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Detection and Identification of Gastrointestinal *Lactobacillus* Species by Using Denaturing Gradient Gel Electrophoresis and Species-Specific PCR Primers

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Denaturing gradient gel electrophoresis (DGGE) of DNA fragments obtained by PCR amplification of the V2-V3 region of the 16S rRNA gene was used to detect the presence of *Lactobacillus* species in the stomach contents of mice. *Lactobacillus* isolates cultured from human and porcine gastrointestinal samples were identified to the species level by using a combination of DGGE and species-specific PCR primers that targeted 16S-23S rRNA intergenic spacer region or 16S rRNA gene sequences. The identifications obtained by this approach were confirmed by sequencing the V2-V3 region of the 16S rRNA gene and by a BLAST search of the GenBank database.

The gastrointestinal tracts of animals, including humans, harbor complex microbial communities comprised of possibly hundreds of bacterial species (13). Members of the genus *Lactobacillus* are commonly present as members of these communities and have received considerable attention with respect to their putative health-conferring properties (as probiotics [6]).

Analysis of gastrointestinal communities has relied traditionally on bacteriological culture methods and microscopy. There is doubtless a bias present in the results of these studies, however, because not all of the members of the communities can be cultured (11). The results of monitoring the composition of gastrointestinal communities may be more reliable if molecular methods were used in addition to traditional approaches (5, 16). Microbiological investigations of the compositions of terrestrial and aquatic microbial communities have shown the versatility of denaturing gradient gel electrophoresis (DGGE) combined with PCR as a molecular analytical method (4, 10). In this technique, DNA is extracted from cells of all of the species represented in the habitat of interest, and a hypervariable sequence region of the 16S rRNA gene is amplified by PCR. The mixture of 16S fragments is subjected to DGGE in order to separate the fragments and thus obtain a profile of the community. This profile is generated because of differences in the chemical stability, and hence the distance into the gradient where denaturation occurs and migration ceases, of the 16S fragments that have different nucleotide base compositions (10). We have for many years maintained a mouse colony devoid of lactobacilli as gastrointestinal inhabitants (14). They provided a suitable system with which to test the efficacy of PCR-DGGE as an analytical method in studies of the gastrointestinal microflora.

Even when they can be cultured reliably, gastrointestinal species of bacteria can be difficult to identify. The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond that of the common fermentation tests (for example, cell wall analysis and electrophoretic mobilities of lactate dehydrogenase [8]). In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (7). The logistics, regardless of the doubtful accuracy of phenotypic identification methods, are daunting when large-scale investigations of the intestinal microflora are pursued. We reasoned that, since modern bacterial classification is greatly influenced by knowledge of 16S rRNA gene sequences, PCR-DGGE could provide a more practical approach to the identification of lactobacilli.

We demonstrate here that addition of *Lactobacillus* species to the stomach microflora of mice could be detected by PCR-DGGE analysis. Further, we demonstrate that *Lactobacillus* isolates cultured from gastrointestinal samples obtained from humans and pigs could be identified, or at least grouped, by PCR-DGGE. The species identities of the isolates were then further investigated with species-specific PCR primers that targeted 16S-23S intergenic spacer region or 16S rRNA gene sequences. These identifications were subsequently confirmed by obtaining 16S rRNA gene sequences that were compared to those in GenBank.

MATERIALS AND METHODS

Bacterial cultures. The lactobacilli used in this study are listed in Table 1. The bacteria were cultured with Lactobacilli MRS medium (Difco Laboratories, Detroit, Mich.) incubated under anaerobic conditions at 37°C. Thirty-six of the strains used in this study were unidentified isolates ("unknowns") originating in gastrointestinal samples (Table 1).

Mouse experiments. Lactobacillus-free mice were maintained in isolators by gnotobiotic technology. The animals harbored a microbial community in their gastrointestinal tracts equivalent to that of conventional mice except that lactobacilli were absent (14). To test the ability of PCR-DGGE to detect changes in the microbial community of the murine stomach, we maintained a group of five lactobacillus-free mice in an isolator. At day 0, we removed one of the mice (mouse 1) as a control. Then we inoculated the remainder of the animals, by mouth, with a culture of strain 100-23. Four days later, we removed one of the mice (mouse 2) and inoculated the remainder with *Lactobacillus* sp. strain 100-5. Then 4 days later, we removed another mouse (mouse 3) and inoculated the remaining mice with *Lactobacillus* sp. strain 21. Another mouse (mouse 4) was removed at day 12, and the remaining animal (mouse 5) was inoculated with strain 20 and examined 4 days later. Upon removal from the isolator, the mice were killed by carbon dioxide anesthesia and cervical dislocation, the stomach was removed from each mouse, and the stomach contents were retained and

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TABLE 1. *Lactobacillus* strains

Strain	Source[a]	Identification by:			GenBank accession no. for sequence
		DGGE	Species-specific primers	16S V2-V3 sequence	
L. acidophilus ATCC 4356^T	ATCC	NA[b]	*L. acidophilus*	NA	NA
L. agilis DSM 20509^T	DSM	NA[b]	NA	NA	NA
L. brevis ATCC 14869^T	ATCC	NA[b]	NA	NA	NA
L. casei ATCC 334	ATCC	NA[b]	*L. casei*	NA	NA
L. casei/*L. zeae* ATCC 393^T	ATCC	NA[b]	*L. zeae*	NA	NA
L. casei ATCC 4684	ATCC	NA[b]	*L. casei*	NA	NA
L. crispatus ATCC 33820^T	ATCC	NA[b]	*L. crispatus*	NA	NA
L. delbrueckii subsp. *bulgaricus* ATCC 11842^T	ATCC	NA[b]	NA	NA	NA
L. fermentum ATCC 14869^T	ATCC	NA[b]	*L. fermentum*	NA	NA
L. gasseri ATCC 33323^T	ATCC	NA[b]	*L. gasseri*	NA	NA
L. helveticus ATCC 15009^T	ATCC	NA[b]	NA	NA	NA
L. johnsonii ATCC 33200^T	ATCC	NA[b]	*L. johnsonii*	NA	NA
L. paracasei subsp. *paracasei* ATCC 25302	ATCC	NA[b]	*L. casei*	NA	NA
L. plantarum ATCC 1988	ATCC	NA[b]	*L. plantarum*	NA	NA
L. plantarum ATCC 14917^T	ATCC	NA[b]	*L. plantarum*	NA	NA
L. rhamnosus ATCC 8530	ATCC	NA[b]	*L. rhamnosus*	NA	NA
L. rhamnosus DSM 20021^T	DSM	NA[b]	*L. rhamnosus*	NA	NA
L. reuteri DSM 20016^T	DSM	NA[b]	*L. reuteri*	NA	NA
L. ruminis ATCC 27780^T	ATCC	NA[b]	NA	NA	NA
L. salivarius subsp. *salicinius* ATCC 11742^T	ATCC	NA[b]	NA	NA	NA
L. salivarius subsp. *salivarius* ATCC 11741^T	ATCC	NA[b]	NA	NA	NA
L. sharpeae DSM 20505^T	DSM	NA[b]	*L. sharpeae*	NA	NA
L. vitulinus ATCC 27783^T	ATCC	NA[b]	NA	NA	NA
L. zeae					
DSM 20178^T	DSM	NA[b]	*L. zeae*	NA	NA
GTP5	Pig	*L. acidophilus*/*L. crispatus*/*L. helveticus*	*L. crispatus*	*L. crispatus*	AF157035

GT3C1	Pig	*L. acidophilus*/*L. crispatus*/*L. helveticus*	*L. crispatus*	*L. crispatus*	AF158588
L8	Human	*L. acidophilus*/*L. crispatus*/*L. helveticus*	*L. acidophilus*	*L. acidophilus*	AF158988
L35	Human	*L. acidophilus*/*L. crispatus*/*L. helveticus*	*L. acidophilus*	*L. acidophilus*	AF159014
L43	Human	*L. acidophilus*/*L. crispatus*/*L. helveticus*	*L. acidophilus*	*L. acidophilus*	AF159021
L10	Human	*L. plantarum*	*L. plantarum*	*L. plantarum* group	AF158990
L12	Human	*L. plantarum*	*L. plantarum*	*L. plantarum* group	AF158992
L13	Human	*L. plantarum*	*L. plantarum*	*L. plantarum* group	AF158993
L33	Human	*L. plantarum*	*L. plantarum*	*L. plantarum* group	AF159012
L45	Human	*L. plantarum*	*L. plantarum*	*L. plantarum* group	AF159023
L44	Human	*L. gasseri*/*L. johnsonii*	*L. gasseri*	*L. gasseri*	AF159022
GTH5	Human	*L. gasseri*/*L. johnsonii*	*L. gasseri*	*L. gasseri*	AF157044
L. johnsonii					
NCK800	Culture collection of T. K. Klaenhammer	*L. gasseri*/*L. johnsonii*	*L. johnsonii*	NA	NA
GTH10	Human	*L. fermentum*	*L. fermentum*	*L. fermentum*	AF158581
GT3S3	Pig	*L. reuteri*	*L. reuteri*	*L. reuteri*	AF158590
GTH18	Human	*L. brevis*/*L. sharpeae*	*L. brevis* (because primer pair 11 negative and aerogenic)	*L. brevis*	AF157038
L4	Human	*L. reuteri*	*L. reuteri*	*L. reuteri*	AF158983
L38	Human	*L. fermentum*	*L. fermentum*	*L. fermentum*	AF159016
L3	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158982
L6	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158986
L9	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158989
L14	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158994
L19	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158998
L20	Human	*L. casei* group	*L. casei*	*L. casei* group	AF158999
L22	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159001
L25	Human	*L. casei* group	*L. casei*	*L. casei* group	AF159004
L27	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159006
L28	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159007
L29	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159008
L30	Human	*L. casei* group	*L. casei*	*L. casei* group	AF159009
L32	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159011
L37	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159015
L39	Human	*L. casei* group	*L. casei*	*L. casei* group	AF159017
L41	Human	*L. casei* group	*L. rhamnosus*	*L. casei* group	AF159019
L42	Human	*L. casei* group	*L. casei*	*L. casei* group	AF159020
L. rhamnosus					
DR20	Culture collection, NZDRI	*L. casei* group	*L. rhamnosus*	NA	NA
GTH1	Human	*L. salivarius*	NA	*L. salivarius* subsp. *salivarius*	AF158557
L5	Human	*L. ruminis*	NA	*L. ruminis*	AF158984

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; NZDRI, New Zealand Dairy Research Institute.
[b] NA, not applicable (these were reference strains).

TABLE 2. Species-specific primers

Primer pair	Species	Primers	Target	Sequence (5′–3′)	PCR annealing temp (°C)	$MgCl_2$ concn (mM)
1	*L. acidophilus*	Aci 16SI 16SII	16S gene	AGCTGAACCAACAGATTCAC ACTACCAGGGTATCTAATCC	62	1.5
2	*L. crispatus*	Cri 16SI 16SII	16S gene	GTAATGACGTTAGGAAAGCG ACTACCAGGGTATCTAATCC	60	1.5
3	*L. gasseri*	GasI GasII	16S-23S spacer	GAGTGCGAGAGCACTAAAG CTATTTCAAGTTGAGTTTCTCT	55	2.5
4	*L. johnsonii*	Joh 16SI 16SII	16S gene	GAGCTTGCCTAGATGATTTTA ACTACCAGGGTATCTAATCC	57	1.5
5	*L. plantarum*	Lfpr PlanII	16S-23S spacer	GCCGCCTAAGGTGGGACAGAT TTACCTAACGGTAAATGCGA	55	2.0
6	*L. casei*	PrI CasII	16S-23S spacer	CAGACTGAAAGTCTGACGG GCGATGCGAATTTCTTTTTC	55	2.0
7	*L. zeae*	ZeaI ZeaII	16S-23S spacer	TGTTTAGTTTTGAGGGGACG ATGCGATGCGAATTTCTAAATT	58	2.0
8	*L. rhamnosus*	PrI RhaII	16S-23S spacer	CAGACTGAAAGTCTGACGG GCGATGCGAATTTCTATTATT	58	2.0
9	*L. reuteri*	Lfpr Reu	16S-23S spacer	GCCGCCTAAGGTGGGACAGAT AACACTCAAGGATTGTCTGA	55	2.0
10	*L. fermentum*	Lfpr FermII	16S-23S spacer	GCCGCCTAAGGTGGGACAGAT CTGATCGTAGATCAGTCAAG	55	3.0
11	*L. sharpeae*	ShaI ShaII	16S-23S spacer	GATAATCATGTAAGAAACCGC ATATTGTTGGTCGCGATTCG	58	1.5

stored at −20°C. To extract bacterial DNA, the stomach contents were homogenized in 1 ml of TN150 buffer (10 mM Tris-HCl, 150 mM NaCl [pH 8]) and centrifuged at 14,600 × *g* for 5 min (5°C). DNA was extracted from the resulting pellet with a FastDNA kit (Bio 101, Vista, Calif.) by using CLS-TC (cell lysis solution for animal tissues and bacteria) and a 1/4-in. sphere plus garnet matrix (see the kit data sheet) according to the manufacturer's instructions.

Extraction of DNA from *Lactobacillus* cultures. Growth from pure cultures on Lactobacilli MRS agar plates was used to prepare a heavy suspension of cells in 1 ml of sterile deionized water. The suspensions were centrifuged at 14,600 × *g* (3 min, 5°C) and washed with 1 ml of TN150 buffer. The pellets were resuspended in 1 ml of TN150 buffer and transferred to sterile tubes containing 0.3 g of sterile zirconium beads (diameter, 0.1 mm). The tubes were placed in a mini-bead beater (Biospec Products, Bartlesville, Okla.), shaken at 5,000 rpm for 3 min, and then stored on ice. Five hundred microliters of this crude DNA solution was extracted sequentially with 500 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.5])-saturated phenol and with chloroform-isoamyl alcohol (24:1). The DNA was precipitated overnight by the addition of 2 volumes of cold ethanol and a 0.1 volume of 3 M sodium acetate at −20°C. The preparations were centrifuged at 14,600 × *g* (20 min, −5°C) and the pellets were dried at 37°C. The pellets were then dissolved in 500 μl of TE buffer (pH 8.5), and 25 μl of DNase-free RNase (2 mg/ml) was added. After incubation at 37°C for 1 h, the phenol-chloroform extraction, precipitation, and drying steps were repeated, and the DNA was dissolved in 20 μl of TE buffer (pH 7.5). The amount of DNA per microliter was measured by spectrophotometry.

Amplification of the DNA target sequence for DGGE. The V2-V3 region of the 16S ribosomal DNA (rDNA) gene (position 339 to 539 in the *Escherichia coli* gene) of bacteria in the stomach contents or from pure cultures of lactobacilli was amplified with primers HDA1-GC (**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′; the GC clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′). The HDA primers were originally described by Turner and colleagues (S. J. Turner, G. D. Lewis, D. J. Saul, C. S. Baker, and A. G. Rodrigo, New Zealand Microbiol. Soc. Ann. Meet., poster paper, 1998). PCR was performed in 0.2-ml tubes in a PCR Express thermal cycler (Hybaid, Teddington, United Kingdom). For the amplification of the target sequence from DNA extracted from pure cultures, the reaction mixture (50 μl) consisted of reaction buffer (final concentrations, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, and 50 mM KCl [pH 8.3]), a 200 μM concentration of each deoxynucleoside triphosphate, 20 pmol of each primer, 500 ng of bacterial DNA, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The amplification program was 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s; and finally 68°C for 7 min. PCR amplifications from DNA extracted from the stomach contents were carried out with the Expand high-fidelity PCR system (Boehringer Mannheim) or *Taq* polymerase as described above, but the reaction mixture (50μl) contained 1 μl of DNA solution. The amplification program was 93°C for 2 min and 30 cycles of 93°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by 2 min at 72°C.

***Lactobacillus* identification ladder.** An identification ladder containing 16S V2-V3 region sequences of 19 *Lactobacillus* reference strains was prepared. The sequences were obtained by PCR from individual pure cultures of the reference strains. The PCR products were then mixed to obtain the identification ladder. It was possible to generate further supplies of the ladder by PCR with the V2-V3 primers and 1 μl of mixture as the template, in the presence of 2.5 mM $MgCl_2$.

DGGE. DGGE was performed with a DCode universal mutation detection system (Bio-Rad, Hercules, Calif.) utilizing 16-cm by 16-cm by 1-mm gels. Eight percent polyacrylamide gels were prepared and run with 1× TAE buffer diluted from 50× TAE buffer (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). The denaturing gradient was formed with two 8% acrylamide (acrylamide-bis, 37.5:1) stock solutions (Bio-Rad). The gels contained a 30 to 50% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7.0 M urea. The electrophoresis was conducted with a constant voltage of 130 V at 60°C for about 4 h 30 min. The run was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide solution (5 μg/ml; 20 min), washed with deionized water, and viewed by UV transillumination.

Species-specific primers. The 11 species-specific primer pairs that we derived were based on the 16S rRNA gene or the 16S-23S rRNA intergenic spacer region (Table 2). A reaction mixture (25 μl) consisted of reaction buffer (10 mM Tris-HCl [final concentration], a variable $MgCl_2$ concentration [Table 2], and 50 mM KCl [pH 8.3]), a 200 μM concentration of each deoxynucleoside triphosphate, 10 pmol of each primer, 50 ng of bacterial DNA (extracted from pure cultures as described above), and 1.75 U of *Taq* DNA polymerase (Boehringer Mannheim). The amplification program was 92°C for 2 min, followed by 30 cycles

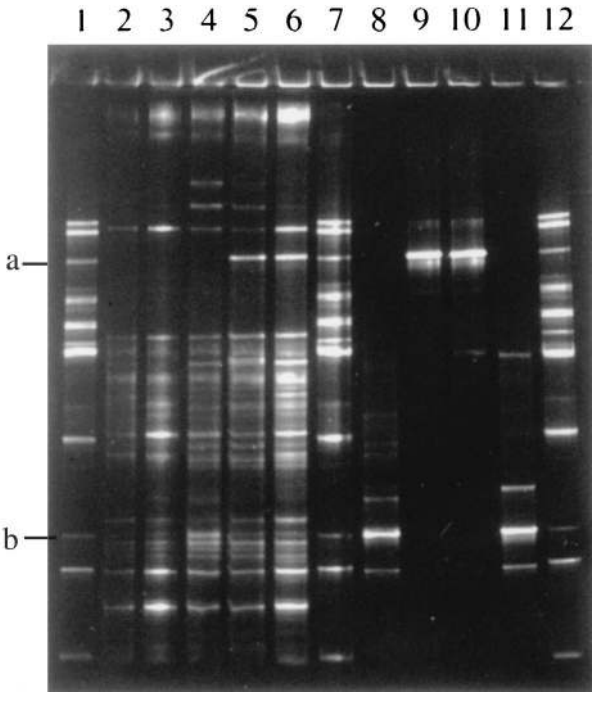

FIG. 1. Detection of *Lactobacillus* strains in stomach contents of mice. Lane 1, *Lactobacillus* identification ladder (from top to bottom, *L. plantarum*, *L. johnsonii*/*L. gasseri*, *L. fermentum*, *L. agilis*, *L. sharpeae*/*L. brevis*, *L. acidophilus*/*L. crispatus*/*L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, *L. salivarius*, *L. ruminis*, *L. reuteri*, *L. casei* group, *L. vitulinus*). The 16S V2-V3 rDNA fragment of *L. fermentum* ATCC 14931^T is fragment a; that of *L. reuteri* DSM 20016^T is fragment b. Lanes 2 to 6, 16S V2-V3 rDNA profiles from stomach contents from mouse 1 (lactobacilli absent) (lane 2); mouse 2, inoculated with strain 100-23 (lane 3); mouse 3, inoculated with strains 100-23 and 100-5 (lane 4); mouse 4, inoculated with strains 100-23, 100-5, and 21 (lane 5); and mouse 5, inoculated with strains 100-23, 100-5, 21, and 20 (lane 6). Lane 7, *Lactobacillus* identification ladder. Lanes 8 to 11, 16S V2-V3 rDNA fragments from pure cultures of strains 100-23, 100-5, 21, and 20, respectively. Lane 12, *Lactobacillus* identification ladder.

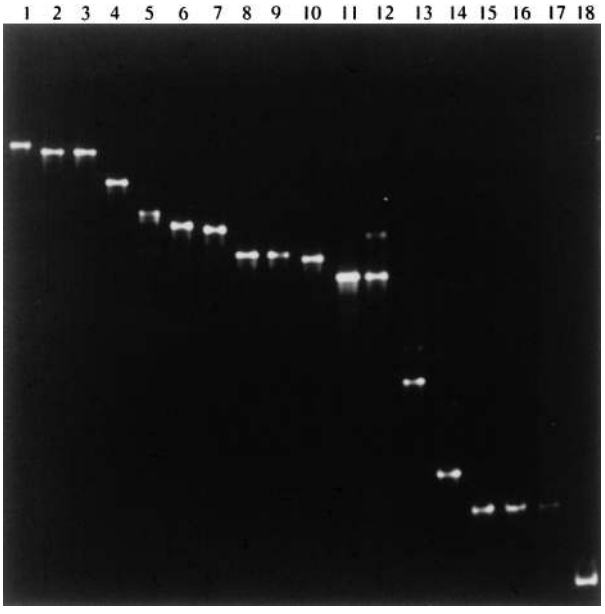

FIG. 2. DGGE of 16S V2-V3 rDNA sequences from *Lactobacillus* reference strains with an 8% polyacrylamide 30 to 50% denaturing gradient gel. Lanes: 1, *L. plantarum* ATCC 14917^T; 2, *L. johnsonii* ATCC 33200^T; 3, *L. gasseri* ATCC 33323^T; 4, *L. fermentum* ATCC 14931^T; 5, *L. agilis* DSM 20509^T; 6, *L. sharpeae* DSM 20505^T; 7, *L. brevis* ATCC 14869^T; 8, *L. acidophilus* ATCC 4356^T; 9, *L. helveticus* ATCC 15009^T; 10, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T; 11, *L. salivarius* subsp. *salivarius* ATCC 11741^T; 12, *L. salivarius* subsp. *salicinius* ATCC 11741^T; 13, *L. ruminis* ATCC 27780^T; 14, *L. reuteri* DSM 20016^T; 15, *L. casei*/*L. zeae* ATCC 393^T; 16, *L. rhamnosus* ATCC 8530; 17, *L. casei* ATCC 4684; 18, *L. vitulinus* ATCC 27783^T. The DNA fragment from *L. crispatus* ATCC 33820^T (not run in this gel) migrated the same distance as those of *L. acidophilus* and *L. helveticus*. The *L. paracasei* subsp. *paracasei* ATCC 25302 fragment (not run in this gel) migrated the same distance as the fragments of *L. casei*, *L. rhamnosus*, and *L. casei*/*L. zeae*.

of 95°C for 30 s, 30 s at the appropriate annealing temperature (Table 2), and 72°C for 30 s. A cycle of 72°C for 1 min concluded the program. Amplification products were detected by agarose gel electrophoresis (5 μl of PCR mixture, 2% agarose gel), ethidium bromide staining, and UV transillumination.

Amplification and sequencing of the 16S V2-V3 region. To confirm the identification of the gastrointestinal lactobacilli examined by using DGGE and species-specific primers, we amplified and sequenced (one polynucleotide strand only) the V2-V3 region of the 16S rRNA gene of each isolate and conducted a search of sequences deposited in the GenBank DNA database by using the BLAST algorithm (1). The identities of the isolates were determined on the basis of highest score. Amplification of the V2-V3 region was accomplished with primers HDA1 (lacking the GC clamp) and HDA2 and the same thermal cycler program as described above for DGGE. Sequencing was carried out by the Centre for Gene Research, University of Otago, by the dideoxy method of Sanger et al. (12) by using the PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Foster City, Calif.) in combination with an Applied Biosystems model 377A automated sequencing system. Analysis of nucleotide sequence data was carried out by using the SeqEd program, version 1.0.3 (Applied Biosystems Inc.).

RESULTS

DGGE detection of *Lactobacillus* populations in mouse stomach contents. Comparison of the 16S V2-V3 rDNA profiles obtained from murine stomach contents showed that this method could detect changes in the microbial community, in this case the intentional addition of lactobacilli (Fig. 1). Multiple DNA fragments were present in the profiles, even from the stomach contents of mouse 1 (Fig. 1, lane 2). These fragments probably represent either fecal bacteria present in the stomach contents of these coprophagous animals or bacterial species not detected previously by culture methods in the stomachs of mice. Inoculation of the mice with *Lactobacillus* strains 100-23 and 20 led to the appearance of a new 16S fragment in the profiles of samples from mice 2, 3, 4, and 5 (fragment b in Fig. 1, lanes 3, 4, 5, and 6). This fragment migrates to the same position as the 16S V2-V3 fragment obtained from pure cultures of strains 100-23 and 20 (lanes 8 and 11). It also coincides with the 16S fragment generated from *Lactobacillus reuteri* DSM 20016^T (fragment b in Fig. 1, lanes 1, 7, and 12). Similarly, inoculation of the mice with *Lactobacillus* strains 100-5 and 21 resulted in their detection in the stomach contents of animals 4 and 5 (fragment a in Fig. 1, lanes 5 and 6). A faint band at this location was also present in the preparation from mouse 3, but it is not visible in Fig. 1 (lane 4). The 16S fragments of these strains (fragment a in Fig. 1, lanes 9 and 10) coincide with that of *Lactobacillus fermentum* ATCC 14931^T (fragment a in Fig. 1, lanes 1, 7, and 12).

Identification ladder for lactobacilli. It was possible to at least group the *Lactobacillus* reference strains according to the migration of their 16S V2-V3 regions in a denaturing gradient gel (Fig. 2). Thus, the fragments from *Lactobacillus johnsonii* and *Lactobacillus gasseri* migrated the same distance in the gel (Fig. 2, lanes 2 and 3). DNA fragments from *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Lactobacillus crispatus* (not shown in Fig. 2) had similar migration distances (Fig. 2, lanes 8 and 9), as did those of *Lactobacillus salivarius* subsp. *salivarius* and *Lactobacillus salivarius* subsp. *salicinius* (Fig. 2, lanes 11 and 12). The fragments from *Lactobacillus casei*, *Lac-*

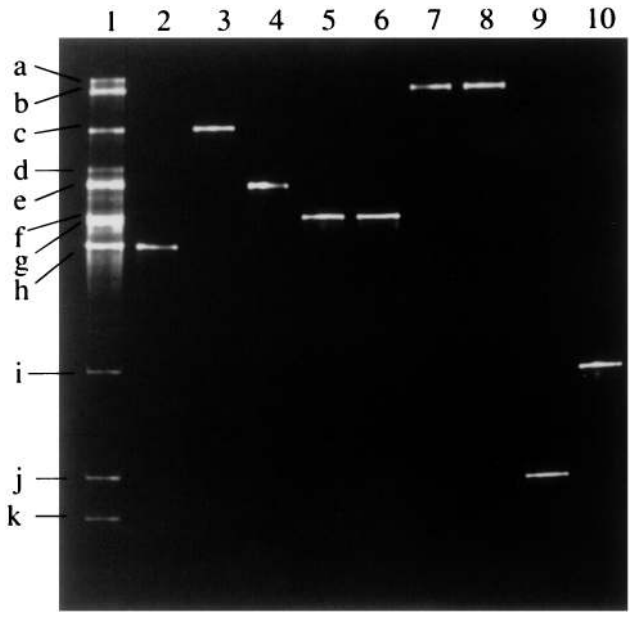

FIG. 3. DGGE of 16S V2-V3 rDNA sequences from gastrointestinal isolates of *Lactobacillus* with an 8% polyacrylamide 30 to 50% denaturing gradient gel. Lane 1, ladder of sequences from reference strains (a, *L. plantarum*; b, *L. johnsonii*/*L. gasseri*; c, *L. fermentum*; d, *L. agilis*; e, *L. sharpeae*/*L. brevis*; f, *L. acidophilus*/*L. crispatus*/*L. helveticus*; g, *L. delbrueckii* subsp. *bulgaricus*; h, *L. salivarius*; i, *L. ruminis*; j, *L. reuteri*; k, *L. casei* group); lane 2, GTH1; lane 3, GTH10; lane 4, GTH18; lane 5, GT3C1; lane 6, GTP5; lane 7, GTH5; lane 8, NCK800; lane 9, GT3S3; lane 10, L5.

tobacillus casei/*L. zeae*, and *Lactobacillus rhamnosus* (Fig. 2, lanes 15, 16, and 17) and *L. paracasei* (not shown in Fig. 2) migrated the same distance in the gel. Fragments from *Lactobacillus brevis* and *Lactobacillus sharpeae* had almost identical migration properties (Fig. 2, lanes 6 and 7). DNA fragments from the remaining seven reference strains that we tested could be distinguished individually by DGGE (Fig. 2).

Identification of unknowns by DGGE. Migration distances of 16S V2-V3 fragments obtained from unidentified isolates of lactobacilli were compared to those of reference strains in the identification ladder. Such comparisons enabled the unknowns to at least be placed in a *Lactobacillus* group, if not identified to the species level (examples are shown in Fig. 3; results are in Table 1).

Identification of unknowns by species-specific primers. Our strategy was to first group or identify each isolate by DGGE and then specifically identify (or confirm) the species to which the isolate belonged by using species-specific primers. This meant that each isolate needed to be tested with only a few primer pairs and that extensive testing of the primers across all known *Lactobacillus* species was unnecessary (Table 3). Thus, isolates grouped as *L. acidophilus*, *L. crispatus*, or *L. helveticus* by DGGE were tested with primer pairs 1 and 2. *L. gasseri* and *L. johnsonii* isolates were tested with primer pairs 3 and 4. Isolates classified as belonging to the *L. casei* group were tested with primer pairs 6, 7, and 8. Isolates identified as *L. reuteri* or *L. fermentum* were tested with primer pairs 9 and 10. *L. brevis* and *L. sharpeae* isolates were tested with primer 11. The results obtained by using these primer pairs with reference strains are shown in Table 3, and identification results for unknowns are given in Table 1.

Comparison of V2-V3 sequences with those of lactobacilli in the GenBank database. Identifications obtained by a BLAST search of the GenBank database with V2-V3 region sequences correlated with those derived by DGGE and species-specific PCR primers (Table 1). Thus, for each isolate, the group or species identification achieved by DGGE or species-specific PCR primers was confirmed by analysis of the V2-V3 sequence. The species-specific primers, however, gave definitive identifications of isolates belonging to *L. rhamnosus* or *L. casei*, whereas V2-V3 sequences did not differentiate between these species but indicated the group (*L. casei*/*L. paracasei*/*L. rhamnosus*/*L. zeae*) to which they belonged.

DISCUSSION

PCR-DGGE clearly has potential in the analysis of gastrointestinal communities. Using the defined system consisting of our unique lactobacillus-free mice, we were able to detect the addition of *Lactobacillus* species to the gastric microflora of the animals. The sensitivity of the method was limited to detection of different *Lactobacillus* species, rather than strains, as expected of a method based on 16S rRNA gene sequences. It will be interesting to apply this method to more complex communities, such as those inhabiting large-bowel ecosystems.

PCR-DGGE also proved a practical addition to available

TABLE 3. Specificity of primer pairs

Species	PCR product obtained with primer pair[a]										
	1	2	3	4	5	6	7	8	9	10	11
L. acidophilus ATCC 4356^T	+	−	−	−	−						
L. crispatus ATCC 33820^T	−	+	−	−	−						
L. gasseri ATCC 33323^T	−	−	+	−	−						
L. johnsonii ATCC 33200^T	−	−	−	+	−						
L. plantarum ATCC 14917^T	−	−	−	−	+						
L. casei ATCC 334						+	−	−			
L. casei ATCC 4684						+	−	−			
L. paracasei subsp. *paracasei* ATCC 25302						+	−	−			
L. rhamnosus ATCC 8530						−	−	+			
L. rhamnosus DSM20021^T						−	−	+			
L. casei/*L. zeae* ATCC 393^T						−	+	−			
L. zeae DSM 20178^T						−	+	−			
L. brevis ATCC 14869^T									−	−	−
L. sharpeae DSM 20505^T									−	−	+
L. fermentum ATCC 14931^T									−	+	
L. reuteri DSM 20016^T									+	−	

[a] See Table 2 for primer details.

identification methods for lactobacilli of gastrointestinal origin. Multiple fragments of different sizes were sometimes present in PCR products from pure cultures (see Fig. 1, lanes 8 and 11, for examples). There was, however, always one major fragment (most dense in the DGGE gel) which indicated the identity of the isolate. The minor (less dense fragments) were probably PCR artifacts resulting from the highly folded (loops and stems) nature of the V2-V3 region (Turner et al., New Zealand Microbiol. Soc. Ann. Meet.). *Lactobacillus ruminis*, *L. reuteri*, *L. fermentum*, *Lactobacillus vitulinus*, and *Lactobacillus agilis* could be identified directly on the basis of the migration of their V2-V3 sequences. DNA fragments from *Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus* had characteristic migration behaviors, but we have not tested representative strains of all of the members of the *L. plantarum* and *L. delbrueckii* taxa.

DGGE results provided a useful initial screen for the remaining gastrointestinal species because it narrowed the possible identities of the isolates. These grouped isolates could then be identified by application of the species-specific PCR primers. This meant that an isolate needed to be tested only with two or three primer pairs to obtain a specific identification. We do not yet have species-specific primers for all of the gastrointestinal species of lactobacilli, but this would be a desirable goal for future research.

The species-specific PCR primers based on the 16S-23S rRNA intergenic spacer regions were particularly valuable in the identification of the *L. casei* group isolates. They permitted discrimination to be made between *L. casei*/*L. paracasei*, *L. rhamnosus*, and *L. zeae*. These species cannot be differentiated by DGGE or BLAST comparisons of V2-V3 sequences. The information that we have obtained using these species-specific primers may be helpful in unraveling the somewhat confused situation regarding the taxonomy of the *L. casei* group. Dellaglio and colleagues (2), on the basis of DNA-DNA homology studies, requested in 1991 that strain ATCC 334 replace ATCC 393 as the neotype strain of *Lactobacillus casei* subsp. *casei*. They rejected the species name *L. paracasei*. In 1996, Dicks et al. (3) proposed that strain ATCC 393 be reclassified as *L. zeae*. Subsequently, Mori and colleagues (9) proposed that the *L. casei* group be reclassified to include three species: *L. zeae* containing strains ATCC 15820^T (DSM 20178) and ATCC 393, a species containing *L. paracasei* and ATCC 334, and *L. rhamnosus*. Our PCR primer pair 7, based on the 16S-23S spacer region sequences of ATCC 393 (which we have considered on the basis of the work of Dicks et al. [3] to be *L. zeae*), produced a PCR product with DNA from both ATCC 393 and ATCC 15820 (DSM 20178), but not with DNA from ATCC 334, *L. paracasei* ATCC 25302, or *L. rhamnosus* strains. PCR primer pair 6, based on the intergenic spacer region of ATCC 334, produced products only from *L. casei* and *L. paracasei* cultures. Primer pair 8, based on the intergenic spacer region of *L. rhamnosus* (15), produced PCR products only from *L. rhamnosus* cultures. The application of our primers to a collection of strains belonging to *L. casei* may therefore assist in future taxonomic considerations of this group. Additionally, it may be possible to differentiate between the members of this group by DGGE if primers that targeted another region of the 16S rRNA gene are used. A potentially useful region is located between nucleotides 73 and 111 (*L. casei* numbering), where variation in sequences has been observed among the members of the *L. casei* group (9).

Identifications obtained by a BLAST search of the GenBank database with V2-V3 region sequences correlated with those obtained by DGGE and species-specific PCR primers. We are therefore confident that the approaches to the detection and identification of *Lactobacillus* species that we have described in this report will contribute to future studies of the composition of the intestinal microflora and to a better understanding of *Lactobacillus* taxonomy.

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