IDENTIFICATION OF LACTIC ACID BACTERIA BY RIBOTYPING[1]

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ABSTRACT

Current methods that can be used for the identification of lactic acid bacteria (LAB) include: biochemical tests, pulsed field gel electrophoresis, and fatty acid analysis. These methods can be costly, time-consuming, and technically difficult. We are investigating the microbial ecology of vegetable fermentations and are interested in the rapid identification of LAB isolates. We have adapted a PCR-based ribotyping method for use with LAB. The PCR product(s) produced contains the sequences from the intergenic spacer regions between the rRNA genes. These products can be resolved by standard agarose or acrylamide gel electrophoresis. Using this method, we have identified PCR product electrophoresis banding patterns for the primary species of LAB found in vegetable fermentations, including those from the genera Lactobacillus, Leuconostoc, Lactococcus, *and* Pediococcus.

INTRODUCTION

A variety of biochemical and molecular methods exist for the identification of lactic acid bacteria (LAB), as reviewed by Teuber (1993), and Dykes and von Holy (1994). Traditional biochemical methods rely on the unique metabolic pro-

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perties of bacterial species, and identification schemes or "diagnostic keys" have been developed for LAB (Cavett 1963; Schillinger and Lucke 1987). Phenotypic differences (morphology, biochemistry) among the LAB, however, may misrepresent the phylogenetic relationships among this group of organisms (Stackebrandt and Teuber 1988), making classification of LAB species difficult. Molecular methods available for identifying LAB include: plasmid typing (Davies *et al.* 1981; Maniatis *et al.* 1982); M13 fingerprinting (Miteva *et al.* 1992); pulse field gel electrophoresis methods (Tanskanen *et al.* 1990); restriction fragment length polymorphism (RFLP) and ribotyping methods (Rodrigues *et al.* 1991; Rodtong and Tannock 1993).

Ribotyping is defined as the use of the position or structure of genes encoding ribosomal RNA to determine the identity of a particular genus or species (Schmidt 1994). We were interested in developing a rapid and simple method for identifying LAB from vegetable fermentations. Toward this end, we adapted a PCR-based ribotyping method for determining the identification of lactic acid bacterial species. This method (Jensen *et al.* 1993) makes use of PCR amplification of the intergenic spacer regions of rRNA operons. The spacer region between the 16S and 23S genes in bacterial rRNA operons has been found to be flanked by highly conserved sequences (Olsen *et al.* 1992). The length of the intergenic spacer sequences and the number of rRNA operons in a cell can vary between species. PCR amplification of this region can, therefore, give a unique set of amplified fragments, specific for a given species. While other ribotyping methods for LAB have previously been developed (Rodrigues *et al.* 1991; Rodtong and Tannock 1993), this method does not require a Southern DNA transfer and requires minimal manipulation of the cellular DNA samples.

MATERIALS AND METHODS

Bacterial Cultures

Strains used in this work are listed in Table 1. All bacterial strains were grown in MRS broth (Difco Laboratories, Detroit MI) supplemented with 1.5% agar for plate medium or APT broth and agar (Difco).

Chromosomal DNA Isolation

Chromosomal DNA was isolated from LAB using a Mini-Beadbeater (model 3110, Biospec Products, Bartlesville, OK) with a modification of the protocol of Wilson *et al.* (Wilson *et al.* 1990). A turbid cell suspension (1.5 ml in a 1.7 ml microfuge tube, type UP2061, United Laboratory Plastics, St. Louis, MO)

was harvested and resuspended in a final volume of 0.4 ml LETS buffer (100 mM LiCl, 10 mM ethylene diamine tetraacetic acid [EDTA], 10 mM Tris-hydrochloride, 1% sodium dodecyl sulfate, pH 7.8, all chemicals from Sigma Chemical Co., St. Louis, MO). The cell suspension extracted with phenol-chloroform-isoamyl alcohol solution (in a ratio of 50:48:2 by volume, all chemicals from Sigma) along with 0.1 g of glass beads (0.1 mM diameter, Tye 11079-101, Biospec Products) in 2 ml screw cap microfuge tubes (Type 10831, Biospec Products). The tubes were then placed in the Mini-Beadbeater and agitated at maximum velocity for 1 min. Following centrifugation in an Eppendorf microcentrifuge (model 5415, Brinkmann Instruments, Westbury, NY) for 3 min at approximately 13,500 × g, at 4C, the aqueous phase was removed and re-extracted with phenol-chloroform-isoamyl alcohol as described above. An additional extraction with an equal volume of chloroform-isoamyl alcohol (48:2, by volume) was carried out, and the DNA was then precipitated with 0.1 volumes of 2 M LiCl (Sigma) and 2.5 volumes of ice cold 95% ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) in a 1.5 ml microfuge tube. The pellet was washed with 70% ethanol and dried for 10 min in a SpeedVac Concentrator (model SVC100 rotor, RT100 condensation trap, VP100 vacuum pump, Savant Instruments, Farmingdale, NY). For RNase treatment, the DNA pellet was resuspended in 100 μl of water, and 5 μl of a 10 mg/ml DNase free stock solution of RNase A (Sigma, prepared as described in Maniatis *et al.* 1982), was added. The RNase treatment was carried out at room temperature for 15 min. The solution was then extracted with an equal volume of phenol, then chloroform and precipitation of the DNA was carried out with 0.1 volume of 3 M sodium acetate (Sigma) and 3 volumes of 95% ethanol. The precipitate was washed with 70% ethanol and dried as described above. The DNA pellet was resuspended in 50 μl of water and the DNA solution was frozen at −20C until used for PCR reactions.

PCR Protocols

A modification of the procedure of Jensen *et al.* (1993) was used for the PCR protocol. The reaction mixture of 100 μl in 0.6 ml microfuge tubes (no. 3437, Continental Laboratory Products Inc., San Diego, CA) contained: 70 μl of water, 10 μl of 10X PCR buffer II (500 mM KCl and 100 mM Tris-HCl, pH 8.3, Perkin-Elmer Corp., Norwalk, CT), 10 μl of 25 mM $MgCl_2$ (Sigma), 4 μl of Primer solution (see below), 1 μl of dNTP mixture (25 mM each dNTP, No. 200415, Stratagene Cloning Systems, La Jolla, CA), 4 μl of DNA preparation as described above, and 0.8 μl of Taq DNA polymerase (5 U/μl, Strategene). Four primers were used in these studies: G1-16S, GAAGTCGTAACAAGG (Genosys Biotechnologies, Inc., The Woodlands, TX); L1-23S, CAAGGCATCCACCGT (Genosys); G2-16S, TGCGGCTGGATCACC (Bio-Synthesis, Inc., Lewisville,

TABLE 1.

BACTERIAL STRAINS

Strain	Source[a]	Calculated band sizes[b]	FFL No.[c]
Lactobacillus plantarum 14917	ATCC	696,443	LA70
Lactobacillus plantarum NC-8	WD	688,431	LA86
Lactobacillus plantarum WSO	FFL	677,424	LA23
Lactobacillus plantarum MOP3	FFL	680,426	B17
Lactobacillus plantarum 340	FFL	702,431	LA90
Leuconostoc mesenteroides C33	JRS	600	LA10
Leuconostoc mesenteroides 23386	ATCC	548	LA108
Leuconostoc mesenteroides NCK293	TRK	605	LA121
Leuconostoc mesenteroides 13146	ATCC	605	LA145
Leuconostoc mesenteroides 10882	ATCC	565	LA147
Leuconostoc mesenteroides 8293	ATCC	594	LA81
Leuconostoc mesenteroides dextranicum 19255	ATCC	600	LA7
Pediococcus pentosaceus 33316	ATCC	723,557	LA76
Pediococcus pentosaceus FFL-48	FFL	706,517	LA3
Pediococcus pentosaceus L-728	FFL	706,517	LA52
Pediococcus pentosaceus L-7230	FFL	710,520	LA61
Pediococcus pentosaceus 25745	ATCC	714,528	LA73
Pediococcus acidilactici 33314	ATCC	755,554	LA74
Pediococcus dextrinicus 33087	ATCC	509,293	LA224
Lactobacillus brevis 14869	ATCC	664,448	LA228
Lactobacillus brevis FBB50	FFL	663,437	LA25

TX); and L2-23S, GGGTTTCCCCATTCGGA (Bio-Synthesis). Equal volumes of the appropriate primers (30 mM) were mixed and then added to the PCR reactions as described above. PCR reactions were carried out using a model GTC-2

TABLE 1.

Lactobacillus brevis MD43	FFL	660,435	LA26
Lactobacillus brevis MD20	FFL	660,435	LA27
Lactobacillus brevis B4006	NRL	660,435	LA188
Lactobacillus brevis 8287	ATCC	657,432	LA200
Lactobacillus cellobiosis 11739	ATCC	593,395	LA31
Lactobacillus fermentum 14931	ATCC	593,398	LA35
Lactobacillus casei B1445	NRRL	699,602,430	LA37
Lactobacillus salivarius salivarius B1949	NRL	709,469	LA39
Lactobacillus coryneformis torquens B4390	NRRL	632,623,556,398	LA41
Lactobacillus xylosus B4449	NRRL	403	LA43
Lactobacillus pentosus 8041	ATCC	683,441	LA136
Lactobacillus curvatus 25601	ATCC	721,531	LA223
Lactobacillus buchneri 4005	ATCC	653,469,437	LA30
Enterococcus faecalis 19433	ATCC	485,358	B174
Lactococcus lactis lactis NCK400	TRK	558	LA138
Lactococcus lactis lactis 7962	ATCC	558	LA123
Lactococcus lactis lactis LM232	LM	561	LA78

[a]ATCC = American Type Culture Collection (Rockville, MD); WD = Dr. W. Dobrogosz, NCSU (Raleigh, NC); FFL = USDA-ARS Food Fermentation Laboratory culture collection (Raleigh, NC); NRRL = USDA-ARS Northern Regional Research Laboratory (Peoria, IL); JRS = Dr. J. R. Stamer, Cornell University; TRK = Dr. T. R. Klaenhammer, NCSU (Raleigh, NC); LM = Dr. L. McKay, Univeristy of Minnesota.

[b]Size predictions in base pairs, as described in Materials and Methods.

[c]USDA-ARS Food Fermentation Laboratory culture collection (North Carolina State University, Raleigh, NC) idenification number.

Genetic Thermal Cycler, with a model LTM-2 refrigeration unit (Precision Scientific Inc., Chicago, IL) with an initial heat denaturation step (Innis *et al.* 1990). The Taq polymerase was then added to the hot solution, and 75 μl of mineral oil was added to overlay the reaction mixture. Twenty five cycles of 94C for 1 min, 55C for 5 min, and 72C for 2 min were then carried out, followed by a final incubation of 5 min at 72C. The aqueous phase of the reaction mixture was then removed from the tubes and stored at -20C.

Polyacrylamide Gel Electrophoresis

PCR products were separated on 5% polyacrylamide gels using a vertical gel electrophoresis box with the glass plates supplied by the manufacturer (BRL Model V16, Life Technologies, Inc., Gaithersburg, MD) with 1.5 mm spacers and 14 or 20 tooth comb (Life Technologies). DNA samples (approximately 0.3 μg) were 10 μl in volume and were mixed with 5 μl bromphenol blue tracking dye solution (0.25% bromphenol blue [Bio-Rad], and 30% glycerol [Sigma], in water) prior to loading onto the gel. Electrophoresis was carried out using a tris-borate buffer (TBE, Maniatis *et al.* 1982). Alternatively, PCR products were electrophoresed in submerged 1.0% agarose (Sigma) gels, using a horizontal submerged gel apparatus (model 1704343, Bio-Rad). After electrophoresis, the gels were stained with ethidium bromide (0.2 μg/ml, in water, Sigma) and photographed with transmitted UV light (UVT Transilluminator, model 3-3100, Haake-Buchler Inst. Inc., Saddlebrook, NJ). The sizes of the DNA fragments produced by the PCR reactions were calculated by measurement of the distance migrated in the gel compared to DNA molecular weight size standards. The sizes were calculated using the log transform of the distance migrated using a linear regression model (The SAS System for Windows, version 3.1, SAS Institute Inc., Cary, NC). In all cases, the R^2 values determined were above 0.98. Electronic images of the DNA gel photographs were prepared for publication using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Restriction Digests

Following PCR amplification, 20 μl of the reaction mixture, containing the DNA products, were removed from the reaction tube and 1 μl of Rsa I enzyme solution (16 U/μl, No. 500890, Stratagene) was added, in a new microfuge tube. The restriction digestion was carried out at 37C for 30 min in a water bath (model 18800, Lab Line Instruments, Inc., Melrose Park, IL). Following the digestion reaction, the samples were placed at −20C prior to electrophoresis.

RESULTS

Selection of PCR Primers

In the initial experiments, the primers G1-16S and L1-23S, described by Jensen *et al.* (1993) were used. These primers gave 12 or more bands with a variety of LAB template preparations (data not shown). Because of the possibility that some of these bands represented nonspecific priming, attempts were made to optimize the PCR reaction conditions (data not shown). An alternative set of primers

was obtained using the rRNA Database (Olsen *et al.* 1992; Dr. J.W. Brown, North Carolina State University, personal communication), by comparing sequence similarities for bacterial 16S genes and for 23S genes. The eubacterial consensus sequences were used to determine the sequences of two new primers, G2-16S and L2-23S. The relative locations of the primers in relation to the rRNA operon intergenic spacer region (transcribed intergenic spacer region, or ITS) between the 16S and 23S rRNA genes, are shown in Fig. 1. The DNA fragments from the PCR reactions were separated on an agarose submarine gel. Subsequent DNA separations were carried out on vertical acrylamide gels as described in Materials and Methods. All four combinations of the original and new 16S and 23S primers were used on two templates (*Pediococcus dextrinicus* and *Enterococcus faecalis*). The results were shown in Fig. 2. Primers G1-16S and L2-23S gave the least amount of background (nonspecific) priming and were selected for further use. Note that, in all cases where primer G2-16S was used, a band of approximately 2 kb in size was produced, regardless of the 23S primer used, indicating nonspecific priming had occurred. The banding patterns produced for the low molecular weight bands with the four sets of primers corresponded to the predicted sizes based on the location of the primers (Fig. 2). The largest set of these low molecular weight bands was seen with the G1-L2 primers (Fig. 2, lanes 4 and 8), and the smallest set of the low molecular weight bands was seen with the G2-L1 primer set (Fig. 2, lanes 3 and 7), as predicted by the location of the primer hybridization sites on the rRNA operon. These results indicate that the low molecular weight bands observed were the products of priming from the intended sites on the rRNA operon, and did not result from nonspecific priming.

Survey of LAB from Vegetable Fermentations

To determine the variation in the size (measured in base pairs, based on the DNA size standards) of the PCR products between strains of a given species, strains from a variety of sources were tested, including the ATCC type strain for that species. The species tested included *Lactobacillus brevis, Pediococcus pentosaceus, Leuconostoc mesenteroides,* and *Lactobacillus plantarum.* These

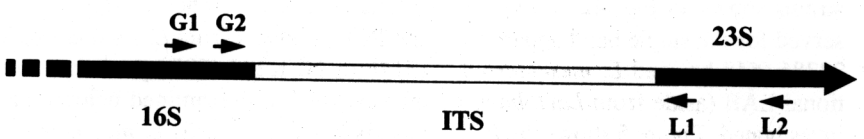

FIG. 1. DIAGRAM OF THE PRIMER LOCATIONS FLANKING THE INTERGENIC TRANSCRIBED SPACER (ITS) REGION BETWEEN THE 16S AND 23S rRNA GENES
Primers G1 and G2 (G1-16S and G2-16S, respectively) are located at the 3′ end of the 16S gene, and primers L1 and L2 (L1-23S and L2-23S, respectively) are located at the 5′ end of the 23S gene.

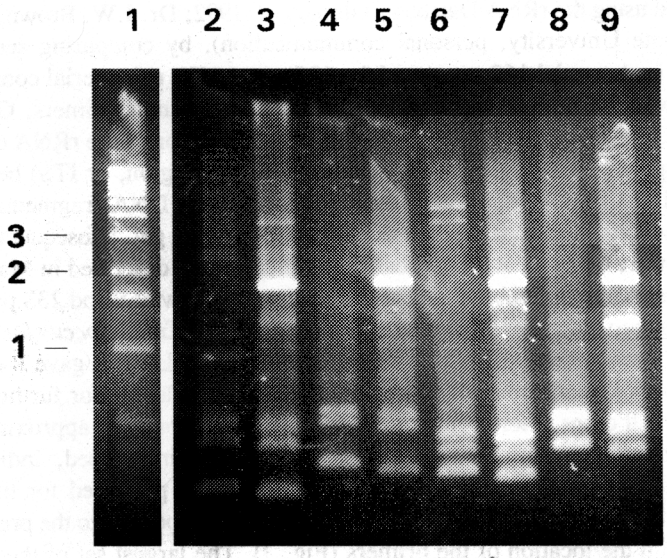

FIG. 2. PCR PRODUCTS FROM THE FOUR COMBINATIONS OF PRIMERS AS DESCRIBED IN THE TEXT

Lane 1: 1 kilobase ladder DNA size standards. Lanes 2–5: *P. dextrinicus* 33087, lanes 6–9: *E. faecalis* 19433. The primers used to produce the DNA fragments were: G1-L1, lanes 2 and 6; G2-L1, lanes 3 and 7; G1-L2, lanes 4 and 8; G2-L2, lanes 5 and 9.

organisms were chosen because they represent the major types of microflora found in vegetable fermentations (Fleming *et al.* 1995; Pederson and Albury 1969). The PCR products for the ATCC type strains of these four species are shown in Fig. 3. The respective PCR DNA fragment sizes were: *L. plantarum*: 696, 443 bp; *L. brevis*: 664, 448 bp; *L. mesenteroides*: 594 bp (possibly 2 fragments of similar size); and *P. pentosaceus* 723, 557 bp. Overall, very little variation (±25 bp) in the PCR fragment sizes was observed between strains of a given species (Fig. 4a-4d). The most variation was observed with the *L. mesenteroides* strains shown in Fig. 4c. As much as 52 base pairs difference in size was observed for the single band apparent for the PCR products from *L. mesenteroides* 23386 (548 bp) and *L. mesenteroides* 13146 or NCK293 (605 bp). Some additional LAB (aside from *Lactobacillus* species which are examined below) were investigated. Figure 5 shows the PCR products from *E. faecalis*, *L. mesenteroides* spp. *dextranicum*, *Pediococcus acidilactici*, *P. dextrinicus*, and 3 strains of *Lactococcus lactis* ssp. *lactis*.

Survey of the Genus *Lactobacillus*

To determine the variation of PCR product sizes between species within a genus, ten species of the genus *Lactobacillus* were tested to determine the size of the PCR products produced. The results are shown in Fig. 6. The pattern of PCR fragment sizes observed was unique for each species, with the exception of *Lactobacillus cellobiosis* and *Lactobacillus fermentum* for which the observed PCR produced DNA fragments were similar in size. Because *L. plantarum* and *L. brevis* also gave fragments that were similar in size (696, 443 and 664, 448, respectively, for the ATCC type strains), we wanted an additional test to clearly distinguish these lactobacilli and other LAB that produced similar PCR banding patterns to each other. Toward this end, we digested the PCR reaction products of some *L. plantarum* and *L. brevis* strains with the restriction enzyme RsaI. This enzyme was active in PCR reaction buffer, eliminating the need to purify the PCR reaction products prior to digestion. Figure 7 shows 4 strains of *L. plantarum* and 3 strains of *L. brevis* digested with RsaI. The patterns observed for the digested fragments clearly distinguished the *L. plantarum* and *L. brevis* species. The

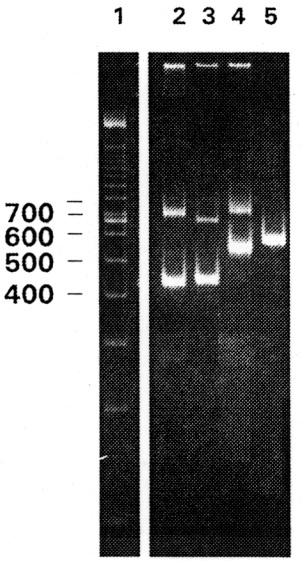

FIG. 3. PCR PRODUCTS FROM THE FOUR SPECIES OF LAB COMMON IN VEGETABLE FERMENTATIONS
Lane 1: 100 bp ladder DNA size standard; lane 2: *L. plantarum* 14917; lane 3: *L. brevis* 14869; lane 4: *P. pentosaceus* 33316; lane 5: *L. mesenteroides* 8293.

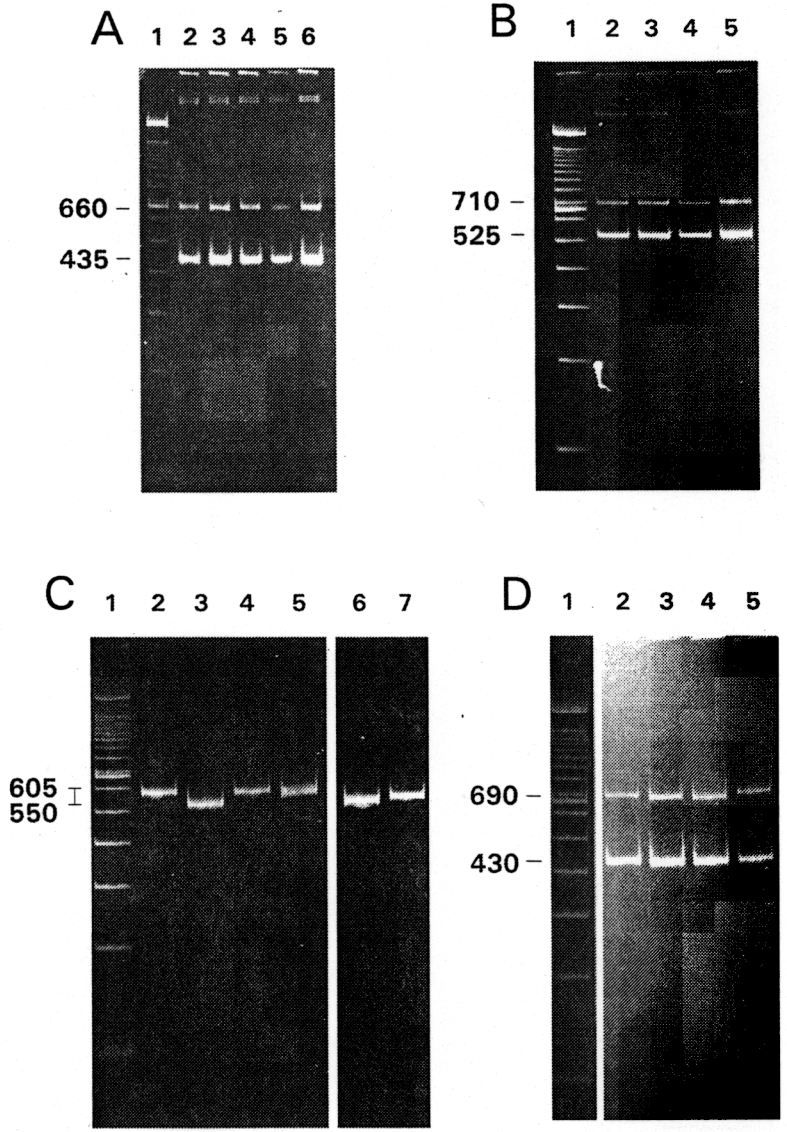

FIG. 4. FOR 4A-4D, LANE 1 IN EACH CASE CONTAINS 100 BP DNA LADDER SIZE STANDARDS

A) *L. brevis* strains in lanes 2-6: FBB50, MD34, MD20, B4006, 8287, respectively. B) *P. pentosaceus* strains in lanes 2-5: FFL-48, L-728, L-7230, 25745, respectively. C) *L. mesenteroides* strains in lanes 2-7: C33, 23368, NCK293, 13146, 10882, 8293, respectively. D) *L. plantarum* strains in lanes 2-5: NC-8, WSO, MOP3, 340, respectively.

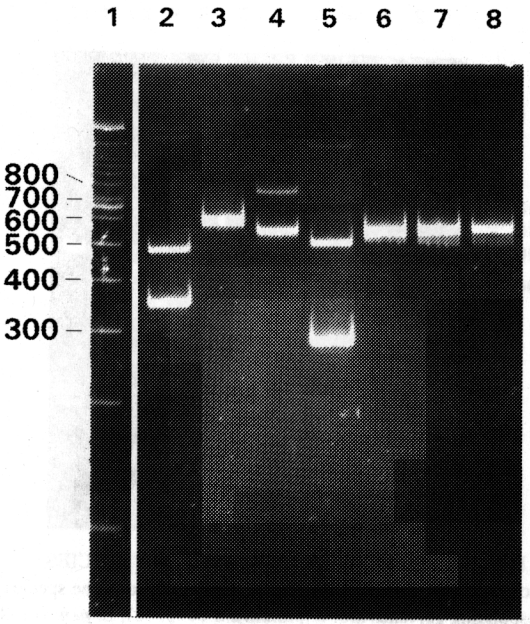

FIG. 5. PCR PRODUCTS FROM ADDITIONAL LAB
Lane 1: 100 BP DNA ladder size standards; lane 2, *E. faecalis* 19433; lane 3, *L. mesenteroides dextranicum* 19255; lane 4, *P. acidilactici* 33314; lane 5, *P. dextrinicus* 33087; lanes 6–8, *L. lactic lactis* strains NCK400, 7962, and LM232, respectively.

digested DNA fragments were determined to be approximately 630 bp and 360 bp for *L. plantarum*, while the *L. brevis* products were approximately 550 bp and 280 bp. The 550 bp fragment from the digest of the *L. brevis* DNA sample was not observed for two of the *L. brevis* strains. This band may represent a partial digestion product from the *L. brevis* 14869 PCR fragments. Alternatively, additional restriction sites may be present in the two strains not showing the bands.

DISCUSSION

The PCR-based ribotyping method described gives a rapid, inexpensive, and simple means for the identification of LAB species in vegetable fermentations. Because we used primers that hybridized to conserved sequences, theoretically present in all bacteria, the method worked only with pure cultures. Mixed culture

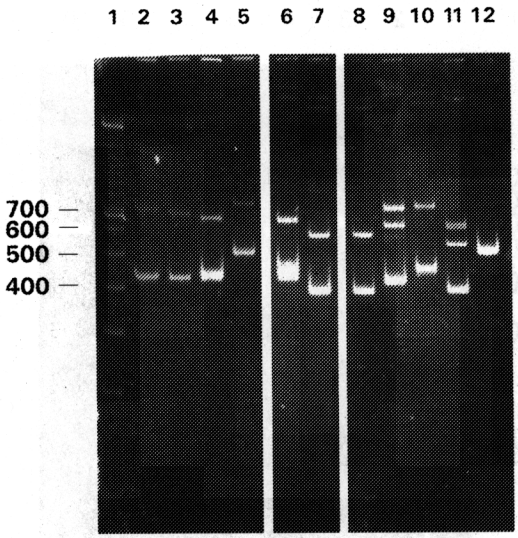

FIG. 6. A SURVEY OF *LACTOBACILLUS* SPECIES
Lane 1 contained the 100 BP DNA ladder size standards. The species and the corresponding gel lanes are: *L. plantarum* 14917 (2), *L. pentosus* 8041 (3), *L. brevis* 14869 (4), *L. curvatus* 25601 (5), *L. buchneri* 4005 (6), *L. cellobiosis* 11739 (7), *L. fermentum* 14931 (8), *L. caseii* B1445 (9), *L. salivarius salivarius* B1949 (10), and *L. coryneformis torquens* B4390 (11), *L. xylosus* B4449 (12).

PCR reactions would result in all the species present contributing to the banding pattern, producing multiple bands that would be difficult to interpret. With pure cultures, two DNA bands were observed for most LAB species. The exceptions were 1 band for *Leuconostoc* species, *L. lactis* and *Lactobacillus xylosis*, and 3 bands for some of the lactobacilli. Because of the small number of bands produced by the PCR reactions, we recommend that cultures for ribotyping by this method should be isolated on a medium that selects for LAB.

It is possible that the bands observed represent the PCR products from multiple operons with the same or similar sized intergenic spacer regions, so the number of rRNA operons/cell can not be directly inferred from the data. The number of bands seen is only an indication of the number of rRNA operons with different 16S-23S spacer region sizes in a given cell.

Some species of LAB gave PCR products with similar sizes to the pattern observed for other species, as observed by similar gel banding patterns. The patterns observed for the PCR-produced DNA fragments may reflect phylogenetic relationships. For example, the *Leuconostoc* strains were found to have two types of banding patterns, with 5 strains giving a single band of approximately 600 bp in size, and 2 strains giving a single band around 550 bp. However, because

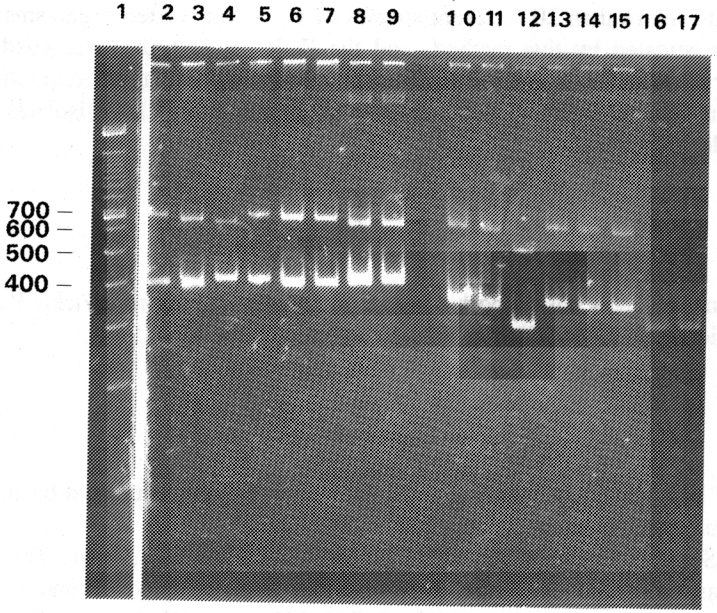

FIG. 7. RESTRICTION DIGESTION PATTERNS FOR *L. PLANTARUM, L. PENTOSUS*, AND *L. BREVIS* STRAINS

Lane 1 contained the 100 BP DNA ladder size standards; lanes 2–9, undigested; lanes 10–17, the corresponding samples digested with *Rsa I.* The bacterial strains and lane numbers for *L. plantarum* were: 14917 (2, 10), NC-8 (5, 13), WSO (6, 14), MOP3 (7, 15); *L. pentosus* 8041 (3, 11); and *L. brevis* were: 14869 (4, 12), B4006 (8, 16), 8287 (9, 17).

only the16S-23S spacer region length is determined by this method, we do not believe that accurate phylogenetic information can be determined. Further characterization of the relationships between these strains would likely require rDNA sequencing (Stackebrandt and Teuber 1988) or other molecular methods.

The use of restriction enzyme digestion of the DNA fragments resulted in distinct banding patterns for *L. plantarum* and *L. brevis*, when the PCR fragment sizes were found to be similar for these species. Because the fragments to be digested were all less than 1 kb, restriction enzymes with 4 base pair recognition sites were chosen, increasing the likelihood that one or more restriction sites would be present on the target DNA. Other restriction enzymes were tested, including AluI and Sau3AI (data not shown). Using one or more of these enzymes to digest the PCR products may serve to further distinguish LAB species giving similar PCR product banding patterns and may distinguish strains within a species. Because the enzymes chosen were functional in PCR reaction buffer, the digestion procedure did not require the DNA to be purified prior to digestion.

To date, more than 20 different species of LAB and related organisms have been investigated by this method, and the PCR product sizes recorded in a database. The database and pattern matching software (F. Breidt, unpublished) will be useful for the rapid determination of the identity of LAB isolates from vegetable fermentations.

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