APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1991, p. 1313–1318
0099-2240/91/051313-06$02.00/0

Vol. 57, No. 5

Development and Application of Oligonucleotide Probes for Identification of *Lactococcus lactis* subsp. *cremoris*†

MAYSOON SALAMA, WILLIAM SANDINE, AND STEPHEN GIOVANNONI*
Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804

Received 4 September 1990/Accepted 2 February 1991

***Lactococcus lactis* subsp. *cremoris* is of considerable interest to the dairy industry, which relies upon the few available strains for the manufacture of cheddar cheese free of fermented and fruity flavors. The subspecies *cremoris* differs from related subspecies by the lack of a few phenotypic traits. Our purpose was to identify unique rRNA sequences that could be used to discriminate *L. lactis* subsp. *cremoris* from related subspecies. The 16S rRNAs from 13 *Lactococcus* strains were partially sequenced by using reverse transcriptase to identify domains unique to *L. lactis* subsp. *cremoris*. All five strains of the subspecies *cremoris* had a unique base sequence in a hypervariable region located 70 to 100 bases from the 5′ terminus. In this region, all *L. lactis* subsp. *lactis* biovar diacetylactis strains examined had a sequence identical to that of *L. lactis* subsp. *lactis* 7962, which was different from other strains of the subspecies *lactis* by only one nucleotide at position 90 (*Escherichia coli* 16S rRNA structural model) (J. Brosius, J. L. Palmer, J. P. Kennedy, and H. F. Noller, Proc. Natl. Acad. Sci. USA 75:4801–4805, 1978). Oligonucleotide probes specific for the genus *Lactococcus* (212RLa) and for the subspecies *cremoris* (68RCa) were synthesized and evaluated by hybridization to known rRNAs as well as fixed whole cells. Efficient and specific hybridization to the genus-specific probe was observed for the 13 *Lactococcus* strains tested. No hybridization was seen with the control species. All five strains of the subspecies *cremoris* hybridized to the subspecies-specific probe.**

Dairy lactococci have been used for centuries in the production of fermented dairy products. Since the work of Vedamuthu and colleagues (23, 24), *Lactococcus lactis* subsp. *cremoris* (previously known as *Streptococcus cremoris*) has been the organism of choice for use in manufacturing fermented milk products, particularly cheddar cheese. All of the strains of this subspecies now in use are believed to be descendants of original isolates taken from cream in Denmark and the United States. The intensive use of these strains has led to problems with bacteriophage infections. Consequently, it is important to the dairy industry to identify new strains of *L. lactis* subsp. *cremoris* suitable for the manufacture of cheddar cheese. Lawrence and coworkers (12) emphasized the great need that exists for more strains of the subspecies *cremoris* for use in starter cultures. Attempts to isolate new strains from nature by using traditional microbiological approaches have not been fruitful (4, 17, 18), possibly because the subspecies *cremoris* occurs naturally in very small numbers. Alternatively, the subspecies *cremoris* phenotype may not occur naturally but rather may have evolved in association with dairy-related practices. With the availability of molecular methods for the study of systematics and microbial ecology (15), molecular probes can now be employed to methodically screen natural isolates of *L. lactis* for the subspecies *cremoris* genotype.

In recent years, rRNA sequences, particularly 16S rRNAs, have been used widely to characterize microorganisms (6, 11, 14, 18). The 16S rRNAs vary in their nucleotide sequences, but they contain some segments that are invariant in all organisms (16). These conserved sequences provide binding sites for primer elongation sequencing protocols (5, 14). Other regions of the 16S rRNA are unique to particular organisms or groups of related organisms. This situation offers the opportunity to design specific hybridization probes to identify an organism or a group of organisms (3, 5, 14). Such probes have potential for use in screening large numbers of natural isolates for commercially significant strains.

On the basis of a comparative analysis of 16S rRNA catalogs, 16S rRNA sequences, and nucleic acid hybridization studies, the mesophilic coccus-shaped lactic acid bacteria are considered to be a monophyletic microbial group. They are now placed in the genus *Lactococcus* (2, 17, 21). This suggested that it might be possible to design phylogenetic genus-specific rRNA probes for the detection of these organisms.

The aim of this study was to design and synthesize two classes of phylogenetic probes, a subspecies-specific rRNA probe for *L. lactis* subsp. *cremoris* and a genus-specific rRNA probe for the lactococci.

MATERIALS AND METHODS

Organisms and growth conditions. Thirteen strains of lactococci were grown in litmus milk (0.75 g of litmus powder per liter of skim milk) and stored at −70°C in litmus milk containing 15% glycerol. The strains used in this study were *L. lactis* subsp. *lactis* ATCC 11955, ATCC 11454, 7962, C2, and f2d2, *L. lactis* subsp. *cremoris* BK5, 107/6, 205, P2, and HP, and *L. lactis* subsp. *lactis* biovar diacetylactis DRC-1, 18-16, and 26-12. The first two were obtained from the American Type Culture Collection in Rockville, Md., and the remaining strains were from the Department of Microbiology culture collection, Oregon State University, Corvallis, Oreg. Active cultures were usually prepared in M-17 broth (22).

Extraction of RNA. Cells from 500 ml of a log-phase culture were harvested at 7,700 × *g* for 15 min and resuspended in 15 ml of ice-cold STE buffer (100 mM NaCl, 50

* Corresponding author.
† Technical report no. 9342 of the Oregon State University Agricultural Experiment Station.

TABLE 1. Primers used for sequencing of 16S rRNAs or for the hybridization experiments

Primer[a]	Hits	*E. coli* nucleotide no.	Sequence (5′ to 3′)
1406F[b]	Negative control	1391–1406	TGYACACACCGCCCGT
1406R[b]	Universal	1406–1392	ACGGGCGGTGTGTRC
519R[b]	Universal	536–519	GWATTACCGCGGCKGCTG
343aR[c]	Eubacteria	357–343	CTGCTGCCTCCCGTA
212RLa	Lactococci	233–212	CTTTGAGTGATGCAATTGCATC
68RCa	*L. cremoris*	87–68	TGCAAGCACCAATCTTCATC

[a] R, Reverse; F, forward.
[b] See reference 11.
[c] Provided courtesy of C. Woese.

mM Tris-HCl [pH 7.4], 1.0 mM Na_2EDTA [pH 7.4]). The suspension was passed twice through a French pressure cell at 20,000 lb/in^2 to disrupt the cells. Cell debris was removed by centrifugation at 7,700 × *g* for 15 min. The nucleic acid was purified from the supernatant fluid by repeated extraction with phenol saturated with STE buffer (pH 6.5), followed by one chloroform-isoamyl alcohol (24:1 [wt/vol]) extraction and precipitation with 1/10 volume of 2.0 M sodium acetate and 2.0 volumes of ethanol. The precipitated nucleic acid was collected by centrifugation at 13,000 × *g* for 10 min, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl [pH 7.4], 1.0 mM Na_2EDTA [pH 7.4]). The bulk cellular RNA was adjusted to a concentration of 2 mg/ml and stored at −70°C in TE buffer. The bulk cellular RNAs prepared by this technique were found to be predominantly 16S and 23S rRNAs when examined by agarose gel electrophoresis and ethidium bromide staining (data not shown). The control 16S rRNAs from *Dermocarpa* strain PCC 7437, *Myxosarcina* strain PCC 7312, *Strongylocentrotus purpureus*, *Halobacterium volcanii*, and *Pseudomonas aeruginosa* IUCC SXI were prepared by isopycnic centrifugation in cesium trifluoroacetate density gradients (5).

Reverse transcription reactions. The sequencing protocol used was the base-specific dideoxynucleotide-terminated chain elongation method of Lane et al. (10, 11) with the following minor changes: the denaturation temperature was 65°C, and microtiter plates were used rather than microcentrifuge tubes.

Oligonucleotide probes and primers. Table 1 lists the primer sequences that were used either for sequencing or hybridization purposes. The subspecies-specific rRNA probe for *L. lactis* subsp. *cremoris* (68RCa) and the genus-specific rRNA probe for the lactococci (212RLa) were synthesized on an Applied Biosystems DNA synthesizer. The oligonucleotides were purified by electrophoresis on 20% polyacrylamide gels and then recovered by elution as described by Lane et al. (11). Oligonucleotides were end labeled with γ-^{32}P by using the protocol of Sgaramella and Khorana (20). Labeled probes were purified on C_{18} reverse-phase Sep-Pak columns (Millipore Corp., Milford, Mass.) as described previously (11).

Nylon membrane hybridization. The bulk cellular RNAs were dot blotted on nylon membranes and hybridized to radiolabeled probes as described previously (5) with minor modifications. A manifold apparatus (Schleicher & Schuell, Keene, N.H.) was used to dot blot appropriate rRNA target molecules (50 ng) onto Nytran nylon membranes (0.45-μm pore size; Schleicher & Schuell). The filters were dried in a vacuum oven at 80°C for 15 to 20 min and then cross-linked by exposure to UV light (200 J/m^2). After this treatment, about 5 to 10 ml of prehybridization buffer (6× SSPE [1.08 M NaCl, 60 mM $NaPO_4$, 60 mM EDTA, pH 7.5], 5× Denhardt solution [0.1% Ficoll {Pharmacia}, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin], and 0.1% sodium dodecyl sulfate [SDS]) was added to the blots in a microseal bag, and prehybridization was carried out for 20 to 30 min at room temperature. The prehybridization buffer was then replaced with 3 to 5 ml of hybridization buffer (6× SSPE, 1× Denhardt solution, 0.1% SDS, and approximately 10^6 cpm of ^{32}P-labeled probe). The bags were sealed and incubated at room temperature overnight. Filters were washed three times for 15 to 20 min at room temperature in 6× SSPE–0.1% SDS and then one time at the predetermined stringency temperature (45°C for both 212RLa and 68RCa probes and 37°C for both 1406R and 1406F probes). After drying, filters were exposed to X-ray film for 6 to 24 h.

Whole-cell dot blot hybridization. Whole cells were hybridized to oligonucleotide probes as described previously (5), with minor modifications. Briefly, the cells were grown in M-17 broth and counted by using a Petroff-Hausser counting chamber. A cell pellet was obtained by centrifugation at 7,700 × *g* for 10 to 15 min. The pellet was suspended in 5 ml of 145 mM NaCl–100 mM sodium phosphate, pH 7.5 (PBS). Formaldehyde was then added at a concentration of 1%. The suspended cells were left on ice for 30 min with occasional shaking. The cells were then washed twice in PBS, suspended in 5 ml of 145 mM NaCl–10 mM Tris-HCl, pH 7.5, plus 5 ml of 100% ethanol with stirring on ice, and held at −20°C. Glass fiber filters (GFC Whatman no. 934-AH) were prepared for blotting by being soaked in poly-L-lysine (50 μg/ml in 10 mM Tris, pH 8), after which they were air dried and sprayed on the back with a thin layer of acrylic spray before being used for blotting. About 5 × 10^7 fixed cells were directly blotted onto the pretreated GFC filters by using a Schleicher & Schuell manifold apparatus. Filters were air dried and hybridized as for rRNAs.

RESULTS

Sequencing of lactococcal 16S rRNAs. The rRNAs from 13 closely related *Lactococcus* strains were sequenced by reverse transcription in the presence of dideoxynucleotides. A conserved site at positions 357 to 343 (here and throughout the article we refer to nucleotide positions relative to the structural model of *Escherichia coli* 16S rRNA; 1) was used to sequence the 5′ region of the 16S rRNAs from the 13 *Lactococcus* strains. About 260 to 280 nucleotides of sequence were obtained with this primer. However, for most of the strains, it was not possible with this primer to sequence accurately through the variable region located at positions 70 to 100. The 212RLa probe, which binds specifically to lactococcal 16S rRNAs at positions 212 to 233 (Fig. 1 and Table 2), enabled us to sequence through the remaining

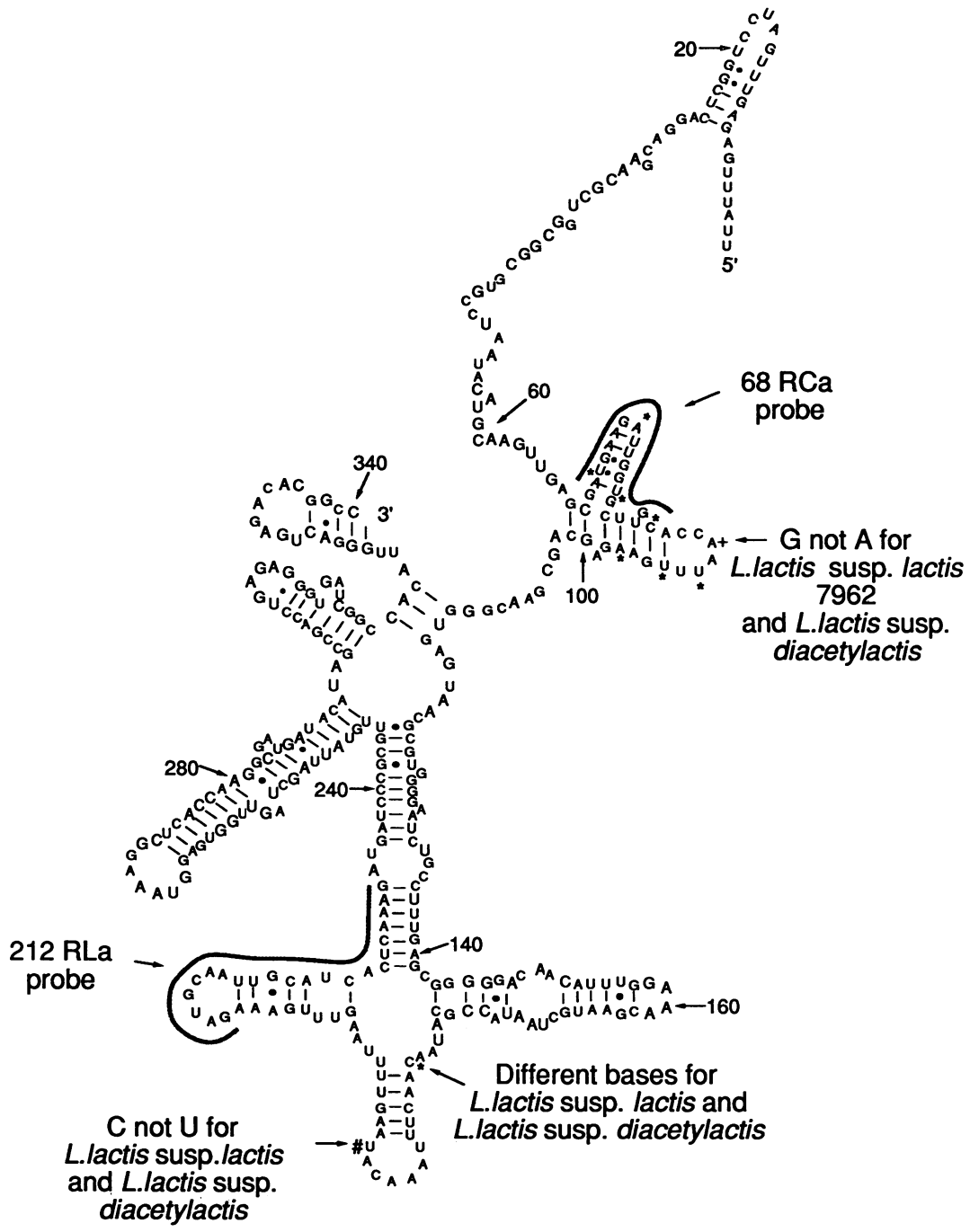

FIG. 1. Secondary-structure model for 5′ region of lactic acid bacterium 16S rRNAs. The positions marked by *, +, and # are the sites of variations within the lactic acid bacteria. The shadowed lines indicate the sites of the genus-specific and subspecies-specific probes. Numbering corresponds to the *E. coli* 16S rRNA structural model (1).

5′ region of the molecule, which included several variable regions of interest. No sequence differences were seen between the five *L. lactis* subsp. *cremoris* strains tested. However, *L. lactis* subsp. *lactis* 7962 differed from the other *L. lactis* subsp. *lactis* strains tested by one base at position 90. Also, the sequence of *L. lactis* subsp. *lactis* C2 was identical to that of the subspecies *cremoris* over 182 nucleotides with the exception of two uncertainties at positions 71 and 80. A complete sequence for the subspecies *lactis* ATCC 11955 at the hypervariable region between positions 98 and 68 could not be obtained accurately and still is being investigated. All of the *L. lactis* subsp. *lactis* biovar diacetylactis strains had exactly the same sequences as *L. lactis* subsp. *lactis* 7962 over about 300 to 320 nucleotides. Figure 1 illustrates a secondary-structure model for the 5′ domain of the *L. lactis* subsp. *cremoris* 16S rRNA. Sites of variations within the *Lactococcus* genus are indicated. The partial sequences of lactic acid bacteria are shown aligned in Fig. 2.

```
Lc 205    UUAUUUGAGAGUUUGAUCCU GGCUCAGGACGAACGCUGGC GGCGUGCCUAAUACAUGCAA GUUGAGCGAUGAAGAUUGGU GCUUGCACCAAUUUGAAGAG 100
Lc BK5    .................... .................... .................... .................... .................... 100
Lc 107/6  .................... .................... .................... .................... .................... 100
Lc P2     .................... .................... .................... .................... .................... 100
Lc HP     .................... .................... .................... .................... .................... 100
Ll 11454  .................... .................... .................... ........C.....G..... A....U.....C.G..U... 100
Ll 7962   .................... .................... .................... ........C.....G..... A....U...G.C.G..U... 100
Ll C2     .................... .................... .................... ..........R........K .................... 100
Ll f2d2   .................... .................... .................... ........C.....G..... A....U.....C.G..U... 100
Ld DRC-1  .................... .................... .................... ........C.....G..... A....U...G.C.G..U... 100
Ld 18-16  .................... .................... .................... ........C.....G..... A....U...G.C.G..U... 100
Ld 26-2   .................... .................... .................... ........C.....G..... A....U...G.C.G..U... 100

Lc 205    CAGCgAACGGGUGAGUAACG CGUgGGGAAUCUGCCUUUGA GCGGGGGACAACAUUUGGAA ACGAAUGCUAAUACCGCAUA ACAACUUUAAACAUAAGUUU 200
Lc BK5    .................... .................... .................... .................... .................... 200
Lc 107/6  .................... .................... .................... .................... .................... 200
Lc P2     .................... .................... .................... .................... .................... 200
Lc HP     .................... .................... .................... .................... .................... 200
Ll 11454  .................... .................... ....                                                     144
Ll 7962   .................... .................... .................... .................... .A..........C...... 200
Ll C2     .................... .........W.......... .................... .                                    161
Ll f2d2   .................... .................... .................... .................... .A..........C...... 200
Ld DRC-1  .................... .................... .................... .................... .A..........C...... 200
Ld 18-16  .................... .................... .................... .................... .A..........C...... 200
Ld 26-2   .................... .................... .................... .................... .A..........C...... 200

Lc 205    UAAGUUUGAAAGAUGCAAUU GCAUCACUCaAAGAUgAuCC CGCGUUGuaUUAGCUAGUUG GUGAGGUaAAGGCUCACCaA GGCGAUGAuACAUAGCCGAC
Lc BK5    .................... .................... .................... .................... .................... 300
Lc 107/6  .................... .................... .................... .................... .................... 300
Lc P2     .................... .................... .................... .................... .................... 300
Lc HP     .................... .................... .................... .................... .................... 300
Ll 7962   .................... .................... .................... .................... .................... 300
Ll f2d2   .................... .................... .................... .................... .................... 300
Ld DRC-1  .................... .................... .................... .................... .................... 300
Ld 18-16  .................... .................... .................... .................... .................... 300
Ld 26-2   .................... .................... .................... .................... .................... 300

Lc 205    CUGAGAGGGUgAUcGGCCAC auuGGGACuGAGACACGGCC 340
Lc BK5    ............           ...................... 312
Lc 107/6  ............           ...................... 312
Lc P2     ............           ...................... 312
Lc HP     .................... .... ..................... 324
Ll 7962   ............           ...................... 312
Ll f2d2   .................... .................... 340
Ld DRC-1  .................... ......              ... 326
Ld 18-16  .................... ......              ... 326
Ld 26-2   .................... ......              ... 326
```

FIG. 2. Nucleotide sequences of 5′ regions of lactic acid bacterium 16S rRNAs. Points indicate nucleotide identity with *L. lactis* subsp. *cremoris* 205. The accumulated positions are given in the right margins. Lowercase letters indicate uncertainty in the determination. Abbreviations: Lc, *L. lactis* subsp. *cremoris*; Ll, *L. lactis* subsp. *lactis*; Ld, *L. lactis* subsp. *lactis* biovar diacetylactis.

Construction of probes and hybridization experiments. On the basis of the analysis of the partial sequence information, two phylogenetic probes were designed and synthesized, a subspecies-specific rRNA probe for *L. lactis* subsp. *cremoris* and a genus-specific probe for the lactococci. The sequences of both probes are shown in Fig. 1. The genus-specific probe is 22 nucleotides in length and is located at positions 212 to 233 of the 16S rRNA. This probe was used to identify members of the lactococci by hybridization of the probe to bulk cellular RNA. Strong, specific hybridization to the probe was noted for all of the lactococci examined (Fig. 3). On the other hand, no cross-reactivity was seen when the probe was tested against other eubacterial (*Dermocarpa* strain PCC 7437, *Myxosarcina* strain PCC 7312, and *P. aeruginosa* IUCC SXI), archaebacterial (*H. volcanii*), and eukaryotic (*S. purpureus*) RNAs (Fig. 3). Identical results were obtained for whole-cell hybridizations. Specific hybridization of the 212RLa probe was observed to all lactococcal bacterial strains (Fig. 4). However, *L. lactis* subsp. *lactis* 7962 hybridized to the probe weakly. The number of cells was increased fourfold for *L. lactis* subsp. *lactis* 7962 to give a signal approximately equivalent to the other strains. The 212RLa probe did not bind to any of the control strains, which included *Enterococcus pyogenes*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Salmonella pullorum*, and *Bacillus subtilis*. The binding of the 1406R universal probe was used as a positive control for the presence of

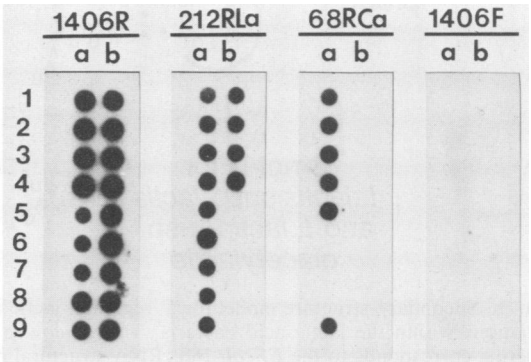

FIG. 3. Autoradiogram of a dot blot hybridization to bulk cellular RNAs from lactic acid bacteria and control strains. The universal (1406R), genus-specific (212RLa), subspecies-specific (68RCa), and negative control (1406F) probes were used. The order of the blotted RNAs is: *L. lactis* subsp. *cremoris* BK5, 107/6, 205, p2, and HP for dot blots 1a to 5a, respectively; *L. lactis* subsp. *lactis* 11955, 11454, 7962, C2, and f2d2 for dot blots 6a to 9a and 1b, respectively; *L. lactis* subsp. *lactis* biovar diacetylactis DRC, 18-16, and 26-2 for dot blots 2b to 4b, respectively; *Dermocarpa* strain PCC 7437 for dot blot 5b; *Myxosarcina* strain PCC 7312 for dot blot 6b; *S. purpureus* for dot blot 7b; *H. volcanii* for dot blot 8b; and *P. aeruginosa* IUCC SXI for dot blot 9b.

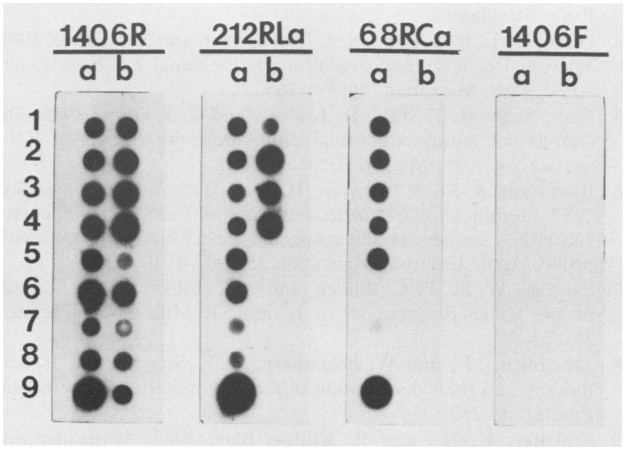

FIG. 4. Autoradiogram of a dot blot hybridization to fixed whole cells of lactic acid bacteria and control strains. The order of the blotted cells is the same as described in the legend to Fig. 3 for the lactic acid bacteria. The control strains, *E. pyogenes*, *E. faecalis*, *S. epidermidis*, *S. pullorum*, and *B. subtilis*, were blotted in wells 5b to 9b, respectively. All control strains were obtained from the Department of Microbiology culture collection, Oregon State University. The number of cells was increased fourfold for *L. lactis* subsp. *lactis* 7962 to give a signal approximately equivalent to that of the other strains.

detectable target sequence. An oligonucleotide that is not complementary to the rRNA (1406F) served as a control for nonspecific binding (Fig. 3 and 4).

The subspecies-specific probe (68RCa) was complementary to a 20-bp region located at positions 68 to 87 of a highly variable domain. This probe was designed to discriminate *L. lactis* subsp. *cremoris* from other lactococci. In RNA-DNA hybridization experiments, this probe bound specifically and efficiently to the RNAs (Fig. 3) as well as to fixed whole cells (Fig. 4) of the five *L. lactis* subsp. *cremoris* strains. All of the control strains, including the other lactococci related to the subspecies *cremoris*, failed to hybridize to the 68RCa probe. The only exception was *L. lactis* subsp. *lactis* C2, which hybridized to the 68RCa probe on all occasions as predicted from sequencing studies. A different source of this strain confirmed these results, indicating that strain C2 has the same sequence as the subspecies *cremoris* at the homologous positions. *L. lactis* subsp. *lactis* ATCC 11955 hybridized weakly to the 68RCa probe. This might be attributed to nonspecific binding. The sequence of the 16S rRNA of this strain at the probe site has not yet been determined.

DISCUSSION

Because of its rapidity and technical simplicity, the reverse transcriptase sequencing method was helpful for determining 16S rRNA partial sequences from the 13 lactococcal strains. The 16S rRNAs of the lactococcal strains showed a high degree of similarity. However, among the eight *L. lactis* subsp. *lactis* and biovar diacetylactis strains studied, only *L. lactis* subsp. *lactis* strain C2 had the same nucleotide sequence as that of *L. lactis* subsp. *cremoris* at the position of the probe target and thus hybridized strongly. The two strains of *L. lactis* subsp. *lactis* C2 originated in Australia; from there they have been dispersed to other laboratories. Phenotypically, the strain behaves like the subspecies *lactis*. However, the 16S rRNA sequence of the strain resembles that of the subspecies *cremoris*. It is possible that the subspecies *cremoris* phenotype could have evolved naturally from the subspecies *lactis*, in association with dairy-related practices, by the loss of certain phenotypic traits. Alternatively, there is a possibility that strain C2 originally had the phenotype of the subspecies *cremoris* but has acquired certain traits of the subspecies *lactis*, perhaps by means of a transducing phage. In this regard, a temperate bacteriophage has been found in the C2 strain which converts lactose-, maltose-, or mannose-negative recipient cells of this strain to the respective carbohydrate-positive phenotype (13). The instability of "pure" cultures of lactic acid bacteria, which would ordinarily be regarded as being constant in properties, has been reported by Hunter (7). This issue could be resolved in the near future if we succeed in obtaining natural isolates of the subspecies *cremoris* genotype and study their phenotypic properties in detail.

Nucleic acid hybridization recently was introduced as a rapid tool for the identification of microorganisms (8, 9, 14). rRNAs are attractive candidates as targets for hybridization probes because of their unique organization, the presence of highly conserved and variable regions, and their presence in high copy number.

The small differences between the 16S rRNA sequences of the lactic acid bacteria were sufficient to allow differentiation between closely related subspecies. Wallace et al. (25) indicated that oligonucleotides that differ in sequence at only one position are potentially useful as sequence-specific probes. The nucleotide sequence that we selected as target site for the genus-specific probe (212RLa) was unique to the lactococci, as indicated by comparisons with a data base of more than 200 known eubacterial 16S rRNA sequences. Furthermore, this uniqueness was verified by the specific hybridization of the probe to all 13 lactic acid strains investigated but none of the control organisms. A 3-bp mismatch in the oligonucleotide probe (68RCa) of 20 bp was sufficient to discriminate *L. lactis* subsp. *cremoris* from the closely related *L. lactis* subsp. *lactis* and its biovar diacetylactis.

The relatively small size of the oligonucleotide hybridization probes used in our study minimizes problems of cellular permeability and access to binding sites. However, the amount of probe that is specifically bound may be influenced by many variables, including the permeability of fixed cells and the accessibility of the rRNAs in fixed-cell preparations (5). One or more such variables could account for the weak hybridization between *L. lactis* subsp. *lactis* 7962 fixed whole cells and the genus-specific probe (212RLa), as opposed to a much stronger signal of the same strain when bulk cellular RNA was hybridized to the probe.

The hybridization probes described here provide a highly sensitive and specific means for the rapid detection and identification of lactic acid bacteria in general and *L. lactis* subsp. *cremoris* in particular. The use of these probes may contribute substantially to the isolation and study of new strains of the subspecies *cremoris* from natural habitats.

ACKNOWLEDGMENTS

We thank Katharine Field for her advice and efforts during the early stages of this work.

This work was supported by a grant from the National Dairy Board and by the Oregon State University Agricultural Experiment Station.

REFERENCES

1. **Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S rRNA gene from *Escherichia*. Proc. Natl. Acad. Sci. USA **75**:4801–4805.
2. **Collins, M. D., C. Ash, J. A. E. Farrow, S. Walbanks, and A. M. Williams.** 1989. 16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. Description of *Vagococcus flavialis* gen. nov., sp. nov. J. Appl. Bacteriol. **67**:453–460.
3. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1362.
4. **Fenton, M. P.** 1987. An investigation into the source of lactic acid bacteria in grass silage. J. Appl. Bacteriol. **62**:181–188.
5. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170**:720–726.
6. **Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. Lane, and N. R. Pace.** 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170**:3584–3592.
7. **Hunter, G. J. E.** 1939. Examples of variation within pure cultures of *Streptococcus cremoris*. J. Dairy Res. **10**:464–470.
8. **Izat, A. L., C. D. Driggers, M. Colberg, M. A. Reiber, and M. H. Adams.** 1989. Comparison of the DNA probe to culture methods for the detection of *Salmonella* on poultry carcasses and processing waters. J. Food Prot. **52**:564–570.
9. **Kapperud, G., K. Dommarsnes, M. Skurnik, and E. Hornes.** 1990. A synthetic oligonucleotide probe and a cloned polynucleotide probe based on the *yopA* gene for detection and enumeration of virulent *Yersinia enterolitica*. Appl. Environ. Microbiol. **56**:17–23.
10. **Lane, D. L., K. G. Field, G. J. Olsen, and N. R. Pace.** 1988. Reverse transcriptase sequencing of ribosomal RNA for phylogenetic analysis. Methods Enzymol. **167**:138–144.
11. **Lane, D. L. B. Pace, G. J. Olsen, D. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequence for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82**:6955–6959.
12. **Lawrence, R. C., T. D. Thomas, and B. E. Terzaghi.** 1976. Reviews of the progress of dairy science: cheese starters. J. Dairy Res. **43**:141–193.
13. **McKay, L. L.** 1982. Regulation of lactose metabolism in dairy streptococci, p. 153–182. *In* R. Davies (ed.), Developments in food microbiology, vol. 1. Applied Science Publishers Ltd., Essex, England.
14. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40**:337–365.
15. **Pace, N. R., R. J. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9**:1–55.
16. **Rhenstam, A.-S., A. Norqvist, H. Wolf-Watz, and A. Hagström.** 1989. Identification of *Vibrio anguillarum* in fish by using partial 16S rRNA sequences and a specific 16S rRNA oligonucleotide probe. Appl. Environ. Microbiol. **55**:1907–1910.
17. **Sandine, W. E., P. C. Radich, and P. R. Elliker.** 1972. Ecology of the lactic streptococci: a review. J. Milk Food Technol. **35**:176–185.
18. **Schieblich, M., and W. Schonherr.** 1957. Streptococci in acid fodders and their distribution in the raw material. Tierernaehr. Fuetter. **11**:232.
19. **Schleifer, K. H., and R. Klipper-Blaz.** 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci, and lactococci: a review. Syst. Appl. Microbiol. **10**:1–19.
20. **Sgaramella, V., and H. G. Khorana.** 1972. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymatic joining of the chemically synthesized polynucleotides to form the DNA duplex representing nucleotide sequence 1 to 20. J. Mol. Biol. **72**:427–444.
21. **Stackebrandt, E., and M. Teuber.** 1988. Molecular taxonomy and phylogenetic position of lactic acid bacteria. Biochimie **70**:317–324.
22. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
23. **Vedamuthu, E. R., W. E. Sandine, and P. R. Elliker.** 1966. Flavor and texture in Cheddar cheese. I. Role of mixed strain lactic starter cultures. J. Dairy Sci. **49**:144–150.
24. **Vedamuthu, E. R., W. E. Sandine, and P. R. Elliker.** 1966. Flavor and texture in Cheddar cheese. II. Carbonyl compounds produced by mixed strain lactic starter cultures. J. Dairy Sci. **49**:151–157.
25. **Wallace, R. B., J. Shaffer, R. F. Murphy, J. Bonner, T. Hirose, and K. Itakura.** 1979. Hybridization of synthetic oligodeoxyribonucleotides to ϕX174 DNA: the effect of a single base pair mismatch. Nucleic Acids Res. **6**:3543–3557.