APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1997, p. 2702–2707 Vol. 63, No. 7
0099-2240/97/$04.00+0

Strain-Specific Differentiation of Lactococci in Mixed Starter Culture Populations Using Randomly Amplified Polymorphic DNA-Derived Probes

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Received 10 February 1997/Accepted 7 May 1997

A hydrophobic grid membrane filtration (HGMF) colony hybridization assay was developed that allows strain-specific differentiation of defined bacterial populations. The randomly amplified polymorphic DNA (RAPD) fingerprinting technique was used to identify potential signature nucleic acid sequences unique to each member of a commercial cheese starter culture blend. The blend consisted of two closely related *Lactococcus lactis* subsp. *cremoris* strains, 160 and 331, and one *L. lactis* subsp. *lactis* strain, 210. Three RAPD primers (OPX 1, OPX 12, and OPX 15) generated a total of 32 products from these isolates, 20 of which were potential strain-specific markers. Southern hybridization analyses revealed, that the RAPD-generated signature sequences OPX15-0.95 and a 0.36-kb *Hae*III fragment of OPX1-1.0b were specific for strains 331 and 210, respectively, within the context of the test starter culture blend. These strain-specific probes were used in a HGMF colony hybridization assay. Colony lysis, hybridization, and nonradioactive detection parameters were optimized to allow specific differentiation and quantitation of the target strains in the mixed starter culture population. When the 210 and 331 probes were tested at their optimal hybridization temperatures against single cultures, they detected 100% of the target strain CFUs, without cross-reactivity to the other strains. The probes for strains 210 and 331 also successfully detected their targets in blended cultures even with a high background of the other two strains.

A variety of molecular techniques have been developed to characterize and differentiate bacterial populations. In recent years, methods focusing on rRNA have been especially useful because the various degrees of sequence conservation within the different regions of 16S rRNA allow the assay to be tailored to the desired specificity. Also, 16S rRNA sequence data has been compiled for a large number of bacterial species. Methods based on rRNA for characterization of bacterial isolates include the sequencing of PCR-generated templates and the use of combinations of genus-, species-, and subspecies-specific probes (4, 15, 17, 20, 29). However, when differentiation below the subspecies level is necessary, methods based on the 16S and 23S rRNA genes are no longer useful because the nucleotide sequences of these genes are too conserved to allow the design of strain-specific probes (18). Other methodologies allow finer discrimination. Many of these are variations of restriction fragment length polymorphism (RFLP) analysis. Ribotyping, including an automated version developed by Qualicon, involves generation of RFLP patterns with probes from the rRNA operons (7, 22, 39). Other researchers have combined RFLP with a variety of probes or have "fingerprinted" organisms by restricting the entire genome (1, 25).

The randomly amplified polymorphic DNA (RAPD) technique is another powerful approach for discriminating among bacterial isolates. RAPD is a modification of PCR that uses single, short primers of random sequence to generate genomic fingerprints (37, 40). This method has been especially useful to epidemiologists attempting to match isolates from potential sources of pathogens to isolates recovered from patients (2, 10, 21), or in some cases, for population monitoring (41). However, RAPD patterns are not always reproducible because they are sensitive to small variations in template concentration and purity, Mg^{2+} concentration, and primer annealing temperature (12, 32, 34).

Our objective was to develop a method to discriminate among the members of a lactococcal population used as a cheese mixed-starter culture. Because dairy starter cultures are blended empirically for the desired characteristics of the final product, maintainance of the optimal strain balance throughout the cheese fermentation process is important. Our approach to this problem was to use RAPD as a screening technique to identify potential strain-specific markers, within the context of a test blend. These markers were further screened, and strain-specific probes were identified and used in a hydrophobic grid membrane filter (HGMF) colony hybridization assay. This is the first report of a method for quantitative strain-specific detection of lactococci in bacterial populations without the need to subculture isolated colonies.

MATERIALS AND METHODS

Bacterial strains and media. Lactococcal strains used in a defined dairy starter culture blend were obtained from Marschall Products (Madison, Wis.). This blend consisted of *Lactococcus lactis* subsp. *lactis* 210 and two *L. lactis* subsp. *cremoris* strains, 160 and 331. Strains were cultured at 30°C in M17 broth or agar (33) with 0.5% glucose (M17G) instead of lactose. Lactococci filtered onto HGMFs (QA Life Sciences Inc., San Diego, Calif.) were grown at 30°C on M17G with 0.05 g of bromocresol purple (BCP; J. T. Baker Chemical Co., Phillipsburg, N.J.)/liter. *Escherichia coli* JM109 [F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/e14$^-$(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* (r_k^- m_k^+) *relA1 supE44 recA1*] used for cloning was grown at 37°C with shaking, in Luria broth (LB; Sigma, St. Louis, Mo.) with 100 μg of ampicillin (Sigma)/ml where appropriate. *E. coli* transformants were selected for on LB plates with 0.12 g of isopropyl-β-D-thiogalactoside/liter and 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (both from Gold Biotechnology, St. Louis, Mo.) and 100 mg of ampicillin/liter.

RAPDs and ribotyping. Crude lysates were prepared from *L. lactis* 160, 210, and 331 according to the method described by Czajka and Batt (10). These lysates were used in RAPD reactions, and a total of 15 random primers (Kit X; Operon Technologies, Alameda, Calif.) were screened individually. RAPDs were performed according to the method described by Czajka and Batt (10), modified

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slightly from that of Williams (40). Briefly, 25-μl reactions were carried out in 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl) with 1 μl of lysate, 14 ng of primer, 1 U of Ampli*Taq* DNA Polymerase (Perkin-Elmer, Foster City, Calif.), 100 μM (each) deoxynucleotide triphosphate, and 4 mM $MgCl_2$. A Hybaid TR1 thermocycler was programmed as follows: 1 cycle of 3 min at 94°C; 45 cycles of 45 s at 94°C, 1 min at 36°C, and 2 min at 72°C; and 1 cycle of 10 min at 72°C. RAPD products were electrophoresed at 100 V for 45 min through a 1.5% TAE (Tris-acetate–EDTA buffer [pH 8.5]) agarose gel.

Lactococcal strains were ribotyped by the Qualicon Molecular Typing Lab (Wilmington, Del.). Briefly, bacterial cells were lysed to release DNA, which was restricted with *Eco*RI, electrophoresed through an agarose gel, and transferred to a nylon membrane. An *E. coli rrnB* rRNA operon probe was hybridized to the membrane and detected by chemiluminescence. The images captured with a CCD camera were processed by using RiboPrinter Analysis software.

Cloning and sequencing. Product mixtures from RAPD reactions yielding at least one polymorphic band were ligated into the pGEM-T vector (Promega, Madison, Wis.) according to the directions of the manufacturer and transformed into *E. coli* JM109 cells made competent with calcium chloride according to the method described by Sambrook et al. (28). The complete nucleotide sequences of the cloned RAPD polymorphisms were determined by the Cornell University Analytical Chemistry and Peptide/DNA Synthesis Facility using the vector primers M13-40 (5′-GTTTTCCCAGTCACGAC-3′) and M13 rev ht (5′-GGAAAC AGCTATGACCATG-3′) and an ABI Prism 373A Stretch automated DNA sequencer (Perkin-Elmer–Applied Biosystems).

Probe preparation. Probes were labeled by PCR incorporation of digoxigenin-dUTP (dig-dUTP; Boehringer Mannheim, Indianapolis, Ind.). One microliter of a 1:50 dilution of plasmid DNA was used together with the vector primers M13-40 and M13 rev ht to generate all the probes except the 210-specific probe, which was generated with internal primers F210-639 (5′-CCGGTGTGGATGA AGATTC-3) and R210-1000 (5′-GGGCACGATGGGACGAGT-3′). The PCRs were carried out in 1× PCR buffer with 1 U of Ampli*Taq* DNA polymerase, 1.5 mM $MgCl_2$, 47.5 μM dTTP, 2.5 μM dig-dUTP, and 50 μM (each) dATP, dCTP, and dGTP. The thermocycler was programmed for 1 cycle of 94°C for 3 min; 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; and 1 cycle of 10 min at 72°C. The dig-labeled probes were purified by ethanol and LiCl precipitation as recommended by the Genius 3 Nonradioactive DNA Labeling Kit (Boehringer Mannheim).

Southern hybridization. Chromosomal DNA was extracted from lactococcal strains according to the method described by Kim and Batt (16). *Eco*RI and *Hin*dIII (New England Biolabs, Beverly, Mass.) digests of the purified DNA were electrophoresed through a 1.5% TAE gel. The DNA was denatured and neutralized, transferred by capillary action, and immobilized on a nylon membrane (MagnaGraph; Micron Separations Inc., Westborough, Mass.), as described by Sambrook et al. (28). Hybridization and nonradioactive detection with alkaline phosphatase and the substrate CDP-Star were conducted according to the Genius 3 Nucleic Acid Detection Kit.

Filtration and bacterial lysis on HGMFs. Dilutions of single lactococcal cultures or blends of various ratios of *L. lactis* 160, 210, and 331 were filtered onto HGMFs with an ISO-GRID HGMF vacuum apparatus, according to the manufacturer's recommendations (QA Life Sciences, Inc.). The HGMFs were placed onto M17G BCP and grown at 30°C for about 36 h. The yellow, glucose-fermenting colonies were counted with a stereomicroscope with 10× magnification.

To prepare the HGMFs for colony hybridization, the procedure developed by Peterkin et al. for *Listeria* spp. (24) was used with some modifications. HGMFs were incubated, colony side up, for 30 min at room temperature on several pieces of 3-mm-thick Whatman filter paper soaked with 5 ml of pretreatment solution (5 mM sodium phosphate buffer [pH 6.0], 100 mM sodium bicarbonate, 0.0066% polyethyleneimine). The resulting purple colonies were photographed with a CCD camera system. Following 10 min of air drying, the HGMFs were incubated for 20 min at room temperature on filter paper soaked with a lysozyme solution (10 mg/ml in a 100 mM Tris [pH 7.5]–50 mM EDTA buffer) and for 5 min on filter paper with 5% sodium dodecyl sulfate (SDS). Complete lysis and DNA denaturation were accomplished by microwaving the HGMFs on filter paper with 150 mM NaOH in a sealed plastic container for 45 s at half power (310 W). The HGMFs were then washed for 5 min in 2× SSC with 0.1% SDS and for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). After being air dried for 30 min, the HGMFs were exposed to UV irradiation at 302 nm for 5 min to cross-link the DNA to them.

Colony hybridizations. The HGMFs were incubated for 1 h at 68°C in 10 ml of prehybridization buffer (2× SSC, 1% Boehringer Mannheim blocking reagent, 0.1% *N*-laurylsarcosine, and 0.02% SDS). The 8-h hybridization at 68°C, in the above buffer containing approximately 25 to 30 ng of dig-labeled probe, was followed by two 15-min washes at 65°C in 2× SSC with 0.5% SDS. Detection was performed according to the Boehringer Mannheim Genius 3 Kit, as described above for the Southern blots.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 210-specific OPX1-1.0b and 331-specific OPX15-0.95 polymorphisms are AF001829 and AF001830, respectively.

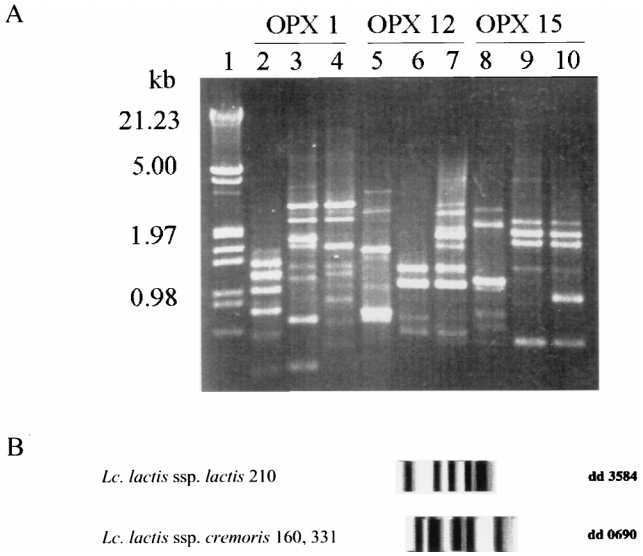

FIG. 1. RAPD and ribotype patterns for *L. lactis* subsp. *lactis* 210, *L. lactis* subsp. *cremoris* 160, and *L. lactis* subsp. *cremoris* 331. (A) RAPD patterns generated by primers OPX 1, OPX 12, and OPX 15. Lane 1, λ *Hin*dIII/*Eco*RI size marker; lanes 2, 5, and 8, lysates from *L. lactis* subsp. *lactis* 210; lanes 3, 6, and 9, lysates from *L. lactis* subsp. *cremoris* 160; lanes 4, 7, and 10, lysates from *L. lactis* subsp. *cremoris* 331. (B) Ribotype patterns generated from *L. lactis* subsp. *lactis* 210 (ribogroup dd3584) and *L. lactis* subsp. *cremoris* 160 and 331 (ribogroup dd0690).

RESULTS

RAPD identification of potential markers and ribotyping. When RAPD primers OPX 1 through 15 were screened with various lactococcal strains, they yielded 0 to 10 products ranging in size from 3 kb to less than 0.5 kb (data not shown). Of those primers which generated a pattern, an average of 3 bands were produced that were polymorphic (absent in at least one of the tested strains). Primers OPX 1, 12, and 15 yielded the most polymorphic products and were selected for screening *L. lactis* subsp. *lactis* 210, *L. lactis* subsp. *cremoris* 160, and *L. lactis* subsp. *cremoris* 331. As shown in Fig. 1A, primer OPX 1 yielded two products unique to strain 210 (1.0 and 0.8 kb) and three products unique to strain 160 (1.7, 1.2, and 0.7 kb). Primer OPX 12 generated two unique 210 markers (0.8 and 1.6 kb) and several unique 331 markers (>1.9 kb). Primer OPX 15 yielded a 0.95-kb potential strain-specific marker for strain 331, in addition to a 1.2-kb product unique to strain 210.

Ribotyping was carried out to determine the genetic relationship among strains 210, 160, and 331. Two different ribotype patterns were detected for the three test blend strains (Fig. 1B). *L. lactis* subsp. *lactis* 210 was classified as ribogroup dd3584. The two *L. lactis* subsp. *cremoris* strains, 160 and 331, belong to the same ribogroup, dd0690. Both ribotype patterns could be grouped with existing *L. lactis* patterns in a Qualicon lactic acid bacteria database (data not shown).

Screening for strain-specific markers. The RAPD products from amplification of *L. lactis* subsp. *lactis* 210 and *L. lactis* subsp. *cremoris* 160 with OPX 1 and from amplification of *L. lactis* subsp. *cremoris* 331 with OPX 15 were shotgun cloned into the pGEM-T vector. Six different cloned inserts were recovered (the size of each insert [in kilobases] is indicated after the primer name): pOPX1-0.7 (strain 160), pOPX15-0.95 (strain 331), pOPX15-0.56 (strain 331), pOPX1-0.57 (strain 210), and two different 1.0-kb inserts from strain 210, pOPX1-1.0a and pOPX1-1.0b. The pOPX1-1.0b insert was initially

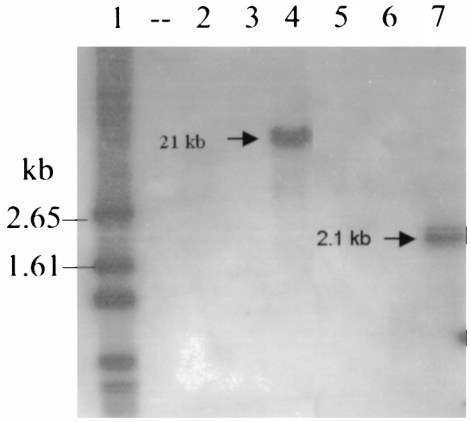

FIG. 2. Southern hybridization with dig-labeled pOPX15-0.95 as a probe. Lane 1, dig-labeled pGEM size marker; lanes 2 to 4, chromosomal DNA from *L. lactis* 160, 210, and 331, respectively, restricted with *Eco*RI; lanes 8 to 10, same as lanes 2 to 4 but restricted with *Hin*dIII.

distinguished from pOPX1-1.0a by an internal *Nde*I site. Because RAPD polymorphisms can arise from as little as a single nucleotide difference at the 3′ end of the primer binding site, Southern hybridizations were performed to determine the degree of strain specificity of the cloned RAPD products. Probes prepared from pOPX1-0.7, pOPX1-0.57, pOPX1-1.0a, and pOPX15-0.56 hybridized to chromosomal DNA from all three strains (data not shown). Typically, the probes recovered from either *L. lactis* subsp. *cremoris* 160 or 331 hybridized identically to chromosomal DNA from these two strains. For example, the pOPX1-0.7 probe hybridized not only to 16-kb and 12-kb *Eco*RI and *Hin*dIII fragments from strain 160 but also to the same-size strain 331 fragments.

The pOPX15-0.95 probe was specific for *L. lactis* subsp. *cremoris* 331, within the context of the three-strain blend. It hybridized only to a 2.1-kb *Hin*dIII fragment and an approximately 21-kb *Eco*RI fragment from strain 331 (Fig. 2). The pOPX1-1.0b probe hybridized predominantly to a 1.9-kb *Eco*RI fragment and a 0.7-kb *Hin*dIII fragment of chromosomal DNA from *L. lactis* subsp. *lactis* 210 (data not shown). However, it also hybridized weakly to a 12-kb strain 160 *Eco*RI fragment, a range of strain 331 *Eco*RI fragments from 1.6 to 2.7 kb, 14- and 3.5-kb strain 160 *Hin*dIII fragments, and a 12-kb strain 331 *Hin*dIII fragment (data not shown). These pOPX1-1.0b results suggested that only a portion of the 1.0-kb probe was hybridizing specifically to 210 DNA. Sequence analysis revealed a number of restriction enzymes that could cut the pOPX1-1.0b probe roughly in half. *Hae*III generates a 0.64-kb fragment and a 0.36-kb fragment; the 0.36-kb portion of pOPX1-1.0b proved to be specific for strain 210. It hybridized only to a 4.8-kb *Hin*dIII fragment and an approximately 21-kb *Eco*RI fragment from strain 210 (Fig. 3). The complete nucleotide sequences of the OPX15-0.98 and OPX1-1.0b polymorphisms were deposited in the GenBank database (see Materials and Methods).

HGMF colony hybridization with pure cultures. The cloned polymorphisms pOPX15-1.0 and the 0.36-kb *Hae*III fragment of pOPX1-1.0b, hereafter referred to as the 331 and 210 probes, were further tested for strain specificity in a colony hybridization format. Dilutions of each member of the blend (*L. lactis* subsp. *lactis* 210, *L. lactis* subsp. *cremoris* 160, and *L. lactis* subsp. *cremoris* 331) were filtered onto HGMFs. The yellow, glucose-fermenting colonies turn purple after the membrane pretreatment step neutralizes the BCP pH indicator, providing a good contrast that allows the HGMFs to be photographed with a CCD camera system. The HGMFs were

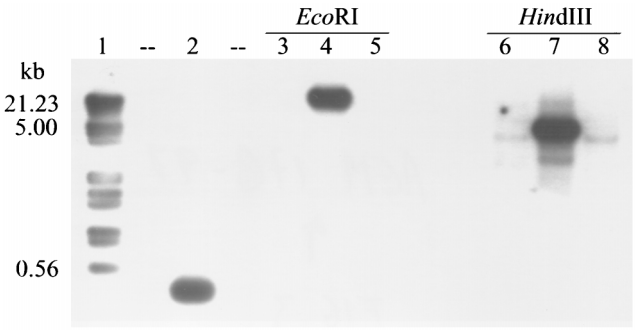

FIG. 3. Southern hybridization with dig-labeled 0.36-kb *Hae*III fragment of pOPX1-1.0b as a probe. Lane 1, dig-labeled λ *Hin*dIII/*Eco*RI size marker; lane 2, pOPX1-1.0b insert restricted with *Hae*III; lanes 3 to 5, chromosomal DNA from *L. lactis* 160, 210, 331, respectively, restricted with *Eco*RI; lanes 6 to 8, same as lanes 3 to 5 but restricted with *Hin*dIII.

treated to lyse the cells and denature and immobilize the DNA, as described in Materials and Methods. To optimize lysis of the lactococcal cells, a lysozyme treatment and a brief SDS incubation were added to the protocol developed by Peterkin et al. (24). Also, to minimize colony washoff from the HGMFs before UV immobilization of DNA, the membranes were cooled to room temperature after the microwave irradiation step.

Hybridization temperatures from 55 to 68°C were tested with the 331 and 210 probes, and strain-specific detection was achieved with both probes at 68°C. Below this temperature, the probes bound to the nontarget strains with signal intensities approaching the lower boundary of the range of signal intensities produced with the target strain (data not shown). In Fig. 4A, the colonies filtered on the HGMF are shown after the membrane pretreatment step. The grid squares which contain growth are referred to as positive squares. The strain 160, 210, and 331 HGMFs had 40, 197, and 154 positive squares, respectively. The 331 probe bound to 100% of the positive squares of *L. lactis* subsp. *cremoris* 331 that were counted on the HGMF prior to treatment but not to *L. lactis* subsp. *cremoris* 160 or to *L. lactis* subsp. *lactis* 210 cells (Fig. 4B). Similarly, the 210 probe enabled *L. lactis* subsp. *lactis* 210-specific detection. The 210 probe detected signal from all 181 positive squares

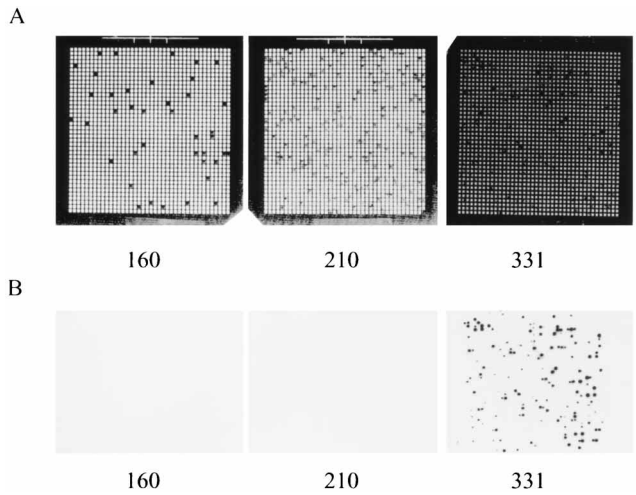

FIG. 4. HGMF colony hybridization of 331 probe with pure cultures. (A) HGMFs with *L. lactis* 160, 210, and 331 after lysis pretreatment. (B) X-ray images of the HGMFs in panel A after hybridization and nonradioactive detection of the 331 probe.

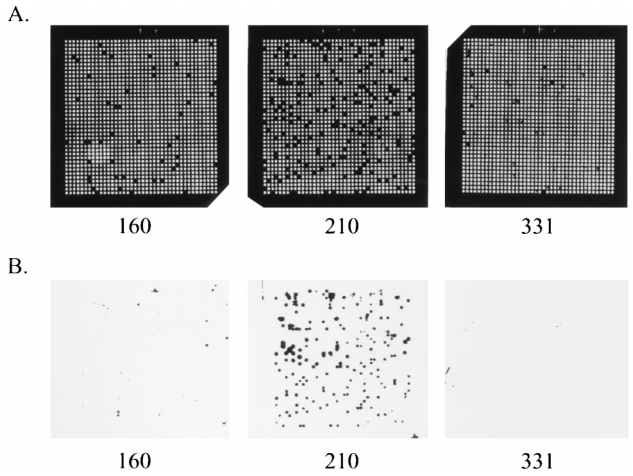

FIG. 5. HGMF colony hybridization of the 210 probe with pure cultures. (A) HGMFs with *L. lactis* 160, 210, and 331 after lysis pretreatment. (B) X-ray images of the HGMFs in panel A after hybridization and nonradioactive detection with the 210 probe.

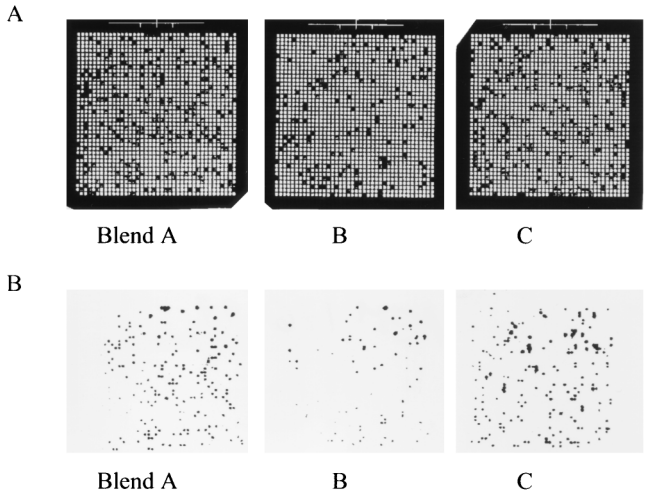

FIG. 6. HGMF colony hybridization of the 210 probe with mixed cultures. (A) HGMFs with blends A, B, and C of *L. lactis* 160, 210, and 331 after lysis pretreatment. (B) X-ray images of the HGMFs in panel A after hybridization and nonradioactive detection of the 210 probe.

counted on the 210 HGMF but did not hybridize to any of the 48 positive squares present on the 160 HGMF or the 78 positive squares from strain 331 (Fig. 5).

HGMF colony hybridization with mixed cultures. Once the HGMF treatment and hybridization conditions were optimized with pure cultures, the ability of the 210 and 331 probes to detect their target strain in a high background of the other two strains in the test blend was examined. Strains 160, 210, and 331 were cultured separately, diluted, and mixed in six different blends (A, B, C, D, E, and F) before filtration. The level of strain 210 in blend B was approximately one-half of that in the A and C blends. The level of strain 331 in blend F was one half of that in the D and E blends. The level of strain 160 was kept the same in all six blends. The initial dilutions of each strain were also filtered individually to serve as controls that could be used to estimate the expected mixed culture results.

The 210 probe successfully detected the predicted number of *L. lactis* subsp. *lactis* 210 cells in all three blends. The blend A HGMF was predicted to consist of 59% 210 cells, and 194 of the 297 positive squares (65%) hybridized to the 210 probe (Fig. 6). A total of 196 of the 245 positive squares on the blend C HGMF hybridized to the probe, and as expected, the number of probe-hybridizing positive squares on the blend B HGMF was approximately one-half of that level (98). The 331 probe also hybridized to the expected number of *L. lactis* subsp. *cremoris* 331 cells, even with high background levels of strains 160 and 210 (Fig. 7). For example, the blend F HGMF was predicted to consist of 37% 331 cells, and the actual percentage of probe-hybridizing positive squares was 39% (133 of 342 positive squares). Although a probe specific for the third strain in the blend, *L. lactis* subsp. *cremoris* 160, was not developed, further cloning and screening of RAPD polymorphisms would achieve this. However, if the other two strains are quantified by the 210 and 331 probes, the level of strain 160 could be estimated by subtraction from the total number of cells, so a 160-specific probe is not necessary.

DISCUSSION

Techniques that have been used to differentiate lactococci and lactobacilli include biochemical and physiological tests (30), phage typing (38), plasmid profiling (13), genomic restriction enzyme analysis (REA) (25), ribotyping (18, 22, 26), and 16S and/or 23S rRNA probes (4, 17, 27). 16S rRNA probes have been used extensively to identify "new" *L. lactis* subsp. *cremoris* and subsp. *lactis* strains from the environment for potential use as dairy starter cultures. However, the 16S and 23S rRNA nucleotide sequences are too conserved to discriminate below the subspecies level (18). Ribotyping, which allows finer discrimination between lactococci, is limited to sampling the sequence diversity of a highly conserved portion of the genome. Strains could differ little around the rRNA locus but have variation in other regions of the genome. For example, our two *L. lactis* subsp. *cremoris* strains had the same ribotype pattern, but variation around other loci was revealed by RAPD analysis. Phage typing and plasmid profiling are techniques that can measure differences below the subspecies level. However, these classifications are not completely stable, because

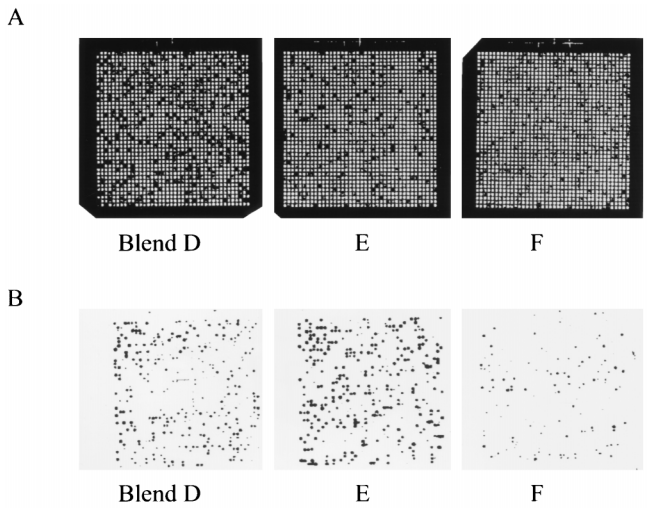

FIG. 7. HGMF colony hybridization of the 331 probe with mixed cultures. (A) HGMFs with blends D, E, and F of *L. lactis* 160, 210, and 331 after lysis pretreatment. (B) X-ray images of the HGMFs in panel A after hybridization and nonradioactive detection of the 331 probe.

plasmids can be lost (or gained through horizontal transfer) and resistance to phages can evolve. One technique that samples the entire genome is genomic REA (25). The major difficulty in analyzing the data is the complex pattern of closely spaced bands, making comparisons difficult. Also, establishing phylogenies may not be possible because a mutation in a methylase-carrying strain would result in a completely different restriction pattern.

The RAPD technique (37, 40) overcomes many of the above disadvantages. RAPD analysis is a sensitive tool for observing diversity among related isolates. This powerful fingerprinting technique can discriminate between otherwise indistinguishable bacterial isolates by sampling the nucleotide sequence of the entire genome (1, 10, 11). Fewer bands (usually 2 to 10) are produced than with genomic REA, and a large number of primers can be quickly and easily screened to further increase discrimination. In our case, potential strain-specific probes were readily identified with only 15 RAPD primers. Three RAPD primers (OPX 1, 12, and 15) tested against three lactococcal strains (160, 210, and 331) yielded a total of 32 different products, 20 of which were amplified from only one of the three strains (Fig. 1A). The *L. lactis* subsp. *cremoris* and *lactis* were easily distinguished on the basis of their RAPD patterns. Only one band, OPX1-1.2, was common to all three starter strains (Fig. 1A). It was more difficult to discriminate between *L. lactis* subsp. *cremoris* strains 160 and 331, which was not unexpected because the two strains are in the same ribogroup (Fig. 1B).

The RAPD patterns reported by Cancilla et al. (8) for seven *L. lactis* subsp. *lactis* and subsp. *cremoris* strains shared more products between the two subspecies, in contrast to our results. When we tested primer OPX 1 with eight additional strains, no common bands were observed across subspecies (data not shown). Three products were shared among the *L. lactis* subsp. *lactis* strains, and four products were shared among the *L. lactis* subsp. *cremoris* strains. The strains used by Cancilla et al. could be more closely related, or this lower degree of observed diversity could be due to more stringent primer and cycling conditions.

Although a number of strain-specific products were evident from our RAPD data, further screening with Southern hybridization showed that at least four of them could not be used as strain-specific probes. These polymorphisms likely arose from limited sequence differences, presumably in the primer binding sites, at these particular loci. Probes developed for *L. lactis* subsp. *lactis* 210 and *L. lactis* subsp. *cremoris* 331 were strain specific within the context of our three-strain blend. It was not essential to develop probes that could identify target strains in a background of any other bacterium. This practical criterion greatly simplified and shortened the probe screening procedure. In general, the broader the desired context for strain specificity, the more RAPD primers and products need to be screened.

We sequenced the OPX1-1.0b and OPX15-0.95 RAPD products. OPX1-1.0b, from which the 210 probe was derived, contains a 759-nucleotide open reading frame (ORF). BLAST searches (3) of the GenBank database with the translated ORF revealed that it was 42% identical to the *Bacillus subtilis* glycerol-3-phosphate dehydrogenase GlpD (GenBank accession no. M34393). Lactococci have not been reported to metabolize glycerol as a sole or cofermentable carbon source, unlike some *Lactobacillus* spp. (6, 35). The 331-specific RAPD product, OPX15-0.95 331, contained no significant ORFs and was not homologous to any sequence in GenBank.

Despite the power of the RAPD technique in laboratory studies, it has been difficult to adapt it for routine use because of reproducibility problems. In order to circumvent these difficulties, several groups have designed conventional PCR primers from sequenced RAPD polymorphisms for subspecies- and pathotype-specific assays (23, 36). Other groups have developed RAPD products as subspecies- or pathotype-specific probes for Southern, dot blot, and in situ hybridization (5, 9, 14, 19). However, with the exception of in situ hybridization, a disadvantage shared by these assays as well as strain-specific differentiation assays (phage typing, ribotyping, RFLP fingerprinting, and RAPDs) is the need to screen subcultures of each isolate individually. Colony hybridization assays are not as limited because many colonies can be probed on a single membrane. However, with traditional colony hybridization methodologies, the number of colonies that can be simultaneously screened is largely dependent on the growth rate and degree of spread by the colonies on the agar. An improvement over the traditional colony hybridization methodology is the use of HGMFs, which increases the theoretical maximum number of colonies that can be simultaneously screened to 1,600 (31). Organisms (such as food pathogens) that normally constitute a small proportion of a bacterial population can be detected and monitored. Also, the technique could be applied to study food and other mixed fermentations. For example, little is known about the changes in the microbial community occurring throughout cheese fermentations that are responsible for texture and complex flavor development.

The RAPD technique and HGMF colony hybridization assays are a synergistic combination for quantitative strain-specific population monitoring. The RAPD technique, with its discriminatory power, is used as a screening tool to rapidly identify potential markers with the desired level of specificity. Then these markers can be developed as probes in colony hybridization assays with HGMFs, or in a variety of other assay formats, for use in epidemiology and population-monitoring studies.

ACKNOWLEDGMENTS

We thank Dennis Romero of Marschall Products and Jeff Kondo for providing commercial lactococcal strains. We also thank Jim Bruce and Eileen Cole from Qualicon for ribotyping our strains.

This work was supported by a grant from the National Dairy Promotion and Research Board.

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