The others hybridize with genomic DNA probes of *L. hilgardii*, *Lactobacillus kefir*, *Lactobacillus confusus* and *Lactobacillus collinoides* (Vescovo *et al.*, 1979; Lonvaud-Funel *et al.*, 1991). Thus, on the basis of the different PCR patterns obtained in this study, the reported

DNA–DNA hybridization data, reflecting the genomic heterogeneity of *L. brevis* was confirmed. It is still undetermined whether this heterogeneity is due to some instability of the *L. brevis* genome, or to genetic rearrangements occurring within the population *in vivo*. According to their genotypes, some strains although able to ferment arabinose, should be reclassified in the *L. hilgardii*. As it has already been shown (Boivin-Jahns *et al.*, 1995), this study provides further evidence that the biochemical and the molecular methods may diverge. These inconsistencies can be attributed to the fact that physiological properties are based on the phenotypic expression possibly influenced by regulation, whereas DNA–DNA hybridization and PCR methods are strictly based on the DNA sequence.

Different PCR methods have been established in order to discriminate *L. hilgardii* and *L. brevis* species. Furthermore, *L. hilgardii*-specific DNA probes have been isolated. They are usable for quality control during winemaking and other food processes where *L. hilgardii* is usually implicated.

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