Characterization of Lactobacilli by Southern-Type Hybridization with a *Lactobacillus plantarum* *pyrDFE* Probe

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Lactobacillus plantarum, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum* (M.-C. Curk, J.-C. Hubert, and F. Bringel, Int. J. Syst. Bacteriol. 46:595–598, 1996) can hardly be distinguished on the basis of their phenotypes. Unlike *L. plantarum* and *L. paraplantarum*, *L. pentosus* ferments glycerol and xylose but not melezitose. We identified two *L. pentosus* strains (CNRZ 1538 and CNRZ 1544) which ferment glycerol and melezitose but not xylose. α -Methyl-D-mannoside was fermented by 66% of the *L. plantarum* strains tested but not by *L. paraplantarum* strains. In this paper we describe a simple method to identify *L. plantarum*, *L. pentosus*, and *L. paraplantarum*. This method is based on nonradioactive Southern-type hybridization between *Bgl*I DNA digests of the lactobacilli tested and a DNA probe (*L. plantarum* *pyrDFE* genes from strain CCM 1904). A total of 68 lactobacilli were classified into five groups on the basis of the bands detected. Two groups contained *L. plantarum* strains; one of these groups contained 31 strains, including the type strain, and was characterized by bands at 7, 4, and 1 kb, and the other group contained strain LP 85-2 and was characterized by bands at 5 and 1.1 kb. Only one band (a band at around 7 kb) was detected in the strains belonging to the *L. pentosus* group, and two bands (at 4 and 1 kb) were found in the strains belonging to the *L. paraplantarum* group. No hybridization was detected in the last group, which contained *Lactobacillus casei*, *Lactobacillus coryniformis*, *Lactobacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus delbrueckii*, and *Lactobacillus leichmannii* strains.

Lactobacilli related to *Lactobacillus plantarum* are lactic acid bacteria that are important in many plant fermentations (silage, pickled vegetables, sourdough) and meat and fish fermentations (17). Some of these organisms, such as the organisms found in beer, also can be food spoilage agents (19), and some lactobacilli are also found in cavities of mammals (16).

The taxonomy of *L. plantarum* is not satisfactory (13); this species contains strains that have similar biochemical characteristics but exhibit little similarity at the DNA level. Dellaglio et al. (9) identified the following three groups on the basis of DNA-DNA hybridization data: *L. plantarum*, *Lactobacillus pentosus*, and an atypical *L. plantarum* group recently reclassified as *Lactobacillus paraplantarum* (7). In agreement with the results of Dellaglio et al. (9), Zanoni et al. (24) revived the name *L. pentosus* for a distinct taxon; *L. pentosus* can be distinguished from *L. plantarum* by its ability to produce acid from D-xylose and glycerol (10, 13). However, these phenotypic characteristics are not sufficient to distinguish *L. plantarum* from *L. pentosus* since some strains ferment glycerol but not D-xylose (this study) or D-xylose but not glycerol (16). Direct sequencing of 16S rRNAs by reverse transcription clarified the phylogenetic status of several groups of lactic acid bacteria but could not be used to distinguish *L. plantarum* from *L. pentosus* since the 16S rRNA sequences of these organisms displayed a very high level of homology (ca. 99%) (6). Fourier transform infrared spectroscopy can be used to identify lactobacilli found in breweries, but *L. pentosus* could not be distinguished from *L. plantarum* by Curk et al. (8) on the basis of the spectra which they studied.

Until now, the only reliable method to discriminate between strains related to *L. plantarum* has been the liquid DNA-DNA hybridization technique (9, 24). Unfortunately, this method is time consuming and cannot be used routinely. A simple meth-

od is necessary. In this paper, we describe a method based on Southern-type hybridization (20). The DNAs of the lactobacilli examined were restricted with *Bgl*I and probed with *L. plantarum* genes of the pyrimidine de novo pathway. Genes of the de novo UMP biosynthetic pathway are conserved in live cells. Six enzymatic steps are required for the biosynthesis of UMP from glutamine, bicarbonate, and ATP. The last three enzymes are dihydroorotate dehydrogenase, orotidine monophosphate (OMP) pyrophosphorylase, and OMP decarboxylase, which are encoded by *pyrD*, *pyrE*, and *pyrF*, respectively. These activities were present in 140 *L. plantarum*-related strains studied for their pyrimidine requirements in the laboratory (unpublished data). The three genes were cloned and sequenced and were shown to be contiguous on the chromosome of *L. plantarum* CCM 1904 (= ATCC 8014) (1, 2). For these reasons, we decided to test whether the *L. plantarum* CCM 1904 *pyrDFE* genes could be used as a taxonomic tool for distinguishing strains related to *L. plantarum*.

MATERIALS AND METHODS

Strains and growth conditions. The sources of the strains which we used are shown in Table 1. Most strains were propagated in MRS broth (Difco) at 30°C; the exceptions were strains ATCC 8008B, ATCC 8008G, ATCC 10776, and ATCC 13649, which were incubated at 37°C. Glycine was added at a concentration of 2.5% when cells were lysed for DNA extraction.

Carbohydrate fermentative profiles. Fermentation of 49 carbohydrates was studied by using API 50 CH strips with API 50 CHL medium (bioMérieux).

DNA preparation. The genomic DNAs used for the Southern-type hybridization experiments and DNA-DNA liquid hybridization experiments were extracted as described by Vanderslice et al. (22) and Champomier et al. (3), respectively.

Southern-type hybridization. The probe used in the Southern-type hybridization experiments consisted of the *pyrDFE* genes (1, 2) of *L. plantarum* CCM 1904. The genomic DNA was released from bacterial cells by heating a preparation in sterile bidistilled water (approximately 10^5 cells in 40 μ l of water) at 94°C for 10 min. The lysate was put on ice, and the cell debris was removed by centrifugation. A 4- μ l portion of the supernatant was used as a template in a PCR performed with *Taq* DNA polymerase (Eurogentec) and the following primers: primer LP-D (5'-CATATGGTCAACAGATGGCTCAACAG-3') and primer LP-R (5'-TGACGCCACGTATGACGCGAAGC-3'). Primer LP-D includes the start codon of *pyrD*, and primer LP-R is located 31 nucleotides from

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TABLE 1. *Lactobacillus* strains

Name as received	Name determined in this study	Strain ^a	Source
<i>L. brevis</i>		CNRZ 425	Isolated from bread dough in France
<i>L. brevis</i>		CNRZ 738B	Isolated from silage
<i>L. brevis</i>		CST 10550	
<i>L. brevis</i>		CST 10932	Isolated from beer wort in France
<i>L. brevis</i>		DSM 20054 ^T	
<i>L. brevis</i>		IOI-O8-75	Obtained from the Centre de Formation API, La Balme-les-Grottes, France
<i>L. casei</i>		64 HG	Obtained from F. Gasser, Institut Pasteur, Paris, France
<i>L. coryniformis</i> subsp. <i>coryniformis</i>		DSM 20001 ^T	
<i>L. delbrueckii</i> subsp. <i>lactis</i>		DSM 20076	
<i>L. leichmannii</i>		NCIMB 8964	
<i>L. paracasei</i>		CST 10927	Isolated from brewery syrup
<i>L. paracasei</i> subsp. <i>paracasei</i>		CIP 53.166	
<i>L. paracasei</i>		DSM 20020	Isolated from saliva
<i>L. plantarum</i>	<i>L. paracasei</i>	NCIMB 3254	Isolated from cheese
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1537	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1538	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1544	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1546	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1547	Isolated from fermented green olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1552	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1555	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1558	Isolated from fermented green olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1561	Isolated from fermented green olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1569	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1570	Isolated from fermented olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	CNRZ 1573	Isolated from fermented green olives in Spain
<i>L. plantarum</i>	<i>L. pentosus</i>	Hansen	Laboratory strain
<i>L. pentosus</i>		NCFB 363 ^T	
<i>L. pentosus</i>		NCIMB 8531	Isolated from waste sulfite liquor
<i>L. plantarum</i>	<i>L. paraplantarum</i>	61 D	Isolated from human feces; obtained from F. Gasser, Institut Pasteur, Paris, France; equivalent to CIP 104445
<i>L. plantarum</i>		Agrano 15b	Laboratory strain isolated from bread dough
<i>L. plantarum</i>	<i>L. paraplantarum</i>	ATCC 10776 ^b	
<i>L. plantarum</i>		ATCC 13649 ^b	Isolated from cheddar cheese; formerly called <i>L. plantarum</i> var. <i>rudensis</i>
<i>L. plantarum</i>		ATCC 14917 ^T	Isolated from pickled cabbage
<i>L. plantarum</i>		CCM 1904	Isolated from corn silage; equivalent to ATCC 8014
<i>L. plantarum</i>		CIP 71.39	
<i>L. plantarum</i>		CNRZ 184	
<i>L. plantarum</i>		CNRZ 424	Isolated from bread dough in France
<i>L. plantarum</i>		CNRZ 738	Isolated from silage in France
<i>L. plantarum</i>		CNRZ 764	Isolated from cheese in France
<i>L. plantarum</i>		CNRZ 1228	Isolated from Domiati cheese in Egypt
<i>L. plantarum</i>		CNRZ 1246	Isolated from Domiati cheese in Egypt
<i>L. plantarum</i>		CST 10928	Isolated from recycled beer bottles
<i>L. plantarum</i>		CST 10952	
<i>L. plantarum</i>	<i>L. paraplantarum</i>	CNRZ 1887	Isolated from beer
<i>L. plantarum</i>	<i>L. paraplantarum</i>	CNRZ 1885 ^T	Isolated from beer; equivalent to CIP 104668
<i>L. plantarum</i>		CST 10967	Isolated from beer
<i>L. plantarum</i>		CST 11019	Isolated from beer
<i>L. plantarum</i>		CST 11023	Isolated from beer
<i>L. plantarum</i>		CST 11031	Isolated from beer
<i>L. plantarum</i>		CST 12009	Isolated from dairy products
<i>L. paracasei</i>	<i>L. plantarum</i>	DSM 2648	Isolated from silage
<i>L. plantarum</i>		Hd4	Obtained from A. Mourey, Nancy University, Villers-les-Nancy, France
<i>L. plantarum</i>		Hd17	Obtained from A. Mourey, Nancy University, Villers-les-Nancy, France
<i>L. plantarum</i>		LP 85-2	Isolated from grass silage; obtained from E. Armau, CNRS, University Paul-Sabatier, Toulouse, France

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the end of *pyrE*. The PCR consisted of denaturation at 94°C for 30 s, hybridization at 61°C for 30 s, and elongation at 72°C for 2 min; this cycle was repeated 40 times. A 2.2-kb fragment was obtained and subsequently labelled with a nonradioactive DIG DNA labelling and detection kit (Boehringer Mannheim). This probe could be stored for more than 1 year at -20°C. (In addition to PCR isolation, the 2.2-kb fragment can also be isolated from plasmid pF5 [1] digested with restriction enzymes *NdeI* and *BstXI*.)

Lactobacillus genomic DNA was digested with restriction enzyme *BglI* (Gibco

BRL) before it was electrophoresed in a 0.7% agarose gel. We used digoxigenin-labelled lambda DNA cleaved with *HindIII* (Boehringer Mannheim) as a DNA molecular weight marker. The DNA was treated with 0.25 M HCl and transferred to a nylon membrane (Hybond-N⁺; Amersham) by the capillary action of a 0.4 M NaOH solution.

We used hybridization conditions which allowed hybridization of DNAs that exhibited a level of similarity of at least 65%. Hybridization was performed at 50°C in the hybridization solution recommended by Boehringer Mannheim (5×

TABLE 1—Continued

Name as received	Name determined in this study	Strain ^a	Source
<i>L. plantarum</i>		NCFB 772	Isolated from cheese
<i>L. plantarum</i>		NCFB 963	Isolated from cheese
<i>L. plantarum</i>		NCFB 965	Isolated from cheese
<i>L. plantarum</i>		NCFB 1042	Isolated from cheese
<i>L. plantarum</i>		NCFB 1204	Isolated from cheese
<i>L. plantarum</i>		NCFB 2171	Isolated from cheese
<i>L. plantarum</i>		NCIMB 1406	Isolated from dental caries
<i>L. plantarum</i>		NCIMB 6461	
<i>L. plantarum</i>		NCIMB 7220	Isolated from pickled cabbage
<i>L. plantarum</i>		NCIMB 8299	Isolated from silage
<i>Lactobacillus</i> sp.		ATCC 8008G ^c	Isolated from butter
<i>Lactobacillus</i> sp.		ATCC 8008B ^c	Isolated from butter
<i>Lactobacillus</i> sp.		CST 10966J	

^a ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; CST, Collection Souches Tepral des brasseries Kronenbourg, Strasbourg, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCFB, National Collection of Food Bacteria, Reading, United Kingdom; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

^b These strains are not included in the current American Type Culture Collection catalog.

^c Two lactobacilli were found in the strain ATCC 8008 tube sent by the American Type Culture Collection. ATCC 8008B and ATCC 8008G produced white dome-shaped and flat grey colonies, respectively. The sugar fermentation profiles of these organisms are given in Table 2.

SSC, 0.1% sarcosine, 0.01% sodium dodecyl sulfate [SDS], 1% blocking agent [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). After hybridization, the membrane was washed with wash solution ($2\times$ SSC, 0.1% SDS) twice at room temperature for 5 min and then twice at 50°C for 15 min. The hybridized DNA was detected as recommended by Boehringer Mannheim.

DNA-DNA liquid hybridization. DNA was labelled by nick translation with [3 H]dCTP (30 Ci $\cdot$ mmol $^{-1}$; Amersham), dialyzed, and treated with S1 nuclease (12). DNA-DNA hybridization experiments were performed at 70°C after treatment with S1 nuclease and precipitation with trichloroacetic acid (12).

Thermal stability of DNA duplexes. After hybridization at 70°C, the temperature was raised in 5°C increments. Each time that the temperature was raised, a sample of reassociated DNA (100 μ l) was treated with S1 nuclease (15 μ l) and then treated as described above for the DNA-DNA liquid hybridization procedure (12). We then extrapolated the temperature at which 50% of the double-stranded DNA was denatured (T_m). The ΔT_m was the difference between the T_m of the homologous duplex and the T_m of the heterologous duplex.

RESULTS

Phenotypic characterization. A total of 53 lactobacilli were tested for the ability to ferment 49 carbohydrates, including glycerol and D-xylose, which are typically fermented by *L. pentosus* but not by *L. plantarum* (24); 15 of these lactobacilli fermented glycerol. A total of 13 of the 15 glycerol-fermenting strains also fermented D-xylose, suggesting that they were *L. pentosus* strains. Two strains, strains CNRZ 1538 and CNRZ 1544, did not ferment D-xylose, which raised the possibility that they were either *L. pentosus* or *L. plantarum* strains. As melezitose was catabolized by 81% of the *L. plantarum* strains tested (Table 2) and by none of the 13 strains

FIG. 1. Discrimination of *Lactobacillus* strains by Southern-type hybridization with an *L. plantarum* probe. (A) Agarose gel electrophoresis of *Bgl*II-restricted *Lactobacillus* genomic DNAs. (B) Hybridization of the *Lactobacillus* DNAs with the digoxigenin-labelled *pyrDFE* probe. Tracks 1, CCM 1904; tracks 2, CST 11019; tracks 3, ATCC 10776; tracks 4, ATCC 13649; tracks 5, CNRZ 1885^T; tracks 6, CNRZ 1887; tracks 7, ATCC 8008G; tracks 8, ATCC 8008B; tracks 9, NCIMB 8964; tracks 10, CNRZ 764; tracks 11, CNRZ 1569; tracks 12, CNRZ 1537. The DNA molecular size marker used was lambda DNA digested with *Hind*III.

TABLE 2. *Lactobacillus* carbohydrate utilization profiles as determined with the API 50CHL system

Taxon	Strain	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β-Methyl-xyloside	Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	α-Methyl-D-mannoside	α-Methyl-D-glucoside	N-Acetylglucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Inulin	Melezitose	D-Raffinose	Starch	Glycogen	Xylitol	β-Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2-Keto-gluconate	5-Keto-gluconate				
L. plantarum	Agrano 15b	- ^a	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	2	-	-	-	-			
	ATCC 13649	-	-	-	2	-	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	1	-	-	-		
	ATCC 14917 ^T	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	2	-	-	-	
	CCM 1904	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	-	-	2	-	-	-	-	-	-	1	-	2	-	-	-	
	CIP 71.39	1	-	-	2	-	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	1	-	-	-	-	
	CNRZ 184	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	1	-	-	-	
	CNRZ 424	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	-	-	-	-	
	CNRZ 738	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	1	-	-	-	-
	CNRZ 764	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	1	-	1	-	-	-
	CNRZ 1228	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	2	-	-	-	
	CNRZ 1246	1	-	-	2	-	-	-	-	-	2	2	2	2	-	2	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	-	-	-	-	-	-	
	CST 10928	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	2	-	1	-	-	-	
	CST 10952	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	1	-	-	-	
	CST 10967	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	2	-	-	-	
	CST 11019	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	2	-	-	-	-	-	
	CST 11023	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	1	-	-	-	-	-
	CST 11031	1	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	1	-	-	-	-
	CST 12009	1	-	-	2	2	-	-	-	-	2	2	2	2	-	2	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	1	-	-	-	-	
	DSM 2648	1	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	2	-	-	-	-	
	Hd4	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	-	-	2	-	-	-	-	1	-	2	-	-	-	-	
	Hd17	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	1	-	2	-	-	-	-	
	NCFB 772	-	-	-	2	-	-	-	-	-	2	2	2	2	-	2	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	1	-	-	-
	NCFB 963	-	-	-	1	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-
	NCFB 965	-	-	-	1	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-
	NCFB 1042	-	-	-	2	-	-	-	-	-	2	2	2	2	-	2	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	1	-	1	-	-	-
	NCFB 1204	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-
	NCFB 2171	-	-	-	1	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-
	NCIMB 1406	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-
NCIMB 6461	-	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	
NCIMB 7220	-	-	-	2	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	2	-	-	-	
NCIMB 8299	-	-	-	2	2	-	-	-	-	2	2	2	2	-	1	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	2	-	2	-	-	-	
LP 85-2	1	-	-	2	-	-	-	-	-	2	2	2	2	-	-	-	-	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	2	-	-	-	-	1	2	-	-	-	-	-	
L. paraplantarum	ATCC 10776	-	-	-	1	2	-	-	-	-	2	2	2	2	-	-	-	-	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	-	-	2	-</												

which fermented D-xylose, melezitose fermentation may also help distinguish *L. plantarum* from *L. pentosus*. Strains CNRZ 1538 and CNRZ 1544 produced acid from melezitose. We also found that ribose was fermented by all of the *L. plantarum* and *L. pentosus* strains tested except CNRZ 1538 and CNRZ 1544. Metabolism of sugars (raffinose and melibiose) has been shown to be temperature sensitive in *L. plantarum* CCM 1904 (21). In strains CNRZ 1538 and CNRZ 1544, different temperatures were used for ribose and D-xylose catabolism, but no fermentation was observed at 25, 30, or 37°C. We concluded that CNRZ 1538 and CNRZ 1544 have atypical biochemical properties and cannot be assigned on the basis of phenotypic characteristics to either *L. plantarum* or *L. pentosus*.

L. paraplantarum (four strains) and *L. plantarum* ATCC 14917^T (T = type strain) could not be distinguished on the basis of biochemical and physiological characteristics (7). When we tested carbohydrate utilization with a larger number of *L. plantarum* strains (32 strains), we observed that α -methyl-D-mannoside was catabolized by 66% of the *L. plantarum* strains but by none of the four *L. paraplantarum* strains (Table 2).

Southern-type hybridization with the *L. plantarum* *pyrDFE* probe. Previous workers have shown that strains classified as *L. plantarum* on the basis of their phenotypes have limited similarity at the DNA level (9, 24), so we assessed two stringencies of hybridization by changing the temperature of hybridization (see Materials and Methods). First, we tested high-stringency conditions at 68°C, but the *L. plantarum* probe did not hybridize with the *L. pentosus* strains (data not shown). When the temperature was lowered from 68 to 50°C, hybridization with *L. pentosus* was detected (Fig. 1, lanes 11 and 12). This corresponded to the theoretical detection of DNA sequences that exhibit at least 65% similarity with the probe (calculated as suggested by the protocol of Boehringer Mannheim). The *L. plantarum* CCM 1904 *pyrDFE* genes have been sequenced, and two *Bgl*I sites are separated by approximately 1 kb (2). In the Southern-type hybridization experiment performed with the entire 2.2-kb *pyrDFE* fragment, we expected three bands, including the 1-kb fragment. When the CCM 1904 genome was digested with *Bgl*I and hybridized, 7-, 4-, and 1-kb bands were detected, as shown in Fig. 1 (lane 1).

We tested 63 lactobacilli with the *L. plantarum* *pyrDFE* probe, and five groups were identified on the basis of the responses obtained (Table 3). Group 1 was characterized by bands at 7, 4, and 1 kb (Fig. 1, lanes 1, 2, 4, and 10) and contained 31 strains, including *L. plantarum* type strain ATCC 14917. Group 2 was characterized by bands at 5 and 1.1 kb and contained strain LP 85-2. Group 3 was characterized by bands at 4 and 1 kb (Fig. 1, lanes 3, 5, and 6) and contained the *L. paraplantarum* strains 61D, CNRZ 1887, ATCC 10776, and CNRZ 1885^T (7). Group 4 was characterized by a band at around 7 kb (Fig. 1, lanes 11 and 12), which was detected in 15 strains, including *L. pentosus* type strain NCFB 363. Group 5 contained the strains which did not interact with the probe and included strains that are not related to *L. plantarum*, such as *Lactobacillus brevis* CNRZ 425, CST 10550, CST 10932, DSM 20054^T, and IOI-08-75, *Lactobacillus casei* 64HG, *Lactobacillus coryniformis* DSM 20001^T, *Lactobacillus delbrueckii* DSM 20076, *Lactobacillus leichmannii* NCIMB 8964 (Fig. 1, lane 7), *Lactobacillus paracasei* CIP 53.166, CST 10927, DSM 20020, and NCIMB 3254, and lactobacilli of undefined species (strains CST 10966J, ATCC 8008B, and ATCC 8008G) (Fig. 1, lanes 8 and 9).

Liquid DNA-DNA hybridization. To test whether the groups based on restriction fragment length polymorphisms of the *pyr* genes had taxonomic significance, liquid DNA-DNA hybrid-

TABLE 3. Southern-type hybridization performed with the *L. plantarum* *pyrDFE* probe and *Bgl*I-digested *Lactobacillus* genomic DNAs

Group	Strain	Size(s) of the band(s) detected (kb)
1	<i>L. plantarum</i> Agrano 15b	7.1, 4, 1
	<i>L. plantarum</i> ATCC 14917 ^T	7.1, 4, 1
	<i>L. plantarum</i> ATCC 13649	7.1, 4, 1
	<i>L. plantarum</i> CCM 1904	7.1, 4, 1
	<i>L. plantarum</i> CIP 71.39	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 1228	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 1246	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 184	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 424	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 738	7.1, 4, 1
	<i>L. plantarum</i> CNRZ 764	7.1, 4, 1
	<i>L. plantarum</i> CST 10928	7.1, 4, 1
	<i>L. plantarum</i> CST 10952	7.1, 4, 1
	<i>L. plantarum</i> CST 10967	7.1, 4, 1
	<i>L. plantarum</i> CST 11023	7.1, 4, 1
	<i>L. plantarum</i> CST 11031	7.1, 4, 1
	<i>L. plantarum</i> CST 11019	7.1, 4, 1
	<i>L. plantarum</i> CST 12009	7.1, 4, 1
	<i>L. plantarum</i> DSM 2648	7.1, 4, 1
	<i>L. plantarum</i> Hd4	7.1, 4, 1
	<i>L. plantarum</i> Hd17	7.1, 4, 1
	<i>L. plantarum</i> NCFB 772	7.1, 4, 1
	<i>L. plantarum</i> NCFB 963	7.1, 4, 1
	<i>L. plantarum</i> NCFB 965	7.1, 4, 1
	<i>L. plantarum</i> NCFB 1042	7.1, 4, 1
	<i>L. plantarum</i> NCFB 1204	7.1, 4, 1
	<i>L. plantarum</i> NCFB 2171	7.1, 4, 1
	<i>L. plantarum</i> NCIMB 1406	7.1, 4, 1
	<i>L. plantarum</i> NCIMB 6461	7.1, 4, 1
	<i>L. plantarum</i> NCIMB 7220	7.1, 4, 1
	<i>L. plantarum</i> NCIMB 8299	7.1, 4, 1
2	<i>L. plantarum</i> LP 85-2	5, 1.1
3	<i>L. paraplantarum</i> 61D	4, 1
	<i>L. paraplantarum</i> ATCC 10776	4, 1
	<i>L. paraplantarum</i> CNRZ 1887	4, 1
	<i>L. paraplantarum</i> CNRZ 1885 ^T	4, 1
4	<i>L. pentosus</i> CNRZ 1537	7.1
	<i>L. pentosus</i> CNRZ 1538	7.5
	<i>L. pentosus</i> CNRZ 1544	7.5
	<i>L. pentosus</i> CNRZ 1546	7.5
	<i>L. pentosus</i> CNRZ 1547	7.1
	<i>L. pentosus</i> CNRZ 1552	7.5
	<i>L. pentosus</i> CNRZ 1555	7.5
	<i>L. pentosus</i> CNRZ 1558	7.5
	<i>L. pentosus</i> CNRZ 1561	7.5
	<i>L. pentosus</i> CNRZ 1569	7.1
	<i>L. pentosus</i> CNRZ 1570	7.1
	<i>L. pentosus</i> CNRZ 1573	7.5
	<i>L. pentosus</i> Hansen	7.5
	<i>L. pentosus</i> NCFB 363 ^T	7.5
	<i>L. pentosus</i> NCIMB 8531	7.5

ization was performed with the DNA of at least one member of each group and radiolabelled DNAs of type strains.

Under our experimental conditions, when *L. pentosus* NCFB 363^T DNA was radiolabelled and probed with DNAs of lactobacilli belonging to the five groups, only the group 4 strains hybridized efficiently (levels of relative binding, >82%) (Table 4), confirming that these strains were *L. pentosus* strains.

When the radioactive DNA was from the *L. plantarum* type strain (ATCC 14917), high levels of relative binding were ob-

TABLE 4. Levels of relative binding between *L. pentosus* NCFB 363^T and *L. plantarum* ATCC 14917^T [³H]DNAs and DNAs of other strains and thermal stabilities of some heteroduplexes

Group based on Southern data	Strain	<i>L. pentosus</i> NCFB 363 ^T		<i>L. plantarum</i> ATCC 14917 ^T		Taxon based on liquid DNA-DNA hybridization data
		% Hybridization	ΔT_m (°C)	% Hybridization	ΔT_m (°C)	
4	NCFB 363 ^T		0	50	8	<i>L. pentosus</i>
	CNRZ 1537	83		32		<i>L. pentosus</i>
	CNRZ 1544	95		35		<i>L. pentosus</i>
	CNRZ 1547	82	1.8	15		<i>L. pentosus</i>
	CNRZ 1552	97		25		<i>L. pentosus</i>
	CNRZ 1555	93		32		<i>L. pentosus</i>
	CNRZ 1569	90		50		<i>L. pentosus</i>
	CNRZ 1570	91		32		<i>L. pentosus</i>
	CNRZ 1573	86		25		<i>L. pentosus</i>
	NCIMB 8531	95		29		<i>L. pentosus</i>
1	ATCC 14917 ^T	20		100	0	<i>L. plantarum</i>
	ATCC 13649	13		98		<i>L. plantarum</i>
	CCM 1904	ND ^a		90		<i>L. plantarum</i>
	CIP 71.39	ND		95		<i>L. plantarum</i>
	CNRZ 764	49		100		<i>L. plantarum</i>
	CST 10952	ND		87		<i>L. plantarum</i>
	CST 11019	20		97		<i>L. plantarum</i>
	CST 11023	ND		90		<i>L. plantarum</i>
	DSM 2648	ND		81		<i>L. plantarum</i>
	NCIMB 6461	ND		80		<i>L. plantarum</i>
2	LP 85-2	18	11.4	79	3	<i>L. plantarum</i>
3	ATCC 10776	23		57		<i>Lactobacillus</i> sp.
	CNRZ 1885 ^T	14		41		<i>Lactobacillus</i> sp.
	CNRZ 1887	14		43		<i>Lactobacillus</i> sp.
	61D	17		41		<i>Lactobacillus</i> sp.
5	ATCC 8008B	0		0		<i>Lactobacillus</i> sp.
	ATCC 8008G	0		0		<i>Lactobacillus</i> sp.
	NCIMB 3254	ND		65	8	<i>Lactobacillus</i> sp.

^a ND, not determined.

tained with the group 1 strains (levels of relative binding, >80%) (Table 4) and with the one group 2 strain, strain LP 85-2 (level relative binding, 79%; ΔT_m , 3°C) (Table 4). When LP 85-2 was hybridized with *L. paraplantarum* CST 10967^T, a level of relative binding of less than 26% was obtained (data not shown). As defined by Wayne et al. (23), two strains belong to the same species when their DNAs have a level of similarity of $\geq 70\%$ and a maximum ΔT_m of 5°C. Therefore, we considered the group 1 and 2 strains *L. plantarum* strains. This was not the case with the group 3, 4, and 5 strains (Table 4) because the levels of relative binding between the DNAs of these strains and the DNA of the *L. plantarum* type strain were less than 65%.

DISCUSSION

Unlike *L. plantarum*, *L. pentosus* typically produces acid from glycerol and D-xylose but not from melezitose. This characteristic was found to be misleading in some cases. Strains CNRZ 1538 and CNRZ 1544 fermented glycerol and melezitose but not the pentose D-xylose. They also did not ferment ribose, another pentose fermented by all of the other lactobacilli tested in this study. These strains were atypical in their sugar fermentation phenotype but not at the DNA level. The results of liquid DNA-DNA hybridization with the type strains (Table 4) and Southern-type hybridization with the *L. plantarum* *pyrDFE* probe (Table 3) showed that CNRZ 1538 and CNRZ 1544 are *L. pentosus* strains. These strains may be isogenic since they had the same sugar fermentation profile

(Table 2), the same origin, and similar plasmid contents. Ruiz-Barba et al. (18) isolated CNRZ 1538 and CNRZ 1544 from Spanish-style fermented green olives (as strains LPC 4 and LPC 16, respectively) and showed that they harbored five plasmids having similar molecular weights. If the genes encoding xylose catabolism are on plasmids, they may be more unstable than chromosome-encoded genes and not appropriate to use as criteria in taxonomic studies. However, at least in *L. pentosus* MD 353, xylose catabolic genes have been cloned from the chromosome and sequenced (15). Erroneous classifications based on plasmid-encoded phenotypes have occurred; the *L. casei* strains which had lost their lactose plasmid (14) were placed in the subspecies *alactosus*. However, on the basis of DNA relatedness studies (13), they were placed in the subspecies *casei* and later renamed *L. paracasei* by Collins et al. (5). We isolated *L. pentosus* strains which do not ferment the pentoses D-xylose and ribose but ferment arabinose, a pentose catabolized by all of the other *L. pentosus* strains studied (Table 2).

In an effort to develop a simple method to distinguish lactobacilli related to *L. plantarum*, we assessed a method based on Southern-type hybridization between an *L. plantarum* *pyrDFE* DNA probe and *Lactobacillus* genomic DNA *Bgl*I digests. A total of 68 lactobacilli were classified into five groups on the basis of the sizes of bands obtained (Tables 3 and 4). All 32 *L. plantarum* strains tested were members of group 1 and group 2. Group 2 contained only strain LP 85-2, which may be a representative of a new subspecies of *L. plantarum* and is discussed below. When strain LP 85-2 was hybridized with the

pyrDFE probe, we detected two *Bgl*I bands (at 5 and 1.1 kb) instead of the three bands (at 7, 4, and 1 kb) detected with *L. plantarum* ATCC 14917^T. We detected no plasmids in strain LP 85-2 (data not shown), although plasmids are frequently found in lactobacilli (13). As determined by *Sfi*I pulsed-field gel electrophoresis macrorestriction (4), the genome size of LP 85-2 was estimated to be 3 Mb, which made this genome the smallest of the *L. plantarum* genomes tested; the genome sizes of strains CCM 1904, CST 11031, CST 11019, and CST 11023 were 3.3, 3.8, 3.9, and 3.9 Mb, respectively. Strain LP 85-2 produced only two of the five bands detected in the *L. plantarum* type strain, compared to the three or four bands detected in other *L. plantarum* strains (a Southern blot of total *Eco*RI genomic DNA digests was hybridized with an *L. plantarum* 5S ribosomal DNA probe [4]). Of the *L. plantarum* DNAs tested, LP 85-2 DNA had the lowest level of similarity with *L. plantarum* ATCC 14917^T DNA (Table 4), so the stability of the heteroduplex (ΔT_m) was determined. According to Grimont (11), DNAs from members of the same species form heteroduplexes with ΔT_m values less than 5°C; if the ΔT_m is between 2.5 and 5.5°C, then the strains belong to different subspecies. We estimated that the ΔT_m of the heteroduplex between ATCC 14917^T and LP 85-2 was 3°C, suggesting that strain LP 85-2 may be a member of a subspecies of *L. plantarum*. Other strains besides LP 85-2 must be found if *L. plantarum* is going to be split into subspecies.

The group 5 lactobacilli did not hybridize under our Southern-type hybridization conditions and were different from *L. plantarum*, *L. pentosus*, and *L. paraplantarum*. For instance, NCIMB 3254 exhibited a relative binding value of 65% when it was hybridized with the *L. plantarum* type strain, and the ΔT_m was 8°C (Table 4), showing that this strain is not an *L. plantarum* strain. When it was hybridized with *L. paracasei* ATCC 334 and *L. casei* NCIMB 11970^T, we obtained levels of relative binding of 100 and 16%, respectively (data not shown), demonstrating that NCIMB 3254 is an *L. paracasei* strain. Strain CST 10966J exhibited less than 4% relative binding with the DNAs of the type strains of *L. plantarum*, *L. pentosus*, *L. casei*, *L. paracasei*, and *L. rhamnosus* (data not shown); therefore, the taxonomic position of this strain remains unknown. Strains ATCC 8008B and ATCC 8008G did not hybridize with either the *L. plantarum* type strain or the *L. pentosus* type strain, confirming the conclusion of Dellaglio et al. (9) that strain ATCC 8008 was not an *L. plantarum* or *L. pentosus* strain. Since no bands were detected with the *L. plantarum pyrDFE* probe, these lactobacilli are not *L. paraplantarum* strains either.

In conclusion, we developed a simple and reliable method to distinguish strains of *L. plantarum* (groups 1 and 2), *L. pentosus* (group 4), and *L. paraplantarum* (group 3). This method may help distinguish additional taxa when it is used with a larger number of lactobacilli.

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