

IDENTIFICATION OF LACTIC ACID BACTERIA BY RIBOTYPING¹

FRED BREIDT² and HENRY P. FLEMING

*Food Fermentation Laboratory
U.S. Department of Agriculture, Agricultural Research Service
and North Carolina Agricultural Research Service
Department of Food Science
North Carolina State University
Raleigh, NC 27695-7624*

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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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²Corresponding author; telephone: 919-515-2979; fax: 919-515-4694; e-mail: fred_breidt@ncsu.edu.

properties 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 $13,500 \times g$, 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 μ l of water, and 5 μ l 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 μ l of water and the DNA solution was frozen at -20°C 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 μ l in 0.6 ml microfuge tubes (no. 3437, Continental Laboratory Products Inc., San Diego, CA) contained: 70 μ l of water, 10 μ l of 10X PCR buffer II (500 mM KCl and 100 mM Tris-HCl, pH 8.3, Perkin-Elmer Corp., Norwalk, CT), 10 μ l of 25 mM MgCl_2 (Sigma), 4 μ l of Primer solution (see below), 1 μ l of dNTP mixture (25 mM each dNTP, No. 200415, Stratagene Cloning Systems, La Jolla, CA), 4 μ l of DNA preparation as described above, and 0.8 μ l of Taq DNA polymerase (5 U/ μ l, Stratagene). 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
<u>Lactobacillus plantarum</u> 14917	ATCC	696,443	LA70
<u>Lactobacillus plantarum</u> NC-8	WD	688,431	LA86
<u>Lactobacillus plantarum</u> WSO	FFL	677,424	LA23
<u>Lactobacillus plantarum</u> MOP3	FFL	680,426	B17
<u>Lactobacillus plantarum</u> 340	FFL	702,431	LA90
<u>Leuconostoc mesenteroides</u> C33	JRS	600	LA10
<u>Leuconostoc mesenteroides</u> 23386	ATCC	548	LA108
<u>Leuconostoc mesenteroides</u> NCK293	TRK	605	LA121
<u>Leuconostoc mesenteroides</u> 13146	ATCC	605	LA145
<u>Leuconostoc mesenteroides</u> 10882	ATCC	565	LA147
<u>Leuconostoc mesenteroides</u> 8293	ATCC	594	LA81
<u>Leuconostoc mesenteroides dextranicum</u> 19255	ATCC	600	LA7
<u>Pediococcus pentosaceus</u> 33316	ATCC	723,557	LA76
<u>Pediococcus pentosaceus</u> FFL-48	FFL	706,517	LA3
<u>Pediococcus pentosaceus</u> L-728	FFL	706,517	LA52
<u>Pediococcus pentosaceus</u> L-7230	FFL	710,520	LA61
<u>Pediococcus pentosaceus</u> 25745	ATCC	714,528	LA73
<u>Pediococcus acidilactici</u> 33314	ATCC	755,554	LA74
<u>Pediococcus dextranicus</u> 33087	ATCC	509,293	LA224
<u>Lactobacillus brevis</u> 14869	ATCC	664,448	LA228
<u>Lactobacillus brevis</u> FBB50	FFL	663,437	LA25

TX); and L2-23S, GGGTTTCCCCATTTCGGA (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.

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

^aATCC = 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, University of Minnesota.

^bSize predictions in base pairs, as described in Materials and Methods.

^cUSDA-ARS Food Fermentation Laboratory culture collection (North Carolina State University, Raleigh, NC) identification 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 μ l 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 $\mu\text{g}/\text{ml}$, 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

