International Journal of Systematic Bacteriology (1999), 49, 997–1007 Printed in Great Britain

Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA

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The genotypic and phenotypic diversity among isolates of the *Lactobacillus curvatus/Lactobacillus graminis/Lactobacillus sakei* group was evaluated by comparing RAPD data and results of biochemical tests, such as hydrolysis of arginine, D-lactate production, melibiose and xylose fermentation, and the presence of haem-dependent catalase. Analyses were applied to five type strains and to a collection of 165 isolates previously assigned to *L. sakei* or *L. curvatus*. Phenotypic and RAPD data were compared with each other and with previous DNA–DNA hybridization data. The phenotypic and genotypic separation between *L. sakei*, *L. curvatus* and *L. graminis* was clear, and new insights into the detailed structure within *L. sakei* and *L. curvatus* were obtained. Individual strains could be typed by RAPD and, after the elimination of similar or identical isolates, two sub-groups in both *L. curvatus* and *L. sakei* were defined. The presence or absence of catalase activity further distinguished the two *L. curvatus* sub-groups. By cloning and sequencing specific RAPD products, pairs of PCR primers were developed that can be used to specifically detect *L. curvatus*, *L. sakei* and each of the *L. sakei* sub-groups.

Keywords: RAPD, PCR primers, genetic diversity, *Lactobacillus sakei*, *Lactobacillus curvatus*

INTRODUCTION

Much taxonomic data on *Lactobacillus sakei* and *Lactobacillus curvatus* strains are currently available as a result of their technological interest. Both species were isolated from diverse habitats such as pre-packaged finished dough and fermented plant and meat material (Kandler & Weiss, 1986). They have been shown to play essential roles in meat preservation and fermentation processes (Egan, 1983; Hammes *et al.*, 1990), in which they are increasingly used as starter cultures. In this context, however, some important points remain to be considered for an objective selection of strains of interest: (i) rapid classification and identification of unknown isolates; (ii) evaluation of genetic diversity among strains and the impact of diversity on the relevant properties of micro-organisms; and (iii) strain typing to assess genetic stability over time.

The phenotypic and genotypic diversity within *L. curvatus* and *L. sakei*, as well as the close relatedness of the two species, have been revealed by many studies. Phenotypically, *L. curvatus* and *L. sakei* were often split into sub-groups (Schillinger & Lücke, 1987; Samelis *et al.*, 1995; Klein *et al.*, 1996) when physiological and/or biochemical data were analysed. The main criteria used to differentiate *L. sakei* from *L. curvatus* varied among laboratories, which resulted in the assignment of a strain to either *L. sakei*, *L. curvatus*, a group of atypical *L. sakei*/*L. curvatus*, or no assignment at all, depending on the criteria used (Schillinger & Lücke, 1987; Döring *et al.*, 1988; Montel *et al.*, 1991; Samelis *et al.*, 1995; Klein *et al.*, 1996). For example, differences in the assigned names were observed for the arginine-positive/melibiose-negative and arginine-negative/melibiose-positive strains. In addition, a protein pattern-based sub-grouping was

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Abbreviations: RAPD, randomly amplified polymorphic DNA; UPGMA, unweighted pair group method using arithmetic means.

Table 1. Relevant characteristics of the strains used in this study

Name as received	Strain number	Isolated from:	Source*	Phenotypic group†	RAPD group‡	Proposed name
L. curvatus	FY 113§	Meat	8	3	A1	*L. curvatus*
L. curvatus	T 402§	Pork meat	5	3	A1	*L. curvatus*
L. curvatus	YMG 348§	Meat	8	3	A1	*L. curvatus*
L. curvatus	CTC 243	Sausage	3	2	A2	*L. curvatus*
L. curvatus	CTC 253	Sausage	3	2	A2	*L. curvatus*
L. bavaricus	CTC 423, CTC 424, CTC 448	Sausage	3	1	A2	*L. curvatus*
L. curvatus	DSM 20019^T	Milk	1	2	A2	*L. curvatus*
L. curvatus	J 116§	Sausage	6	2	A2	*L. curvatus*
L. bavaricus	LTH 1143, LTH 1782, LTH 2053	Sauerkraut	2	1	A2	*L. curvatus*
L. curvatus	LV 19§	Pork meat	7	2	A2	*L. curvatus*
L. curvatus	R 57§, R 79, R126		9	2	A2	*L. curvatus*
L. curvatus	N 151§, N153§, N 177§, N 178§	Pork meat	12	2	A2	*L. curvatus*
L. curvatus	T 411§, T 662	Pork meat	5	2	A2	*L. curvatus*
L. sakei	CTC 041, CTC 287, CTC 427	Sausage	3	7	B1	*L. sakei*
L. sakei	J 3, J 20, J 23, J 25, J 28¶, J 30, J 32, J 45, J 55, J 62, J 66, J 69¶, J 70, J 72	Sausage	6	7	B1	*L. sakei*
L. sakei	J 38, J 52¶, J 53¶, J 56	Sausage	6	5	B1	*L. sakei*
L. sakei	J 321	Sausage	6	4	B1	*L. sakei*
L. sakei	LV 5	Pork meat	7	5	B1	*L. sakei*
L. sakei	LV 34¶	Beef meat	7	7	B1	*L. sakei*
L. sakei	T 300¶	Beef meat	5	5	B1	*L. sakei*
L. sakei	T 504	Pork meat	5	7	B1	*L. sakei*
L. sakei	T 518, T 557	Pork meat	5	5	B1	*L. sakei*
L. sakei	T 732¶	Meat	5	7	B1	*L. sakei*
L. sakei	TISTR 911		10	6	B1	*L. sakei*
L. sakei	V 553¶, YME 344¶, YMW 54¶	Meat	8	7	B1	*L. sakei*
L. sakei	CTC 014, CTC 335, CTC 429	Sausage	3	7	B2	*L. sakei*
L. curvatus	CTC 163	Sausage	3	5		*L. sakei*
L. sakei	DSM 6747¶		1	7	B2	*L. sakei*
L. sakei	J 14¶, J 18¶, J 19¶, J 21¶, J 22, J 26¶, J 29, J 31, J 33¶, J 35¶, J 36, J 37, J 43, J 44, J 48¶, J 60, J 63¶, J 67¶, J 71, J 81, J 82, J 91, J 93, J 95, J 112 -J 114, J 117, J 119–J 122, J 126, J 129, J 131, J 134, J 135, J 137¶, J 149¶–J 152, J 155–J 158, J 171–J 173, J 17¶, J 175¶, J 179¶–J 181¶, J 184¶, J 195, J 200, J 207¶, J 319	Sausage	5	7	B2	*L. sakei*
L. sakei	J 64¶	Sausage	6	4	B2	*L. sakei*
L. sakei	J 54, J 186¶	Sausage	6	5	B2	*L. sakei*
L. sakei	J 160x1¶	Horse meat	6	7	B2	*L. sakei*
L. sakei	J G3¶, J G13¶	Beef meat	6	7	B2	*L. sakei*
L. sakei	J GV1	Pork meat	6	7	B2	*L. sakei*
L. sakei	LTH 675, LTH 677, LTH 938, LTH 939, LTH 945	Sausage	2	7	B2	*L. sakei*
L. sakei	LTH 1764	Sauerkraut	2	7	B2	*L. sakei*
L. bavaricus	LTH 2068, LTH 2069, LTH 2070, LTH 2071, LTH 2076	Sauerkraut	2	6	B2	*L. sakei*
L. sakei	LV 21¶	Pork meat	7	7	B2	*L. sakei*
L. sakei	LV 52, LV 59	Lamb meat	7	7	B2	*L. sakei*
L. sakei	LV 82¶, LV 92	Bacon	7	7	B2	*L. sakei*
L. sakei	L 110¶	Starter	11	7	B2	*L. sakei*

Table 1 *(cont.)*

Name as received	Strain number	Isolated from:	Source*	Phenotypic group†	RAPD group‡	Proposed name
L. sakei	T 205¶, T 475	Beef meat	5	7	B2	*L. sakei*
L. sakei	T 214¶, T 240¶	Beef meat	5	4	B2	*L. sakei*
L. sakei	T 324¶, T 331¶, T 332¶, T 378¶, T 495¶, T 511, T 689, T 741¶	Pork meat	5	7	B2	*L. sakei*
L. sakei	T 381¶	Pork meat	5	5	B2	*L. sakei*
L. sakei	TISTR 890		10	7	B2	*L. sakei*
L. sakei	Y 216¶, YMH 243¶, YMW 557¶	Meat	8	7	B2	*L. sakei*
L. sakei	ATCC 15521^T	Sakei starter	4	7	B2	*L. sakei*
L. graminis	DSM 20719^T	Silage	1	10	C	*L. graminis*
L. plantarum	ATCC 14917^T		4	ND	D	*L. plantarum*
L. pentosus	ATCC 8041^T	Silage	4	8	E	*L. pentosus*
L. curvatus	T 497§	Pork meat	5	8	E	*L. pentosus*
Lactobacillus sp.	T 720	Starter	?	8	E	*L. pentosus*
L. curvatus	R 102e§	Meat	9	8	E	*L. pentosus*
L. sakei	T 392¶, T 589	Pork meat	5	9	F	?
Lactobacillus sp.	T 692¶		5	9		?

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† This work (Table 2).

‡ This work (Fig. 1).

§ Genetically identified by DNA–DNA hybridization or rDNA probing and affiliated with *L. curvatus* DNA (Montel *et al.*, 1991; Nissen & Dainty, 1995).

¶ Genetically identified by DNA–DNA hybridization and affiliated with *L. sakei* DNA (Montel *et al.*, 1991; M.-C. Montel, personal communication).

ND, Not determined.

conducted on 50 strains (Klein *et al.*, 1996) and two sub-groups were described for each species. The *L. sakei* sub-groups were not differentiated by biochemical features, whereas the *L. curvatus* sub-groups were defined according to their utilization or not of melibiose. Genotypically, numerical analysis of RAPD patterns from 39 strains of *L. sakei* and *L. curvatus* also revealed two sub-groups within *L. curvatus* and *L. sakei*, with one *L. curvatus* sub-group more closely linked to one of the two *L. sakei* than to the other *L. curvatus* sub-group (Torriani *et al.*, 1996). Protein pattern- and RAPD-based sub-groups coincided in the 24 strains that were analysed by both methods. At present, it is not possible to rapidly assign strains to these *L. sakei* genetic sub-groups. Comparison of 16S rRNA sequences from *L. curvatus*, *Lactobacillus graminis* and *L. sakei* showed that these species form a tight and distinct phylogenetic sub-group of the *Lactobacillus casei* group (Schleifer & Ludwig, 1995). These highly related species could be clearly delineated by DNA–DNA hybridization experiments (Beck *et al.*, 1988; Montel *et al.*, 1991; Klein *et al.*, 1996). *Lactobacillus bavaricus*, the previous name of a fourth species, was shown to be the junior synonym of either *L. sakei* or *L. curvatus* (Kagermeier-Callaway & Lauer, 1995).

The objectives of this study were to evaluate the genetic and biochemical diversity of the *L. sakei*/*L. curvatus* group, to compare its genetic and biochemical diversity, and to provide rapid and reliable tools for differentiating and detecting the group(s) and sub-group(s). For this, we used RAPD for several reasons: (i) it has proven to be a sensitive and efficient molecular method for distinguishing between different strains of a species or of related species; (ii) its rapidity renders it extremely attractive given the large size of our strain collection; (iii) with numerical analysis of data, it allows the grouping and the identification of strains (Vauterin & Vauterin, 1992); and (iv) RAPD products can serve as probes or can be sequenced to provide oligonucleotide probes and primers for specifically

Table 2. Phenotypic groups among the collection of strains

Phenotype no.	Hydrolysis of arginine	Fermentation of melibiose	Presence of catalase activity	Production of D-lactate	Fermentation of xylose
1	−	−	−	−	−
2	−	−	−	+	−
3	−	−	+	+	−
4	+	+	−	+	−
5	+	−	+	+	−
6	+	+	+	−	−
7	+	+	+	+	−
8	−	+	+	+	+
9	+	−	−	−	−
10	ND	−	ND	+	+

ND, Not determined.

Table 3. List of primers, PCR conditions, size of PCR products and specificity

Primer pair(s)	Sequence 5′–3′*	Duration of annealing/number of cycles	Size of PCR product(s) (bp)	Specificity
A1a/A1a′†	CTGCTGGGACGATTTG/CTGCTGGGACCATGTG	0 min/20 cycles	1840	*L. curvatus*
A1b/A1b′†	CTGCTGGGACCATTATTG/CTGCTGGGACACAATATG	1 min/30 cycles	1470	Not tested
A1c/A1c′†	GGAGGGTGTTCAGGAC/GGAGGGTGTTGATAGG	0 min/20 cycles	260	*L. curvatus*
B1a/B1a′†	CTGCTGGGACCAATT/CTGCTGGGACGAAAAG	0 min/20 cycles	750	*L. sakei* sub-group B1 (absent in B2)
B2a/B2a′†	CTGCTGGGACCTTAA/CTGCTGGGACTGAAG	1 min/30 cycles	1700	*L. sakei* sub-group B2
16/Lc‡	GCTGGATCACCTCCTTTC/TTGGTACTATTTAATTCTTAG	0 min/20 cycles	220	*L. curvatus*
16/Ls‡	GCTGGATCACCTCCTTTC/ATGAAACTATTAAATTGGTAC	0 min/20 cycles	220	*L. sakei*

* The first 10 nucleotides in the sequence (underlined) correspond to the sequence of the 10-mer RAPD primer.

† This work.

‡ Berthier & Ehrlich (1998).

detecting groups of strains (Manulis *et al.*, 1994; Rodriguez *et al.*, 1995; Bazzicalupo & Fani, 1996; Pooler *et al.*, 1996). RAPD analysis was applied to a collection of 165 isolates and five type strains representing different lactic acid bacteria. The isolates were isolated from diverse biotopes and geographic origins and were previously assigned to the *L. curvatus*/*L. sakei* group. This work provides new insights into the phenotypic and genotypic diversity of *L. curvatus* and *L. sakei*.

METHODS

Strains used. The bacterial isolates examined and their sources are listed in Table 1. Many of them were previously included in the DNA–DNA hybridization studies of Montel *et al.* (1991). They were routinely grown in MRS broth or on MRS agar plates (de Man *et al.*, 1960) at 30 °C.

Phenotypic analysis

For each strain, a liquid culture was grown from a single colony. Subcultures were used either for phenotypic analysis or for extracting DNA.

Production of D-lactate. D-Lactate was detected enzymically in the supernatant fluid of 3 d cultures using D-lactate dehydrogenase as indicated by the manufacturer (Boehringer Mannheim).

Presence of haem-dependent catalase. Cells were grown aerobically at 30 °C in MRS broth containing 5 g glucose l^{-1}, supplemented with 30 μg haematin l^{-1}. After 24 h, catalase activity was assessed by the presence of an effervescence when adding 0·6 % H_2O_2.

Melibiose and xylose fermentation. Cells were grown anaerobically for 3 d at 30 °C, under a H_2/CO_2 atmosphere (GasPack System; BBL) in MRS broth without glucose, either unsupplemented or supplemented (1 g l^{-1}) with xylose or melibiose. Fermentation was positive when the culture

growth improved and/or the pH of the culture broth dropped in the presence of the carbohydrate.

Arginine hydrolysis. Cells were grown anaerobically for 3 d at 30 °C under a H_2/CO_2 atmosphere (Gas-Pack System; BBL), in a broth containing (l^{-1}): 10 g meat extract (Difco); 10 g proteose peptone no. 3 (Difco); 5 g universal peptone (Merck); 1 ml Tween 80; 1 g glucose; and 30 mg $MnSO_4$. Broths were either unsupplemented or supplemented with 3 g arginine l^{-1}. Production of ammonia was detected by the rise of pH in the culture containing arginine.

RAPD analysis

DNA isolation. Total DNA was extracted from a culture inoculated with a single colony as described previously (de los Reyes-Gavilan *et al.*, 1992). The quantity of DNA was estimated by comparison with known standards in ethidium bromide-stained 0·7% agarose gels.

Primers used. Fifteen arbitrarily-designed primers of 10 nucleotides were tested initially by screening DNA from three isolates obtained from different sources in RAPD-PCR amplification assays. The suitability of each primer was scored on the basis of intensity and distribution of bands. Some primers gave reproducible banding profiles with a sufficient number of bands to be discriminatory for each isolate. Two of them were selected for performing subsequent RAPD analysis. Their sequences were 5′ CTGCTGGGAC 3′ for primer 1 and 5′ GGAGGGTGTT 3′ for primer 2.

PCR amplification. PCR amplification was performed in 100 µl volumes containing 20–100 ng DNA, 1·5 mM $MgCl_2$, 0·5 µM primer, 200 µM each dNTP and 2·5 U *Taq* DNA polymerase (Appligène) in 10 mM Tris/HCl (pH 9·0). PCR reactions were carried out in a thermal cycler (Perkin Elmer 9600) programmed for 30 cycles of amplification of 1 min at 94 °C, 2 min at 36 °C and 2 min at 72 °C.

Electrophoresis. The PCR reaction mixes (20 µl) were electrophoresed in 1% Seakem GTG agarose (Tebu) gels in TBE (90 mM Tris/borate, 1 mM EDTA). Each gel contained 10 lanes of PCR products and three lanes of a 123 bp ladder (Gibco-BRL) as normalization reference. This normalization reference was applied every five samples. Gels were then stained with ethidium bromide and photographed with Polaroid film 665. Negative photographs were digitized with a laser densitometer (Hewlett Packard ScanJet IIcx/T).

Computer analysis. The data set was analysed with the software package GelCompar (version 3.1; Applied Maths, Kortrijk, Belgium). The analysis included: (i) registration of the electrophoretic patterns; (ii) normalization of the densitometric traces and subtraction of background noise; (iii) grouping of strains by the Pearson product-moment correlation coefficient; and (iv) cluster analysis by UPGMA (unweighted pair group method using arithmetic means). PCR products larger than 2460 bp were not included in the analysis because of their poor electrophoretic resolution. The normalized densitometric traces obtained with each of the primers were assembled to obtain a single combined densitometric trace for each strain.

DNA sequence analysis. The RAPD-PCR fragments were gel-purified and cloned into a pBluescript II SK(+) plasmid vector (Stratagene). Both strands of DNA of four different recombinant clones were at least partially sequenced. The nucleotide sequence of DNA was determined with the 373 automated DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Nucleotide sequence data were analysed with version 8 of the GCG software package (Genetics Computer Group, Madison, WI, USA).

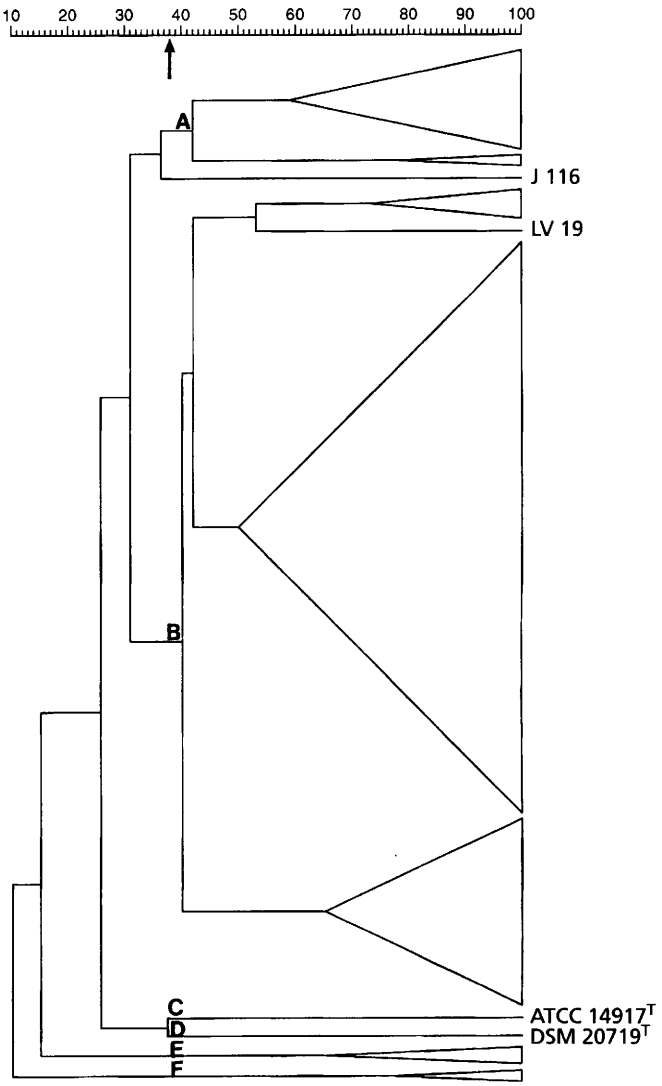

Fig. 1. Dendrogram generated from RAPD profiles of 170 isolates. The scale indicates the similarity level (Pearson coefficient level × 100). Profiles were grouped using the UPGMA method. Groups of profiles were reduced to white triangles and lettered A–F. The arrow marks the cut-off value at 38% to define the RAPD groups within the dendrogram. The names of some strains are indicated on the right.

PCR assays for diagnostic amplification. PCR was performed with 20 µl volumes containing 1 × PCR buffer without $MgCl_2$ (Boehringer Mannheim), 2·5 mM $MgCl_2$, 0·3 µM each dNTP, 0·5 U *Taq* DNA polymerase (Boehringer Mannheim), 20 ng template DNA and 0·3 µM each primer (Table 3). The amplification cycles always comprised a denaturation step of 1 min at 94 °C, a synthesis step of 1 min at 72 °C and an annealing temperature of 53 °C. The number of cycles and the annealing time depended on the primers used and are listed in Table 3. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide.

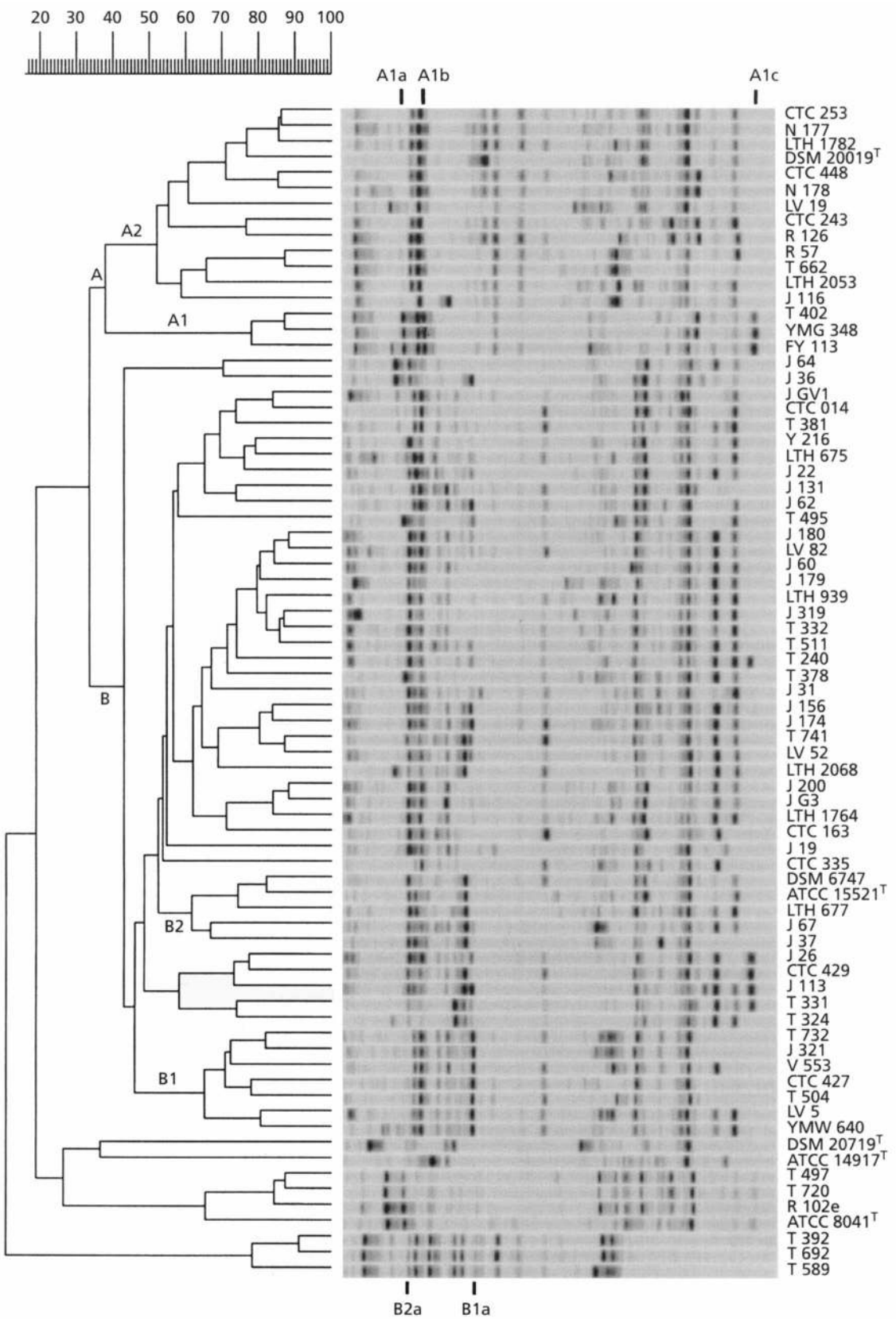

Fig. 2. RAPD profiles of 75 isolates and generated dendrogram. The scale indicates the similarity level (Pearson coefficient level × 100). Profiles were grouped using the UPGMA method. RAPD groups and sub-groups, as well as selected RAPD fragments, are lettered.

RESULTS

Biochemical analysis: phenotypic diversity among isolates

We examined five biochemical features usually used to differentiate between *L. sakei*, *L. graminis* and *L. curvatus* species: arginine hydrolysis in the presence of a low glucose concentration; melibiose and xylose fermentation; the presence of haem-dependent catalase; and the production of D-lactate. Typically, (i) *L. sakei* hydrolyses arginine, ferments melibiose and has catalase activity, but *L. curvatus* does not, (ii) *L. graminis* ferments xylose, but *L. sakei* and *L. curvatus* do not, (iii) the three species produce L- and D-lactate (Beck *et al.*, 1988; Wolf & Hammes, 1988; Montel *et al.*, 1991). As shown in Table 2, atypical phenotypes were found for some of the *L. sakei* or *L. curvatus* isolates studied. Novel atypical phenotypes were observed: three isolates (T 402, YMG 348 and FY 113) identified as *L. curvatus* by DNA–DNA hybridization, exhibited *L. curvatus* features, except that catalase activity was present (phenotypic group 3; Table 2) and inversely, four *L. sakei* isolates (J 64, J 321, T 214 and T 240) exhibited *L. sakei* features, except that catalase activity was absent (phenotypic group 4; Table 2). We did not find any melibiose-positive *L. curvatus* isolates, although some of the isolates given to us were believed to be positive. Two *L. curvatus* isolates (R 102e and T 497) were found to be melibiose-positive and also exhibited all biochemical features of *Lactobacillus pentosus*. As previously mentioned (Kagermeier-Callaway & Lauer, 1995; Samelis *et al.*, 1995), we observed melibiose-negative *L. sakei* isolates, as well as D-lactate-negative *L. curvatus* and *L. sakei* isolates. The latter were formerly named *L. bavaricus* (Kagermeier-Callaway & Lauer, 1995). Three isolates given to us as *L. sakei* (T 392, T 589 and T 692) exhibited a phenotype completely different from that of *L. sakei* (phenotypic group 9; Table 2).

RAPD-PCR analysis: genotypic diversity among isolates

Reproducibility. To ensure reproducible RAPD results, we strictly controlled the parameters of RAPD-PCR and electrophoresis and we assessed the level of variation among different profiles from a single isolate. Total DNA from three isolates was prepared independently five times and assayed by independent RAPD. All profiles of an isolate yielded similarity levels of 88% or higher when computed and clustered by UPGMA (not shown).

RAPD groups in the isolate collection: delineation of species. Numerical analysis of the 170 combined PCR profiles clustered by UPGMA enabled the plotting of the dendrogram shown in Fig. 1. Profiles differing by more than 88 %, termed 'selected' here, are displayed in Fig. 2.

Two major groups, A and B, were defined at the similarity level of 38%. Group A comprised the *L. curvatus* type strain and 20 isolates, including nine that were previously affiliated with *L. curvatus* by DNA–DNA hybridization or rDNA probing (Table 1). Group B comprised the *L. sakei* type strain and 138 isolates, including 49 that were previously affiliated with *L. sakei* by DNA–DNA hybridization (Table 1). Isolates formerly named *L. bavaricus* were placed in either the *L. sakei* or the *L. curvatus* groups.

In addition to the two major groups, four minor groups were defined. They were weakly linked to the major groups and comprised from one to four strains. One of these groups was composed of the *L. graminis* type strain. Another minor group comprised the *L. pentosus* type strain and three isolates, including two that were previously affiliated with *L. curvatus*. Given their *L. pentosus*-like phenotypic features (phenotypic group 8; Table 2), especially their xylose utilization and present RAPD grouping, the three isolates were very likely misnamed and were members of the *L. pentosus* species. Another minor group comprised three isolates, from which two were previously affiliated with *L. sakei*. They formed a distinct phenotypic group, exhibiting features very different from those typical of *L. sakei* or *L. curvatus* (phenotypic group 9; Table 2). They also were most likely misnamed. For the misnamed isolates, we do not know whether the cultures we received were named incorrectly or whether they became contaminated during our experiments.

Isolate J 116, identified as *L. curvatus* by DNA–DNA hybridization, was closely linked to group A (Fig. 1). As shown in Fig. 2, its profile was placed in group A when a selection of strains was analysed. Therefore, it was included in group A. Isolate LV 19, identified as *L. curvatus* by DNA–DNA hybridization, was located peripherally in group B (Fig. 1). As shown in Fig. 2, its profile was placed in group A when a selection of strains was analysed. Visual inspection of its profile clearly showed that it was misplaced within group B and better placed in group A.

The above results show that, after numerical analysis of the combined profiles obtained with two different primers, RAPD analysis could clearly differentiate between the species *L. curvatus* (RAPD group A), *L. sakei* (RAPD group B) and *L. graminis* strains previously delineated by DNA–DNA hybridization. Therefore, these conditions were routinely used for further RAPD analysis.

RAPD sub-groups: genetic diversity within *L. sakei* and *L. curvatus* isolates. Groups A and B could be subdivided into many sub-groups defined at different similarity levels.

Sub-groups defined at similarity levels of 88% or higher could be obtained. These levels corresponded to the lowest similarity level obtained by repeated RAPD analyses of the same isolate. In some cases, these sub-groups comprised different isolates originating from the same sample and containing similarly sized plasmids (not shown). In this regard, they probably corresponded to isolates of the same strain. In other

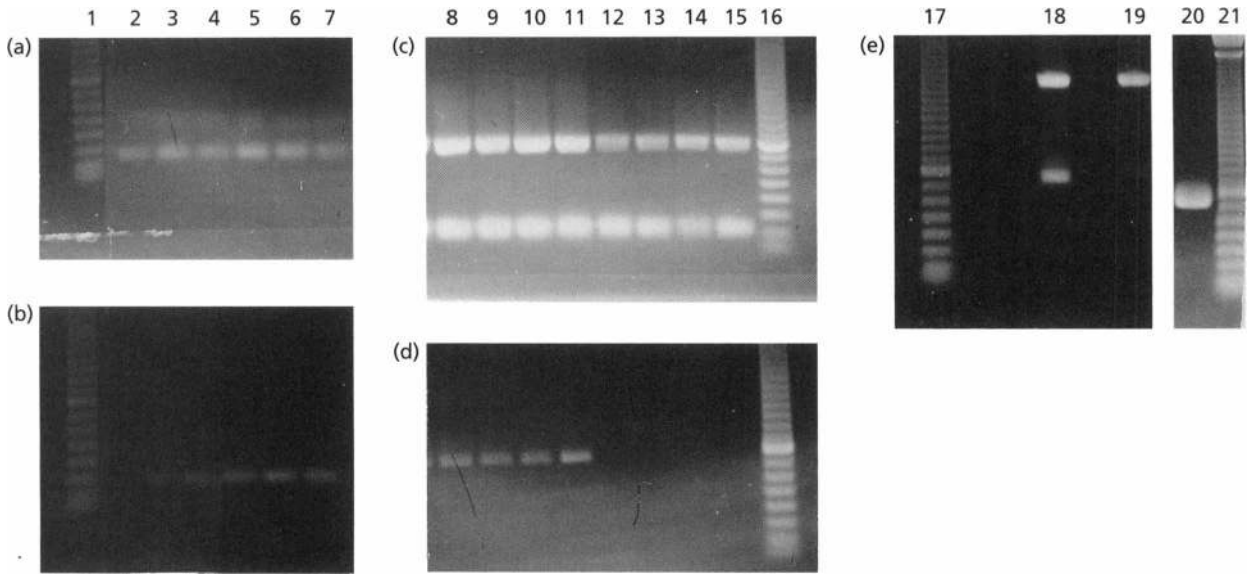

Fig. 3. PCR specificity. Amplification of *L. curvatus* [(a) and (b)] and *L. sakei* [(c), (d) and (e)] DNAs primed by the following oligonucleotides: (a) 16/Lc; (b) A1c/A1c′; (c) B1a/B1a′ and 16/Ls, separately then pooled; (d) B1a/B1a′; and (e) B1a/B1a′+B2a/B2a′. Reactions were carried out under conditions described in Table 3 except for (c) and (e), in which PCR conditions were 1 min annealing, 30 cycles. Lanes: 1, 16, 17, 21:100 bp ladder; 2–4, *L. curvatus* FY 113, T 402, YMG 348; 5–7, *L. curvatus* CTC 243, J 116, R 57; 8–11, *L. sakei* YMW 540, V 553, CTC 427, LV 5; 12–15, *L. sakei* LTH 2068, Theix 378, LV 82, Theix 495; 18, *L. sakei* LV 82; 19, *L. sakei* J 60; 20, *L. sakei* T 732.

cases, the sub-groups were composed of isolates originating from different samples, sometimes obtained from different countries and at different times. This indicates that similar strains can be widely dispersed.

Profiles differing by more than 88% are displayed in Fig. 2. Group A comprised 16 such profiles and group B comprised 50. Similarity values indicated that the *L. sakei* and *L. curvatus* strains were genetically diverse. Similarity values among the *L. sakei* and *L. curvatus* isolates were 43–87% and 52–87%, respectively. The new groups A and B were also composed of sub-groups.

Two sub-groups, A1 and A2, were defined for group A. A1 was composed of 13 profiles, including that of the *L. curvatus* type strain. A2 was composed of the three profiles of isolates which were phenotypically catalase-positive, a feature common to *L. sakei*.

Two sub-groups, B1 and B2, were defined for group B. B1 was composed of seven profiles and B2 of 40 profiles, including that of the *L. sakei* type strain. Sub-groups B1 and B2 did not correlate with any of those based on the phenotypic features we analysed, including trehalose fermentation. At this stage of analysis, the profiles of three isolates, CTC 335, J 36 and J 64, were not included in any sub-group. Indeed, the profile of isolate CTC 335 was linked to sub-group B1 within the selected set of profiles (Fig. 2) and was within B2, when the entire collection was considered (Fig. 1). The profiles of isolates J 36 and J 64 separated these isolates from all others.

Diagnostic PCR analysis

Characterization of the sub-group-specific RAPD fragments. Five RAPD products were chosen on the basis of their size, intensity and specificity to sub-groups A1 (fragments A1a, A1b, A1c), B1 (fragment B1a) and B2 (fragment B2a). They are marked with pointers in Fig. 2. These DNA products were cloned and partially or totally sequenced.

Design of sub-group-specific PCR primers. Sequence information was used for selecting the five PCR primer pairs listed in Table 3. Oligonucleotide primers specific for the detection of RAPD fragments consisted of the original 10-base RAPD primer plus the next 5 to 8 nucleotides as determined by sequence analysis. All primers amplified a specific product identical in size to the original cloned RAPD product (Table 3). Amplification with primer pair A1b/A1b′ was poor and this primer pair was rejected.

Specificity of the PCR assays. The PCR primer pairs were tested for their specificity by using DNA from strains of *L. graminis* and the RAPD groups A and B, selected for diversity analysis (Fig. 2). All amplification assays from one DNA were conducted simultaneously with the same DNA-containing PCR mixture and aliquots were mixed together with the primer pair(s) tested. One aliquot was mixed with primer pair 16/Lc or 16/Ls for species identification (Berthier & Ehrlich, 1998).

In theory, PCR with pairs A1a/A1a′ and A1c/A1c′ should selectively amplify the 1840 bp and 260 bp fragments, respectively, from DNAs of only the *L.*

curvatus sub-group A1. However, as seen in Fig. 3, for pair A1c/A1c′, it also amplified the same fragments from DNAs of *L. curvatus* sub-group A2, albeit less intensely, even when the number of cycles and the annealing time were low. Specificity was not enhanced by the addition of formamide at various concentrations (not shown). PCR with pairs A1a/A1a′ and A1c/A1c′ did not amplify any fragment from DNAs of *L. sakei* or *L. graminis* (data not shown). Thus, strains could be specifically identified as *L. curvatus* by testing the presence/absence of a fragment after PCR amplification with primer pairs A1a/A1a′ and A1c/A1c′, but not assigned reliably to a sub-group.

We expected that PCR with pairs B1a/B1a′ would selectively amplify a 750 bp fragment from DNAs of *L. sakei* sub-group B1. Such a fragment was indeed amplified only from DNAs of this sub-group under appropriate conditions (0 min annealing, 20 cycles; Table 3). However, under different conditions (1 min annealing, 30 cycles), amplification from some DNAs of sub-group B2 was also observed (Fig. 3). PCR with pairs B2a/B2a′ should selectively amplify a 1700 bp fragment from DNAs of *L. sakei* sub-group B2. A fragment of the expected size was indeed amplified from all the DNAs of sub-group B2, except one (LTH 2068). No fragment was amplified from DNAs of sub-group B1. PCR with primer pairs B1a/B1a′ and B2a/B2a′ did not amplify any fragment from DNAs of *L. curvatus* or *L. graminis* when the number of cycles was 30 and the annealing time was 1 min (data not shown). Thus, strains could be simultaneously identified as *L. sakei* and assigned to *L. sakei* RAPD sub-group B1 or B2 by PCR. To screen DNAs rapidly, we recommend the following procedure: primer pairs B1a/B1a′ and B2a/B2a′ used together with 30 cycles of amplification and 1 min of annealing. If a fragment of 1700 bp is amplified, the strain belongs to *L. sakei* sub-group B2. However, if a fragment of 752 bp is amplified, a PCR with pair B1a/B1a′ is run with 20 cycles of amplification and 0 min of annealing. If the fragment of 752 bp is still amplified, then the strain belongs to *L. sakei* sub-group B1. If not, the strain belongs to *L. sakei* sub-group B2. Isolates CTC 335, J 64 and J 36, which could not be assigned by RAPD, were included in sub-group B2.

DISCUSSION

The work presented here shows that RAPD combined with appropriate analysis tools can be used for detailed analysis of the structure of a strain collection. We found that arbitrarily selected 10-mer oligonucleotide primers, when used under well-defined conditions, are capable of reproducible amplification of random DNA fragments. With the numerical analysis of RAPD profiles, the inter- and intra-relatedness of species could be determined objectively. We used the Pearson correlation coefficient to obtain a measure of similarity for RAPD profiles, rather than other coefficients, which suffer from subjective band detection. The validity of classification by RAPD was assessed by comparing it with classifications obtained by phenotypic analysis and by the method of reference, DNA–DNA hybridization. We found an excellent agreement between these three approaches in terms of species delineation. The three methods allowed the same clear separation of the *L. sakei*, *L. curvatus* and *L. graminis* species. In comparison to the other two methods, RAPD offers the additional advantage of strain typing, since it simultaneously allows the determination of species affiliation and the differentiation of individual strains. This last property enabled us to analyse the genetic diversity within *L. curvatus* and *L. sakei* after elimination of similar isolates, and thus to delineate two *L. sakei* and two *L. curvatus* sub-groups. Affiliation of strains to one of these sub-groups can be readily determined by PCR with the diagnostic primers developed here for *L. sakei* strains and by testing for catalase activity for *L. curvatus* strains.

The detailed genetic structure of the *L. curvatus*/*L. sakei* group revealed by this work is somewhat different from that previously found (Klein *et al.*, 1996; Torriani *et al.*, 1996). Firstly, unlike the previously cited authors, we clearly separated *L. sakei* from *L. curvatus* by RAPD analysis. This finding is in agreement with results obtained in previous DNA–DNA hybridization studies which had shown differences between the whole genomes of *L. sakei* and *L. curvatus* (Montel *et al.*, 1991; Torriani *et al.*, 1996). Secondly, we did not find any melibiose-positive strains which could be regarded as *L. curvatus*, although the strains originated from several laboratories. The four strains N 151, N 153, N 177 and N 178, reported as melibiose-positive *L. curvatus* (Nissen & Dainty, 1995), were melibiose-negative in this study and clustered in the RAPD sub-group which includes the *L. curvatus* type strain. Strain W 54d, one of the strains belonging to the *L. curvatus* melibiose-positive group described by Klein *et al.* (1996) and Torriani *et al.* (1996), also fermented melibiose and hydrolysed arginine, but clustered in our *L. sakei* RAPD group. The phenotypic characterization of this strain was in accordance with our RAPD clustering, hydrolysis of arginine being a valid criterion for separating *L. sakei* from *L. curvatus*. Thirdly, the two sub-groups we identified in *L. curvatus* represent another subdivision to that proposed previously (Klein *et al.*, 1996; Torriani *et al.*, 1996), because of the different phenotypic features of the sub-groups in the two studies.

In addition to the information allowing strain classification, RAPD results helped us to develop tools for the rapid characterization of the *L. sakei*/*L. curvatus*/*L. graminis* strains, both at the species and subspecies levels. By searching for subspecies-specific PCR primers from sequences of some selected RAPD fragments, we designed primer pairs which specifically amplify DNA segments from strains of *L. curvatus* or from each of the two *L. sakei* RAPD sub-groups. We did not succeed in designing a diagnostic primer set for the *L. curvatus* sub-groups. This could be due to a failure to optimize PCR conditions or to the fact that

a primer-template mismatch near the 5′ end of the 10-mer primers caused RAPD polymorphism. This last explanation is supported by the hybridization studies of *L. sakei* RAPD fragments (not shown), for which we observed that polymorphism was caused by differences in priming sites rather than insertions or deletions.

It could be advantageous for laboratories to use a single diagnostic DNA fragment obtained by PCR with a pair of diagnostic primers, instead of the complex RAPD technique. However, RAPD remains necessary and useful in the search for identical isolates of the same strain.

In conclusion, RAPD can be used to evaluate the genetic diversity within species, as long as the validity of the RAPD classification at the species level is assessed. The number of primers to be used, i.e. the information needed, and the treatment of the information, i.e. the choice of the similarity coefficient and the clustering method to be used, are important parameters to be considered in elaborating a RAPD analysis protocol. RAPD then is a highly efficient method for evaluating diversity, because redundant strains can be eliminated. The practical implications of this study are numerous. For instance, the phenotypic identification key for *L. sakei* and *L. curvatus* was refined. Three phenotypic features, i.e. the fermentation of melibiose, the presence of catalase and the hydrolysis of arginine, were previously regarded as the main criteria for differentiating *L. sakei* from *L. curvatus* (Wolf & Hammes, 1988; Montel *et al.*, 1991; Samelis *et al.*, 1995). Melibiose fermentation was considered to be a useful criterion for differentiating subspecies in *L. curvatus* (Torriani *et al.*, 1996). In view of our results from RAPD and biochemical analyses, as well as previous results on DNA–DNA hybridization, a novel identification key can be proposed. It clearly appears that: (i) hydrolysis of arginine is the only phenotypic feature that can always differentiate *L. sakei* from *L. curvatus*; (ii) *L. sakei* exhibits at least two of the three phenotypic features; (iii) *L. curvatus* does not ferment melibiose; and (iv) the presence of catalase is the phenotypic criterion for differentiating two sub-groups in *L. curvatus*. Another implication of this study is that strains can be readily assigned to *L. sakei* or *L. curvatus* by using different PCR assays. The diversity of the data that can be obtained will be helpful for the rapid and reliable identification of unknown organisms.

ACKNOWLEDGEMENTS

We thank all the laboratories which supplied strains and P. Tailliez for help in designing RAPD experiments.

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