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Title: The Isolation of *Lactococcus lactis* subsp. *cremoris* From Nature With Probes for 16S Ribosomal RNAs

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Lactococcus lactis subsp. *cremoris* is of considerable interest to the dairy industry, which relies upon the limited number of strains available the manufacture of Cheddar cheese free of fermented and fruity flavors. Our purpose was to identify unique ribosomal RNA sequences that could be used to discriminate *L. lactis* subsp. *cremoris* from related subspecies *lactis*. The 16S rRNAs from 13 *Lactococcus* strains were partially sequenced using reverse transcriptase in order to identify domains unique to *L. lactis* subsp. *cremoris*. Oligonucleotide probes specific for the species *Lactococcus lactis* (212RLa) and the subspecies *cremoris* (68RCa) were designed, synthesized and evaluated for ability to discriminate lactococci and *L. lactis* subsp. *cremoris* from closely related strains.

These probes were used in colony hybridizations to rapidly screen large numbers of colonies for *L. lactis* subsp. *cremoris*. Thirty-eight plant and vegetable species, twelve other samples from

dairy farms, twenty-one individual raw milk and milk product samples from the United States, China, Morocco, and Yugoslavia, were examined for lactic acid bacteria by the colony hybridization method using the 68RCa and 212RLa probes.

Lactococcus lactis subsp. *lactis* was found to occur on potato, cucumber, sweet peas, beans, cantaloupe, corn, cow's body and tail and in colostrum, goat and cow raw milk, cottage cheese and cream. *Lactococcus lactis* subsp. *diacetylactis* was isolated from cow raw milk obtained from Morocco, as well as goat raw milk and cottage cheese from Yugoslavia. *Lactococcus lactis* subsp. *cremoris* was isolated from raw milk obtained from Morocco, Yugoslavia and China and from cottage cheese obtained from Yugoslavia. The phenotypical, morphological, and physiological characteristics of the newly isolated lactococcal strains generally agreed with the standard description for the genus *Lactococcus*.

The isolation of *L. lactis* from different plant sources confirmed that plants are a natural source of this bacterium. The fact that a few strains of *L. lactis* subsp. *cremoris* were isolated from raw milk and cottage cheese from Morocco and Yugoslavia, but not from plants, suggests that a natural habitat of the subspecies *cremoris* could be raw milk and milk products and prepared by traditional dairy practices.

The biochemical and physiological characteristics of the new *L. lactis* isolates, their resistance to bacteriophage preparations obtained from cheese factories, and their acid producing capabilities, indicate the potential usefulness of these strains as dairy starter cultures.

The Isolation of *Lactococcus lactis* subsp. *cremoris* From Nature With Probes for 16S Ribosomal RNAs

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Dedicated with love ....

To my dear husband Mohammad A. Alayan, my beloved son Atta M. Alayan, my parents Qadriya Odeh and Subhi Salama (God bless his soul), and to both dear families, the Salamas and the Alayans.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 5

The Isolation of *Lactococcus lactis* subsp. *cremoris* From Nature With Probes for 16S Ribosomal RNAs

CHAPTER 1

Introduction

Ecology of lactic acid bacteria.

In the early years of the science of dairy bacteriology it was thought that freshly drawn milk was sterile. It was not until 1891 that Schultz (as cited by Harding (21)) found that milk contained a large number of bacteria. At that time it was thought that this was an indication of udder disease. Later, Moore confirmed the findings of Schulz, and concluded that bacteria do occur in normal milk. Once this idea was firmly established, the species of bacteria occurring in milk were brought under examination. Walker (67) conducted one of the earliest studies in this regard. He found that *Streptococcus lactis* (now known as *Lactococcus lactis* subsp. *lactis* and herein referred to as *L. lactis*) constituted at least 95% of the organisms present in all the milk samples he studied. In more recent times Sherman and Hastings (58) found streptococci in the milk of 31.1% of 48 cows and in 15.1% of the samples from 161 cows. *Streptococcus cremoris* (now known as *Lactococcus lactis* subsp. *cremoris* is found in even lower numbers in milk. Of 3,000 isolates from 59 samples of commercial raw milk, only 4% were *L. lactis* subsp. *cremoris* according to a study conducted by Nelson and Thornton (38). In that same year 35 strains of *Lactococcus* were isolated from raw milk samples in a

remote area of the Jura mountains in France (25). Only 2 of the isolates were *L. lactis* subsp. *cremoris*. This supported the idea that this organism occurs naturally and is only an environmental variety of *L. lactis* subsp. *lactis*.

Since lactic acid bacteria (LAB) are found most often in milk it was only natural that the body of cows, including the udder and the mouth, would be suspected as being a natural habitat for these microorganisms. LAB have been reported to occur on milking utensils (4), and in the udders (15, 46), surfaces, mouths, and feces of cows (14). However, these reports were made without the identification of lactococci to species and subspecies. Later investigators, who had more refined differential tests for the identification of the LAB, failed in many cases to isolate the organisms from these sources. Plant material, not cattle, was then suggested to be the natural habitat for lactococci (14, 58, 61). One of the most fruitful studies carried out to investigate the presence of LAB on plant material was that done by Stark and Sherman (61). Two hundred cultures were isolated from different plant samples and identified as *L. lactis* subsp. *lactis*. Samples of fresh corn and corn silks tested were found in every instance to contain only *L. lactis* subsp. *lactis*. Esten (14) tried to isolate these organisms from various plant materials but none were found. Several types of grain feeds were also tested. Only one culture of *L. lactis* subsp. *lactis* was isolated from corn meal. Pinter (43) was able to find *L. lactis* subsp. *lactis* on clover, beans, and grass. Out of 50 samples of plant materials studied, 20% showed the presence of various streptococci. Out of the 20%, 70% were *Streptococcus faecalis* (now known as

Enterococcus faecalis) and 30% were *L. lactis* subsp. *lactis*. Attempts to isolate *L. lactis* subsp. *cremoris* from plant material have been made by a few investigators, with no success. Yawger (73) screened 60 samples of plant material for *L. lactis* subsp. *cremoris*. He managed to isolate 16 cultures of *L. lactis* subsp. *lactis*, but no strains of *L. lactis* subsp. *cremoris*. Even though the results of his experiments were negative, he felt that plant materials still represented the most logical source for *L. lactis* subsp. *cremoris*.

Much of the early work on the ecology of LAB had little value, because reliable methods of distinguishing these organisms from fecal streptococci did not exist (25). In an attempt to determine the natural habitat of *Lactococcus* organisms, Radich (44) examined 27 different species of vegetables, 18 species of fruits, and many individual cow raw milk samples. *L. lactis* subsp. *lactis* was found on potatoes, corn, cucumber, peas, beans, and cantaloupe. In each case the organism was isolated in low numbers. All fruits examined failed to yield any lactococci with the exception of cantaloupe. From 31 individual cow raw milk samples, 4 isolates of *L. lactis* subsp. *cremoris* were obtained, but all proved to be slow acid producers. Three of the remaining strains were *L. lactis* subsp. *lactis* biovar. *diacetylactis*, and 28 were *L. lactis* subsp. *lactis*. The isolation of *L. lactis* subsp. *lactis* from several plant sources confirmed the belief that plants are the natural habitat for this bacterium. Failure to isolate *L. lactis* subsp. *cremoris* from plant material suggested that this species may be a variant of *L. lactis* subsp. *lactis*, with milk as its natural habitat. King and Koburger (27) characterized Group N streptococci isolated from meats, frozen vegetables, dairy products,

bran-trough water, and poultry feed. From 18 samples, 184 isolates of *L. lactis* were obtained. These were generally more resistant (94.6%) to 20 bacteriophages than dairy starter culture isolates (77% resistant). No *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains were isolated and the subspecies *cremoris* was recovered from only cottage cheese and raw milk. Unfortunately, the *L. lactis* subsp. *cremoris* strains found in milk were lost because they failed to survive freezing.

Even more recently, Fenton (16) studied the role of farm machinery in harboring LAB that were present on grass. Sixteen different groupings or species of LAB were isolated from grass, but no *L. lactis* subsp. *cremoris* was found. *Pediococcus acidilactici* and *Streptococcus faecium* were the predominant organisms. *Lactococcus lactis* subsp. *cremoris* was isolated from two items of farm machinery, especially the forest harvester. Fourteen percent of the isolates were identified as *L. lactis* subsp. *cremoris* based on the their ability to grow at 10°C but not 45°C and their inability to produce ammonia from arginine. It is questionable whether or not that represents sufficient testing to identify an organism as *L. lactis subsp. cremoris*. In another study *L. lactis* subsp. *cremoris* was reported to have been isolated from frozen peas (7), but subsequent work (8) showed that these cultures had been incorrectly designated and were in fact unusual Group N streptococci with properties different from those of both *lactis* and *cremoris* subspecies. The natural habitat of subsp. *cremoris* thus remains unknown. More studies similar to that of Hirsch (25) are needed to understand its ecological relationship to other lactococci. Several scientists believe

that *L.* lactis *subsp. cremoris* may only be isolated from nature very infrequently (1, 25, 27, 35, 37, 39, 52, 72). Lawrence and co-workers (35) also emphasized the great need that exists for more strains of *L. lactis* subsp. *cremoris* for use in starter cultures. These authors further emphasized that most strains of *L. lactis* subsp. *cremoris* in use in starter cultures these days are related because they are descendants of strains that were originally isolated 70 years ago from cream in Denmark and the United States. Thus it is of utmost importance to isolate from nature new strains of this bacterium that are suitable for use in fermented milk products.

Economic importance.

LAB are of great economic importance to the dairy and other fermented food industries. Their application makes possible the manufacture of thousands of fermented foods, especially when used in mixed cultures with other types of bacteria, yeast and molds (49). They have been shown also to enhance the nutritional quality of certain foods. They are also beneficial in inhibiting pathogens (11) and spoilage bacteria in foods, food products, and animal feed (12, 13, 68). The importance of LAB in the health of newborn human infants is generally accepted (6, 74). In addition, much of research attention is now given to the possible usefulness of these organisms in intestinal health (50), reduction of blood cholesterol levels (20, 24), cancer prevention (3, 19), elevating immunocompetence (59), and antibiotic production (50, 57).

Dairy lactococci have been used for centuries in the production of fermented dairy products. The need to isolate *Lactococcus* starter culture strains has been emphasized by cheese makers, industry consultants, and research workers (51). Undesirable flavors encountered in cultured dairy products, insufficient development of acid during fermentation and frequent culture failures resulting from virus infection of existing strains are some factors which have contributed to this need for new strains of lactococci.

Over the past several decades, cultures of *L. lactis* subsp. *cremoris* were found to be the most suitable for producing high quality Cheddar cheese. The temperature tolerance, proteolytic, lypolytic, and acid producing properties of this organism allow its use to manufacture aged Cheddar cheese free of flavor and body defects (35, 64). The dairy industries in the United States, Australia and Canada have suffered heavy economic losses during the past decade, due to the occurrence of slit openness and fruity flavor in Cheddar cheese (63). Perry (42) reported the occurrence of fruity flavor in Cheddar cheese when certain strains of *L. lactis* subsp. *lactis* were used as starters in New Zealand. Since flavor and body defects result in a large economic loss to the dairy industry each year, the isolation and selection of starter cultures, especially *L. lactis* subsp. *cremoris*, is important.

Bacteriophage infection of starter cultures represents the most challenging problem to the cheese industry, as well as the greatest source of economic loss. There are no known cultures in use in the dairy industry which are "permanently" resistant to lysis by bacteriophage. Some LAB are lysed by several known races of

phage, while others are susceptible to only one race (51). The intensive use of the same cultures has increased the phage problem, and therefore, newly isolated cultures resistant to existing bacteriophages are in great demand.

Taxonomy and phylogenetic position of Lactic acid bacteria.

Lactic acid fermentation was already known to humans when they invented writing. However, it took several thousand years from these early applications before Louis Pasteur recognized, in 1857, the microbial nature of lactic fermentations, as described by Stackebrandt and Teuber (60). Fortuitously, the first bacterial pure culture (on earth) obtained by Joseph Lister in 1878, turned out to be *Bacterium lactis* which is now called *L. lactis* subsp. *lactis* (60).

In 1942, S. Orla Jensen (41) established a systematic order for LAB on the basis of morphological and cultural features, source of energy and nutritional needs, agglutination, and growth response toward different temperatures. In his classification, genera of LAB were placed in 3 groups. The first group included rod and sphere forms without catalase, which produced only traces of by-products in addition to lactic acid. *Thermobacterium*, *Streptobacterium*, and *Streptococcus* were members of this group. The second group included rod and sphere forms without catalase; detectable amounts of gas and other by-products in addition to lactic acid were produced. *Betabacterium* and *Betacoccus* belonged to this group. The third group included rod and sphere forms with catalase. *Microbacterium* and *Tetracoccus* belonged to this group.

With the development and application of modern biochemical and molecular methods, evidence was provided that the traditional identification scheme for LAB only partially correlated with their natural phylogenetic relationships (60). Schleifer (55) reported, based on extensive nucleic acid hybridization studies (29, 30, 54) and comparative oligonucletide cataloguing of 16S rRNA (36), catalase negative, facultatively anaerobic Gram positive cocci could be classified into 3 genetically distinct groups. The majority of streptococci including pyogenic (29), the 'mutans-like' (55), 'milleri-like' (31) and 'viridans' (54) streptococci were placed in the first group. The second group is composed of fecal streptococci and has been described as a new genus, *Enterococcus* (53, 9). The third group is formed by a few representatives of the lactic streptococci such as *S. lactis* and *S. raffinolactis* (30). DNA/23S rRNA hybridization and superoxide dismutase studies have shown that lactic streptococci form a group distinct from the pyogenic streptococci and enterococci (56). Nucleic acid hybridization studies and immunological relationships of superoxide dismutase showed that *Streptococcus lactis* (and its subspecies), *Lactobacillus* (*Lb.*) *xylosus*, *Lb. hordniae*, *S. gravieae*, *S. plantarum* and *S. raffinolactis* are closely related to each other but not to other streptococci. Therefore Schleifer and co-workers (56) proposed a new genus, *Lactococcus*, to accomodate the lactic or Group N species. Currently four species, *Lactococcus lactis*, *L. gravieae*, *L. plantarum*, and *L. raffinolactis*, are recognized. Similarity in lipoteichoic acid structures, lipid pattern, fatty acid and menaquinone composition also demonstrated the relatedness of these organisms (56).

Lactococcus picium was recently described as a new *Lactococcus* species (70). This was based on chemical and taxonomic studies performed on a representative strain of LAB of unknown taxonomic position isolated from Salmonid fish. However, not all Group N strains are members of the genus *Lactococcus*. Some motile *L. lactis*-like strains from chicken feces and river water (22, 23) which react with Group N antiserum, have been shown to be unrelated to lactococci (56). Of the many molecular properties tested (e.g, cell wall composition and structure, immunological relationships of lactic dehydrogenase and enzymes of the glycolytic pathway), DNA and ribosomal RNAs have been the molecules most useful in phylogeny (60, 62). Construction of meaningful phylogenetic trees is usually the outcome of the analysis of these nucleic acids. Such trees offer the opportunity to establish a phylogenetically comprehensive classification scheme for bacteria (60). Collins and co-workers (10) examined the phylogenetic status of members of the genus *Lactococcus* and similar motile strains which reacted with Group N antiserum using reverse transcriptase sequencing of 16S rRNA (RT sequencing). Their data clearly demonstrated that lactococci represent a distinct phylogenetic group equivalent in rank to the genera *Enterococcus* and *Streptococcus*. This was in agreement with earlier nucleic acid hybridization and immunological studies of superoxide dismutase (56). Within the genus *Lactococcus* it was evident that *L. plantarum* is closely related to *L. raffinolactis* whereas *L. lactis* shows a closer affinity with *L. gravieae* (13). Results of DNA-DNA and DNA-RNA hybridization studies (17, 56) are in agreement with these intrageneric relationships. Also, *Lactococcus*

and *Streptococcus* proved to be more closely related to each other than to *Enterococcus* (10). The motile Group N strains from chicken feces and river water, however, were found to be phylogenetically unrelated to lactococci but were closely related to members of the genus *Enterococcus*. Based on RT sequencing and DNA-rRNA hybridization, together with phenotypic criteria, it was proposed that these motile strains be classified in a new genus *Vagococcus* - *Vagococcus fluvialis* sp.nov. (10)

Nucleic acid sequencing and hybridization techniques.

The identification of microorganisms is essential in basic as well as applied research. The classification of organisms traditionally has been based on similarities in their morphological, physiological, and biochemical characteristics (5, 33, 40). It is now clear that classification based on these criteria does not necessarily correlate well with natural (i.e., evolutionary) relationships as defined by macromolecular sequence comparisons (33). Several molecular methods for evaluating phylogenetic relationships are available (e.g., genomic DNA/DNA and genomic DNA/rRNA hybridization, 5S rRNA and protein sequencing, 16S rRNA oligonucleotide cataloguing, enzymological patterning, etc.). All of these methods have advantages as well as limitations (40). Macromolecular sequencing seems preferred because it permits quantitative analysis of relationships (18, 33). Until recently 16S rRNA cataloguing was the most powerful technique for determining the phylogenetic relationships of microorganisms. Sequencing of 16S rRNA by reverse

transcriptase (RT-sequencing) has recently been introduced as an improved alternative to cataloguing (69). Of the macromolecules used for phylogenetic analysis, the ribosomal RNAs, particularly 16S rRNA, have proved the most useful for establishing distant relationships because of their universal distribution, high information content (their size is about 1,500 nucleotides), and conservative nature (18, 33, 40, 62). In recent years the use of rRNA sequences for identification and phylogenetic analyses has been generally accepted (39). RT sequencing now supersedes oligonucleotide cataloguing as the most rapid and powerful technique for determining the phylogenetic relationships of microorganisms (10, 69, 71). In contrast to generation and comparison of rather short oligonucleotides (e.g. 6-20 bases in length), this method produces long stretches of sequence (> 95% of the total sequence) which facilitates more precise phylogenetic determinations (66, 69).

Within the last decade, the application of nucleic acid sequencing techniques to microbial systematics has led to significant practical and theoretical advances. It is bringing a much-needed phylogenetic perspective into microbiology (40). There is no more fundamental and straightforward way to classify and relate organisms than by appropriate nucleic acid sequence comparisons (40). Recent chemical and molecular systematic studies, for example, have done much to clarify the phylogeny of 'lactic' or group N streptococci (10).

The 16S rRNAs vary in their nucleotide sequence but they also contain regions that are conserved among all organisms so far investigated (34, 40, 45, 71). These conserved sequences represent

broadly applicable initiation sites for primer elongation sequencing techniques. By analysing partial 16S rRNA sequences, it is possible to design specific probes directed against rRNA or the genes that encode them (rDNA). These probes can be designed specifically for different levels of phenotypic groups ranging from kingdom to species (5, 18, 45, 47, 62) and even to subspecies (48).

Diagnostic bacteriology is entering a new era marked by the application of gene probes in addition to other classical identification methods (2). Current methods of detecting microorganisms by nucleic acid hybridization with DNA probes have been used as rapid, sensitive, specific, and powerful diagnostic techniques for infectious diseases (18, 28, 32, 65). DNA probes based on highly variable rRNA regions have been applied successfully for the identification, detection, and quantification of microorganisms in soil, intestinal tract, and rumen (18, 28, 62). Nucleic acid hybridization probes have broad applications for detection of genetically altered microorganisms in the environment and the study of population structure and dynamics in microbial ecosystems (18). In general, hybridization probes used for microbial identification are highly specific synthetic oligonucleotides, or cloned genes from particular organisms, usually used after radioactive labeling (32).

DNA colony hybridization allows rapid and reliable identification of microorganisms directly on the primary plate without the need for classical identification protocols (26). The probes thus offer an alternative to traditional isolation and identification methods. Non-radioactive labeling of suitable probes

would make the application of this technology more convenient and routine in laboratory studies.

Because LAB have similar nutritional and growth requirements, it is very difficult to identify them by classical methods. With the development of such new genetic techniques, it is now possible to isolate *L. lactis* subsp. *cremoris*. Thus, the main objectives of this study were:

1. To isolate 16S rRNA from lactococci and study their nucleotide sequences in comparison to those of each other and other lactococci. The hypothesis being tested here is that there are specific sequences of ribosomal RNA that are conserved and unique to each of the organisms being studied.
2. To develop a replica plating technique for screening large numbers of bacterial colonies for isolates of *L. lactis* subsp. *cremoris*. The hypothesis being tested is that these organisms are present in nature and can be isolated, provided rapid screening methods are developed.
3. To extend the phenotypic characterization of the newly isolated strains, including screening for carbon source utilization and phage sensitivity.
4. To examine the newly isolated lactococcal strains to insure that they possess suitable acid-producing and flavor properties for their successful use in fermented milk product manufacture.

The following chapters describe our effort to achieve these objectives. Chapters 2, 3, 4, and 5 of this thesis have all been either published, sent for publication or are being prepared for publication.

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CHAPTER 2

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

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ABSTRACT

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 ribosomal RNA sequences that could be used to discriminate *L. lactis* subsp. *cremoris* from related subspecies. The 16S rRNAs from 13 *Lactococcus* strains were partially sequenced using reverse transcriptase in order to identify domains unique to *L. lactis* subsp. *cremoris*. All 5 strains of 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 an identical sequence 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 (*E. coli* 16S rRNA structural model; 1). Oligonucleotide probes specific for the genus *Lactococcus* (212RLa) and 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 subspecies *cremoris* hybridized to the subspecies-specific probe.

INTRODUCTION

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 using traditional microbiological approaches have not been fruitful (4, 17, 18), possibly because subsp. *cremoris* occurs naturally in very small numbers. Alternatively, the *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 subsp. *cremoris* genotype.

In recent years ribosomal RNA (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 (13). 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 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 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 species-specific rRNA probe for the lactococci.

MATERIALS AND METHODS

Organisms and growth conditions. Thirteen strains of lactococci were grown in litmus milk (0.75 gm litmus powder/L skim milk) and stored at -70°C in litmus milk containing 15% glycerol. The strains used in this study were *Lactococcus lactis* subsp. *lactis* ATCC 11955, ATCC 11454, 7962, C2, and f2d2, *L. lactis* subsp. *cremoris* BK5, 107/6, 205, P2, and HP, *L. lactis* subsp. *lactis biovar. diacetylactis* DRC-1, 18-16, and 26-2. 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. Active cultures were usually prepared in M-17 broth (22).

Extraction of RNA. Cells from five hundred ml of a log phase culture were harvested at 8000 x g for 15 min and resuspended in 15 ml ice cold STE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1.0 mM sodium ethylenediaminetetraacetic acid, [Na EDTA], pH 7.4). The suspension was passed twice through a French pressure cell at 20,000 psi to disrupt the cells. Cell debris was removed by centrifugation at 8000 x 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, w:v) extraction, and precipitation with $1/10^{th}$ volume of 2.0 M sodium acetate and 2.0 volumes of ethanol. The precipitated nucleic acid was collected by centrifugation at 13,000 x g for 10 min, washed with 70% ethanol and resuspended in TE buffer

(10 mM Tris-HCl, pH 7.4, 1.0 mM Na EDTA, 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* PCC 7437, *Myxosarcina* 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 microfuge 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 species-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. (10). Oligonucleotides were end-labeled with $\partial^{32}P$ according to the protocol of Saramella and colleagues (18). Labeled probes were purified on C18 reverse-phase

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

Primer*	Hits	*E. coli* No.	Sequence (5'to 3')
1406F#	negative control	1391-1406	TGYACACACCGCCCGT
1406R#	universal	1406-1392	ACGGGCGGTGTGTRC
519R#	universal	536-519	GWATTACCGCGGCKGCTG
343aR@	eubacteria	357-343	CTGCTGCCTCCCGTA
212RLa	lactococci	233-212	CTTTGAGTGATGCAATTGCATC
68RCa	*L. cremoris*	87-68	TGCAAGCACCAATCTTCATC

* R, Reverse; F, Forward

See reference 11.

@ Provided courtesy of C. Woese.

Sep-Pak columns (Millipore Corporation, Milford, Massachusetts) as described previously (10).

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. An S&S manifold apparatus (Schleicher & Schuell, Keene, NH 03431) was used to dot blot appropriate rRNA target molecules (50 ng) onto Nytran nylon membranes (0.45 mm; Schleicher & Schuell). The filters were dried in a vacuum oven at 80°C for 15-20 min, and then cross-linked by exposure to UV light (200 J/m^2). After this treatment, about 5-10 ml of prehybridization buffer (6x SSPE [1.08 M NaCl, 60 mM $NaPO_4$, and 60 mM EDTA, pH 7.5], 5x Denhardt's solution [0.1% Ficol, 0.1% polyvinylpyrolidone, and 0.1% BSA], and 0.1% SDS) were added to the blots in a microseal bag and prehybridization was carried out for 20-30 min at room temperature. The prehybridization buffer was then replaced with 3-5 ml of hybridization buffer (6x SSPE, 1x Denhardt's 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 3 times for 15-20 min at room temperature in 6x SSPE, 0.1% SDS, 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 using a Petroff Hausser counting chamber. A cell pellet was obtained by centrifugation at 800 rpm for 10-15 min. The pellet was suspended in 5 ml 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. Then the cells were washed twice in PBS, suspended in 5 ml of 145 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 5 ml of 100% ethanol while stirring on ice, and held at -20°C. Glass fiber filters (GFC Whatman No. 934-AH) were prepared for blotting by soaking in poly L-lysine (50 mg/ml in 10 mM Tris, pH 8), following which they were air dried and sprayed on the back with a thin layer of acrylic spray before being used for blotting. About 5 x 10^7 fixed cells were directly blotted on the pretreated GFC filters using the S&S 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 357-343 (here and throughout the manuscript we refer to nucleotide positions relative to the structural model of *E. coli* 16S rRNA; 1) was used to sequence the 5' region of the 16S rRNAs from the 13 *Lactococcus* strains. About 260-280 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 70-100. The 212RLa probe, which binds specifically to lactococcal 16S rRNAs at positions 212-233 (Fig. 1 or Table 1), enabled us to sequence through the remaining 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 98-68 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 300-320 nucleotides. Figure 1 illustrates a secondary structural model for the

Fig. 1. Secondary structure model for 5' region of lactic acid bacteria 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 species-specific and subspecies-specific probes. Numbering corresponds to the *Escherichia coli* 16S rRNA structural model (1).

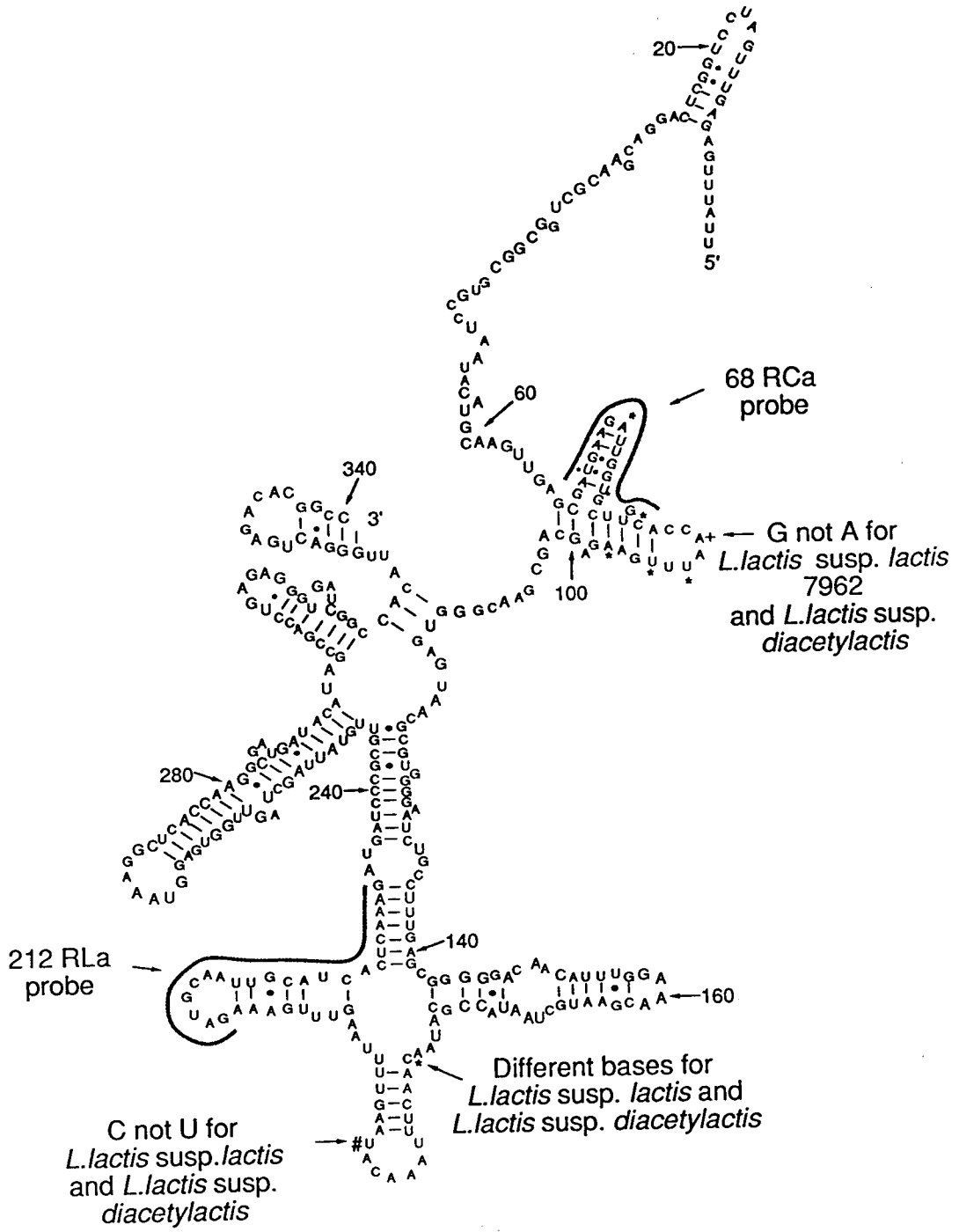

Figure 1 (Cont.)

5' domain of the *Lactococcus 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 Figure 2.

Construction of probes and hybridization experiments. Based on 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 species-specific probe for the lactococci. The sequences of both probes are indicated in Table 1. The species-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 the lactococci examined (Fig. 3). On the other hand, no cross reactivity was seen when the probe was tested against other eubacterial (*Dermocarpa* PCC 7437, *Myxosarcina* PCC 7312, and *Pseudomonas aeruginosa* strain IUCC SXI), archaebacterial (*Halobacterium volcanii*), and eukaryotic (*Strongylocentrotus 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 4-fold 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,*

Fig. 2. Nucleotide sequences of 5' regions of lactic acid bacteria 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.
Lc: *L. lactis* subsp. *cremoris*, Ll: *L. lactis* subsp. *lactis*, Ld: *L. lactis* subsp. *lactis* biovar. *diacetylactis*.

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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
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Figure 2 (Cont.)

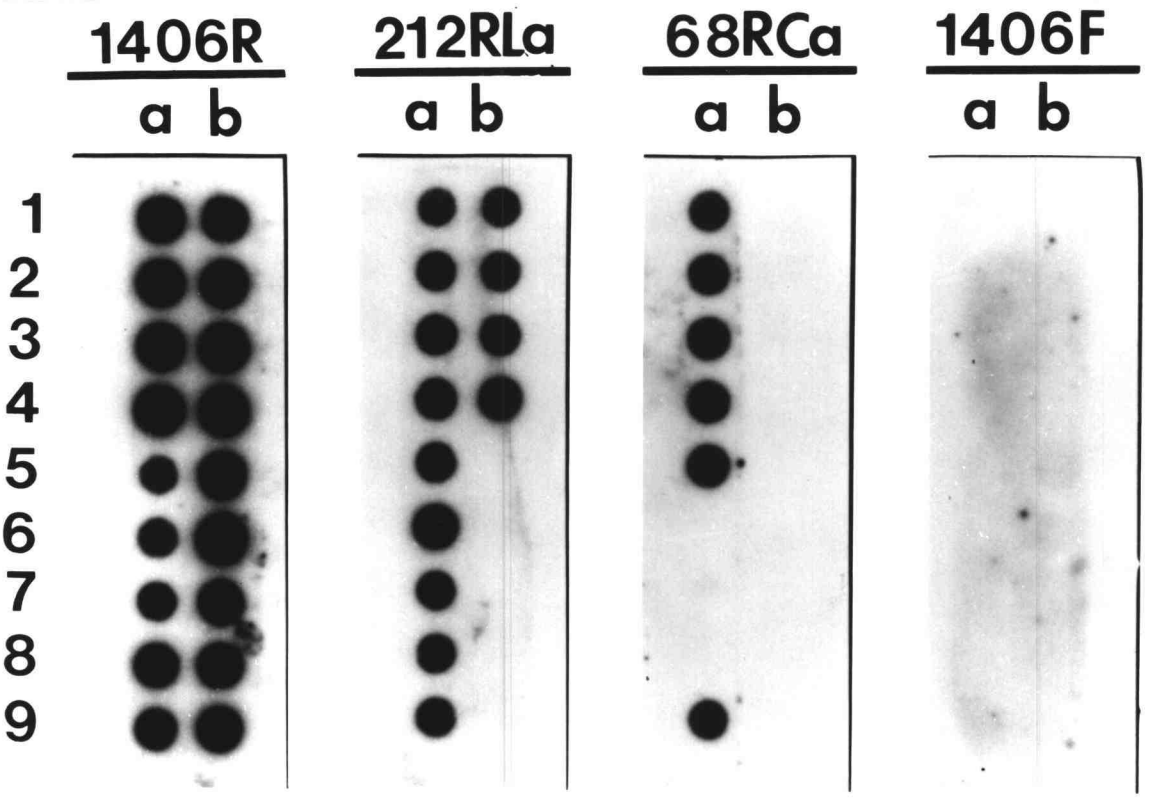

Fig. 3. Autoradiogram of a dot blot hybridization to bulk cellular RNAs from lactic acid bacteria and control strains. The universal (1406R), species-specific (212RLa), subspecies-specific (68RCa) and a negative control (1406F) probe were used. The order of the blotted RNAs is: 1a-5a (*L. lactis* subsp.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

Staphylococcus epidermidis, Salmonella pullorum, and *Bacillus subtilis*. The binding of the 1406R universal probe was used as a positive control for the presence of detectable target sequence. An oligonucleotide that is not complementary to the rRNA (1406F) served as a control for non-specific binding (Fig. 3 and 4).

The subspecies-specific probe (68RCa) was complementary to a 20-base pair 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 subsp. *cremoris* at the homologous positions. *L. lactis* subspecies *lactis* strain ATCC 11955 hybridized weakly to the 68RCa probe. This might be attributed to non-specific binding. The sequence of the 16S rRNA of this strain at the probe site has not yet been determined.

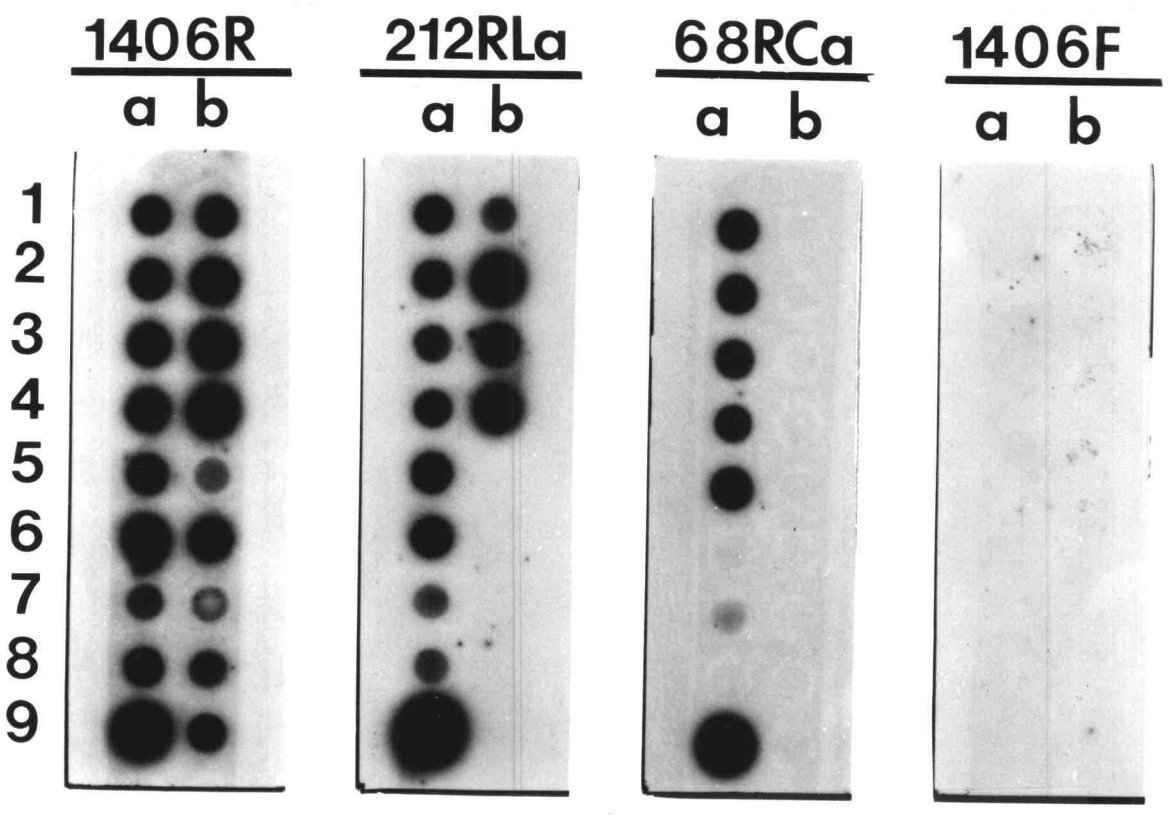

Fig. 4. Autoradiogram of a dot blot hybridization to fixed whole cells of lactic acid bacteria and control strains. The order of the blotted cells was same as in Fig. 2 for the lactic acid bacteria. The control strains, *Enterococcus pyogenes, Enterococcus faecalis, Staphylococcus epidermidis, Salmonella pullorum,* and *Bacillus subtilis,* were blotted in wells 5b-9b respectively. All control strains were obtained from the Department of Microbiology culture collection, Oregon State University. The number of cells was increased 4-fold for *L. lactis* subsp. *lactis* 7962 to give a signal approximately equivalent to the other strains.

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 *diacetylactis* biovar. strains studied, only the subsp. *lactis* C2 had the same nucleotide sequence as that of the subspecies *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 *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 et al. (7). This issue could be resolved in the

near future if we succeed in obtaining natural isolates of the *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). Ribosomal RNAs are attractive candidates as targets for hybridization probes due to 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 species-specific probe (212RLa) was unique to the lactococci, as indicated by comparisons to a data base of more than 200 known eubacterial 16S rRNA sequences. Furthermore, this was verified by the specific hybridization of the probe to all 13 lactic acid strains investigated, but none of the control organisms. A 3-base-pair mismatch in the oligonucleotide probe (68RCa) of 20 base pairs was sufficient to discriminate the subspecies *cremoris* from the closely related subspecies *lactis* and its *diacetylactis* biovar.

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

of 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.

ACKNOWLEDGEMENTS

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CHAPTER 3

Isolation of Lactococci From Nature by Colony Hybridization With Ribosomal RNA Probes

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ABSTRACT

Previously we described oligonucleotide probes based on unique sequence domains in 16S rRNAs which could be used to discriminate *L. lactis* subsp. *cremoris* from related strains. These probes were used in colony hybridizations to rapidly screen large numbers of colonies. Inocula from green plant surfaces and raw milk samples were enriched in skim milk and plated on PMP agar (19). Colonies were lifted from plates with poly-l-lysine coated glass micro fiber filters, treated with sodium dodecyl sulfate, and hybridized to four oligonucleotide probes: 68RCa, specific for *L. lactis* subsp. *cremoris*; 212RLa, specific for *L. lactis* species; 1406R, universal probe used as positive control; and 1406F, negative control probe. The method discriminated *Lactococcus lactis* subsp. *cremoris* from other closely related Gram positive organisms, such as *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar. *diacetylactis*, and *Enterococcus faecalis*, in mixed culture. The colony hybridization approach described here generally will be applicable to screening large numbers of colonies from natural environments for specific rRNA genotypes.

INTRODUCTION

Dairy lactococci have been used for centuries in the production of fermented dairy products. The need to isolate new *Lactococcus lactis* subsp. *cremoris* starter culture strains has been emphasized by cheese makers, industry consultants, and research workers (10, 15). Undesirable flavors encountered in cultured dairy products, insufficient development of acid during fermentation, and frequent culture failures resulting from virus infection are some factors which have contributed to the need for new strains of *Lactococcus lactis* subsp. *cremoris.*

The isolation of bacteria from nature traditionally has been dependent on the design of selective media and the screening of isolates using arrays of tests for phenotypic characteristics. Among the commercially significant subspecies of *Lactococcus lactis,* traditional methods for isolation and identification limit the number of new strains which can be evaluated. Various studies have emphasized the virtual impossibility of isolating new strains of *L. lactis* subsp. *cremoris* from nature with conventional approaches (7, 15, 16). Two alternative explanations for the failure to isolate new strains of *L. lactis* subsp. *cremoris* by these approaches are: 1) that *Lactococcus lactis* subsp. *cremoris* is very rare in nature, or 2) that these strains do not occur in nature at all but co-evolved in association with dairy practice.

In a previous study (13) we described a 16S rRNA-targeted species-specific probe (212RLa) for *L. lactis* and a subspecies-specific

probe (68RCa) for *L. lactis* subsp. *cremoris*. *Lactococcus* strains hybridized to these probes as predicted, with the exception of an atypical strain, *L. lactis* subsp. *lactis* C2, which had the same nucleotide sequence as *L. lactis* subsp. *cremoris* at the positions of the 68RCa probe target, and thus hybridized strongly. Since then, we have sought to develop a colony hybridization technique which would allow the direct identification of the *L. lactis* subsp. *cremoris* rRNA genotype in a mixed environmental population.

Here we describe a colony hybridization method which permits large numbers of isolates from natural environments to be screened efficiently. The method is analogous to approaches used routinely in molecular biology to screen libraries of molecular clones, and offers similar advantages to microbiologists seeking to identify rare cellular clones in large populations.

MATERIALS AND METHODS

Bacterial strains and environmental samples. The bacterial strains used as controls for colony hybridization experiments and whole cell dot blot hybridizations were obtained from the Oregon State University (OSU) culture collection. The non-starter lactococcal strains, *Lactococcus gravieae*, *Lactococcus plantarum*, and *Lactococcus raffinolactis*, were ATCC strains. *Lactococcus lactis* subsp. *hordniae* was obtained from Dr. R. E. Kunkee, College of Agricultural and Environmental Sciences, University of California, Davis. *Lactococcus piscium* was obtained from Dr. R. L. Holt, Microbiology Department, OSU. All strains were either patched on M17 agar plates (19) or grown in M17 broth at 30°C. Environmental enrichments were obtained from a plant growing on the OSU campus (*Prunus laurocerasus*) and a fresh corn sample from local produce. Weighed portions of collected material were added to stomacher bags (Lab-Blender 400, Tekmar company, Cincinnati, Ohio) containing 99 ml of sterile 11% skim milk and stomached for 1 min. The bags were then held at 21°C for 2 days to enrich for lactococci. Dilutions of the enrichments were spread on the surfaces of PMP agar plates, a modified formulation of M17 (19) using the insoluble trimagnesium phosphate instead of disodium-ß-glycerophosphate. Plates were incubated anaerobically at 21°C for 2 to 3 days. For the plant samples, several bright yellow colonies surrounded by halos of clearing contrasted against the purple medium were picked, streaked for purification, and patched on PMP

plates. Dilutions from the corn sample enrichment were directly spread onto large (150 X 15 mm) PMP agar plates and the plates were incubated anaerobically at 21°C for 2 to 3 days. The large plates allowed the use of lower dilutions of the sample with less crowded plates, thus facilitating return to those colonies needed for further investigation following the colony hybridizations. Colonies from the corn sample enrichment were lifted directly from PMP agar plates with glass microfibre filters, and screened by colony hybridization. After colony lifting, the plates were reincubated for an additional 2 days to allow regrowth.

RNA-DNA colony hybridization. Colonies were lifted from M17 or PMP agar plates onto glass microfibre filters (Whatman no. 934-AH). The glass fiber filters were previously soaked in poly-L-lysine (50 mg /ml in 10 mM Tris-HCl, pH 8), air dried and sprayed on the back with a thin layer of acrylic spray (Krylon 1301, Crystal Clear Acrylic) for mechanical support during subsequent treatments. Colony hybridizations were performed using a modified combination of the Anderson and McKay (2) and Kapperud (8) methods. In the modified procedure we describe, the glass fiber filters with the lifted colonies were placed, colony side up, on Whatman no. 1 paper soaked with 5% SDS and left for 3 to 5 min. The filters were then dried at 80°C in a vacuum oven for 10 to 15 min. After this treatment, 5-10 ml of prehybridization buffer (6x SSPE [1.08 M NaCl, 60 mM $NaPO_4$, and 60 mM EDTA, pH 7.5], 5x Denhardt's solution [0.1% Ficol, 0.1% polyvinylpyrolidone, and 0.1% BSA], and 0.1% SDS) were added to the filters in a Petri plate or HB-1 hybridizer (Techne Ltd., Duxford,

Cambridge CBZ 4PZ. U. K). Prehybridization was carried out for 45 to 60 min at 65°C. The prehybridization buffer was then replaced with 5-7 ml of hybridization buffer (6x SSPE, 1x Denhardt's solution, 0.1% SDS, and approximately 10^6 cpm of ^{32}P-labeled probe). The hybridization was carried out at room temperature overnight. Filters were washed 3 times for 20-30 min at room temperature in 6x SSPE, 0.1% SDS, then one time at the predetermined stringency temperature (45°C for 212RLa probe, 50°C for 68RCa probe and 37°C for both 1406R and 1406F probes). Filters were then exposed to X-ray film for 6 to 24 h.

Glass fiber filters with lifted colonies were hybridized three times to three different probes in the following order: 68RCa, 212RLa, 1406R. After each hybridization and X-ray film exposure, the filter was washed at 65°C for 4 to 5 h, then exposed to X-ray film to insure that all of the hybridized probe was washed off completely before performing the next hybridization.

Whole-cell dot blot hybridization. After regrowth of the lifted colonies on the PMP plates, those cells which hybridized to the 212RLa probe or both 68RCa and 212RLa probes were picked and streaked for purification on PMP plates. The pure colonies were grown in M17 broth for whole-cell dot blot hybridization to confirm their relatedness to lactococci. Whole cells were hybridized to oligonucleotide probes as described previously (13).

Phenotypic characterization. The environmental isolates which were proved to belong to the *Lactococcus* genus by genotypic

testing were further tested phenotypically for arginine hydrolysis and gas production from citrate utilization using the differential broth described by R. S. Reddy and co-workers (11), growth at pH 9.2, growth in 4% NaCl and growth at 40°C. These criteria are very important for distinguishing lactococci from other related microorganisms as well as differentiating between *L. lactis* subsp. *lactis* and subsp. *cremoris*.

RESULTS AND DISCUSSION

Different approaches were considered (2, 6, 8, 12) in attempts to render bacterial cells permeable to nucleic acid probes and to develop and optimize a method of rRNA-DNA colony hybridization. From the results obtained with modifications of the methods of Beitz and co-workers (3), Anderson and McKay (2) and Kapperud (8), we arrived at an approach which used glass fiber filters to detect colonies of Gram positive bacteria, particularly the dairy lactococci. The resolution of the autoradiograms with glass fiber filters was greater than with nitrocellulose, Nytran, or Zeta probe membranes using two different procedures for colony hybridizations (data not presented).

The results of a colony hybridization experiment using the method we developed are shown in Fig. 1. The cells were patched on PMP agar plates, incubated, and then lifted onto glass fiber filters as described earlier. Filters were hybridized to 4 different probes (Table 1). The 1406R, a universal probe complementary to a highly conserved region of 16S rRNAs, was used as positive control. All strains hybridized to this probe, indicating that the cells were efficiently lifted and permeabilized by the treatment (Fig. 1a). The 1406F, a universal probe complementary to the 1406R probe, was used as a negative control for non-specific binding (Fig. 1b). Hybridization of the 212RLa probe, specific for the *L. lactis* species, is shown in panel 1c. This probe hybridized to *L. lactis* subsp. *lactis*

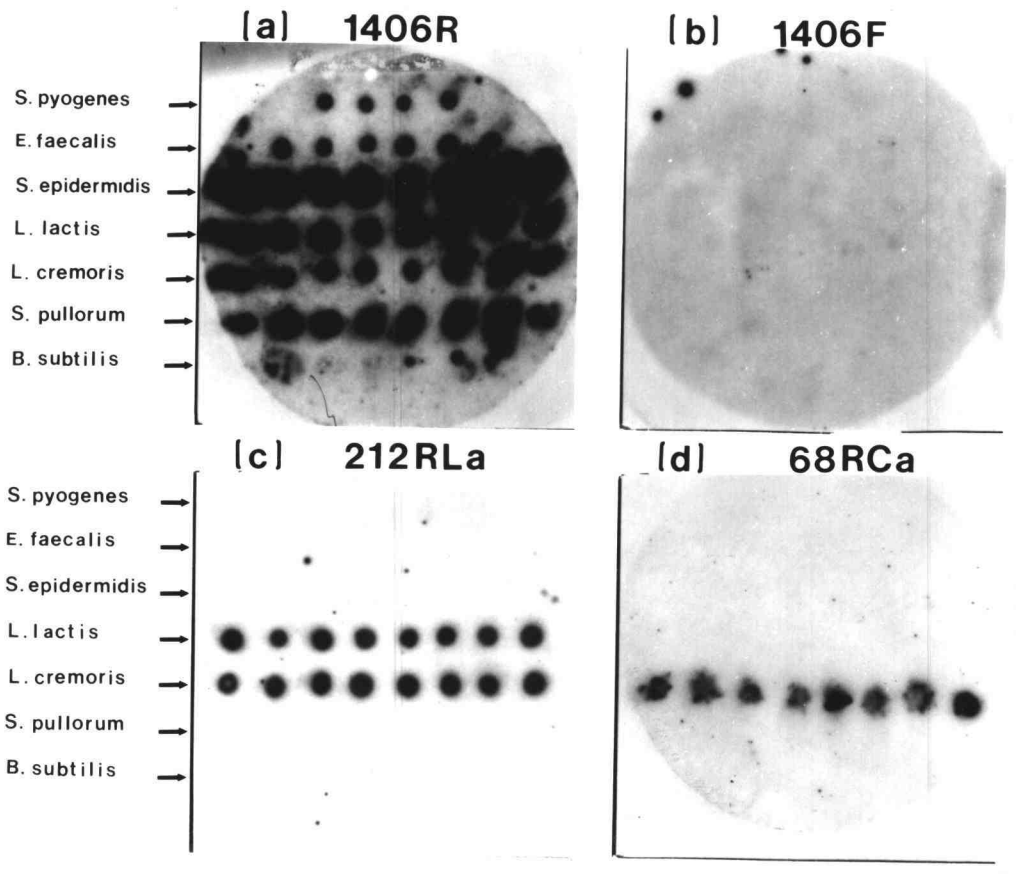

Fig. 1. Colony hybridization to bacteria on glass fiber filters. (a) Cells hybridized to the universal probe 1406R; (b) cells hybridized to the negative control 1406F; (c) cells hybridized to the species-specific probe 212RLa; (d) cells hybridized to the subsp.-specific probe 68RCa. The bacterial strains included: *Streptococcus pyogenes*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Salmonella pullorum*, and *Bacillus subtilis*. The dots in each row are replicates of one strain of the species indicated.

TABLE 1. List of oligonucleotides used.

Oligo.#	Target	Position*	Sequence
1406F	negative control	1391-1406	TGYACACACCGCCCGT
1406R	universal	1406-1392	ACGGGCGGTGTGTRC
212RLa	lactococci	233-212	CTTTGAGTGATGCAATTGCATC
68RCa	*L. cremoris*	87-68	TGCAAGCACCAATCTTCATC

R: Reverse
F: Forward
Oligonucleotide
* Position numbers refer to *E. coli* 16S rRNA, Brosius et. al. (5).

and *L. lactis* subsp. *cremoris*, but not to *Streptococcus pyogenes, Enterococcus faecalis, Staphylococcus epidermidis, Salmonella pullorum,* or *Bacillus subtilis*. The specificity of the 68RCa probe for the subsp. *cremoris* is illustrated in Fig. 1d. Of the known strains tested, only *L. lactis* subsp. *cremoris* hybridized to this probe.

To ascertain whether the probes were able to differentiate lactococci and subsp. *cremoris* from other strains occurring in natural populations, several environmental isolates from a plant sample enrichment (*Prunus laurocerasus*) were patched on PMP agar together with known *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains. After incubation, colonies were lifted onto glass fiber filters and hybridized to three probes: 1406R, 212RLa, and 68RCa. As documented in Fig. 2, the environmental isolates hybridized to the 1406R universal probe, as did *L. lactis* subsp. *lactis* and *L. lactis* subsp *cremoris*. (Fig. 2a). However, in this experiment the environmental isolates did not hybridize with either the 212RLa or 68RCa probes, while the subsp. *cremoris* showed specific binding to both probes and subsp. *lactis* hybridized to the 212RLa probe, but not to the 68RCa probe (Fig. 2b and 2c). This indicated that none of the environmental isolates from this sample were *L. lactis*. Further testing of these isolates supported this finding. Unlike lactococci, these isolates were able to grow at 45°C. Thus, the probes were able to discriminate between *L. lactis* and other organisms appearing in these enrichment cultures.

The first environmental sample harboring microbial flora that would hybridize to both probes (212RLa and 68RCa probes) was a fresh corn sample (Fig. 3). Thirty percent of the colonies which

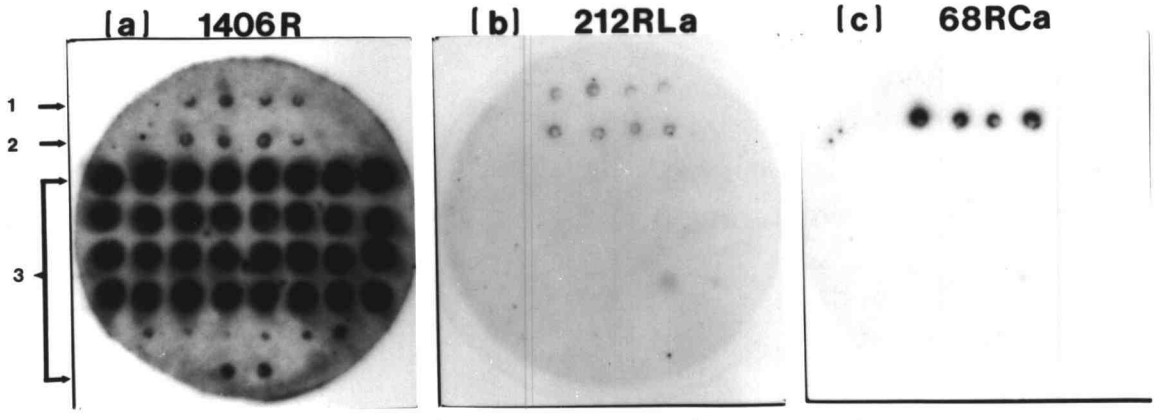

Fig. 2. Colony hybridization of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and unknown environmental isolates from a plant sample (*Prunus laurocerasus*). (a) Hybridization to the universal probe 1406R; (b) hybridization to the species-specific probe 212RLa; (c) hybridization to the subsp.-specific probe 68RCa. Lane 1, *L. lactis* subsp. *lactis* (the four dots represent one strain); lane 2, *L. lactis* subsp. *cremoris* (the four dots represent one strain); lanes labeled 3, environmental isolates (each dot represents a different isolate).

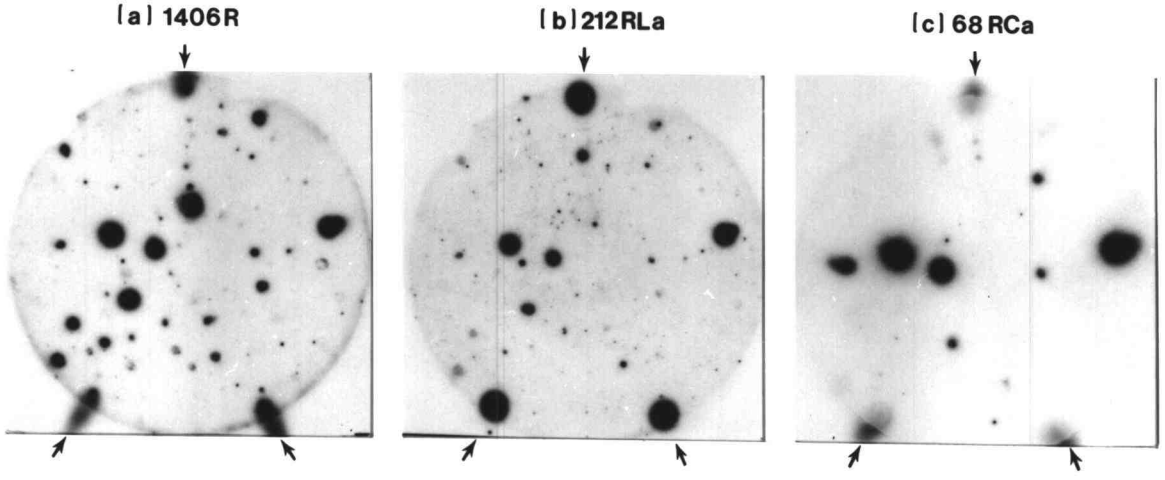

Fig. 3. Colony hybridization of environmental flora from a fresh corn sample. The sample was plated on a PMP agar plate after enrichment, incubated anaerobically, lifted onto a sterile glass fiber filter, and hybridized three times to three different probes. (a) Hybridization to the universal probe 1406R; (b) hybridization to the species-specific probe 212RLa; (c) hybridization to the subsp.-specific probe 68RCa. The glass fiber filter was washed at 65°C after each hybridization to prepare it to be hybridized to the next probe. Arrows indicate filter orientation marks.

hybridized to the universal 1406R probe (Fig. 3a), also hybridized to the 212RLa probe. Six (about 60%) of the colonies which hybridized to the 212RLa probe also hybridized to the 68RCa probe, indicating that these were lactic acid bacteria of the *L. lactis* subsp. *cremoris* rRNA genotype (Fig. 3c). All of the colonies (Co-1 through Co-6) which hybridized to the 68RCa probe were picked from the reincubated original agar plate, streaked for purification and confirmed for positive hybridization to this probe by means of whole-cell dot blot hybridization (13, Table 2). These strains had the phenotypic characteristics of *L. lactis* subsp. *lactis*. They were positive for arginine dihydrolase, growth at pH 9.2, growth in 4% NaCl, and growth at 40°C (Table 2). In this respect they resemble *L. lactis* subsp. *lactis* strain C2, which has the rRNA genotype of *L. lactis* subsp. *cremoris*, but resembles *L. lactis* subsp. *lactis* in phenotype (13). In a recent report (1), it was found that lithium ion inhibited the growth of *L. lactis* subsp. *cremoris* strains and *L. lactis* subsp. *lactis* strain C2, but not other *L. lactis* subsp. *lactis* strains. We have found that natural isolates from the corn sample which hybridized to the 68RCa probe were not sensitive to lithium inhibition. Further screening of environmental samples will be needed to fully understand the relationships among the subspecies of *L. lactis*.

While the *cremoris* and *lactis* subspecies are the only lactococci used as starters to manufacture fermented milk products, other lactococci are recognized (14). To gain further knowledge of the molecular systematics of all members of the *Lactococcus* genus, additional rRNA probing of these non-starter species was carried out. Our findings support the recent classification of the

TABLE 2. Genotypic and phenotypic characteristics of some of the environmental strains isolated from a fresh corn sample using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	#Hybridization to:		Source:	Growth at					Hydrolysis
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2	of Arginine
L. lactis	-	+	Laboratory strain	+	+	-	+	+	+
L. crem*	+	+	Laboratory strain	+	-	-	-	-	-
Co1	+	+	corn	+	+	-	+	+	+
Co2	+	+	corn	+	+	-	+	+	+
Co3	+	+	corn	+	+	-	+	+	+
Co4	+	+	corn	+	+	-	+	+	+
Co5	+	+	corn	+	+	-	+	+	+
Co6	+	+	corn	+	+	-	+	+	+

* *L. lactis subsp. cremoris*

Whole-cell dot blot hybridization

lactococci (17). When hybridized to the 68RCa probe, *L. lactis* subsp. *hordniae*, *L. gravieae*, *L. plantarum*, and *L. raffinolactis* (all non-starter lactococci) failed to hybridize to this *cremoris* subspecies-specific probe. However, only *L. lactis* subsp. *hordniae* was able to hybridize to the 212RLa probe which is specific to the *Lactococcus lactis* species. *Lactococcus piscium* was recently described as a new *Lactococcus* species (20). This organism did not hybridize to either the 68RCa or the 212RLa probes as expected.

We have described a colony hybridization approach which may provide a general method for the isolation of bacteria from natural environments using glass microfibre filters and ribosomal RNA probes. We have presented a specific use of this technique for the general identification and isolation of the *L. lactis* species from mixed cultures. The technique may also be applicable to the isolation of novel species from nature using rRNA genetic markers cloned from natural microbial populations (4, 9, 18).

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CHAPTER 4

Isolation of New Strains of *Lactococcus lactis* subspecies *cremoris*

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ABSTRACT

Previously (25) we described a colony hybridization method for isolation of bacteria from natural environments using rRNA probes. Here we report the successful use of the method in the isolation of dairy lactococci.

Thirty-eight plant species and vegetables, twenty-one individual raw milk and milk product samples from the United States, China, Morocco, and Yugoslavia, and twelve other samples from dairy farms were examined for lactic acid bacteria by the colony hybridization method. Two types of probes were used for hybridization, the 68RCa (specific for *L. lactis* subsp. *cremoris*) and 212RLa (specific for *L. lactis* species).

Lactococcus lactis subsp. *lactis* was found to occur on potato, cucumber, sweet peas, beans, cantaloupe, corn, cow's body and tail, colostrum, goat and cow raw milk, cottage cheese and cream. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* was isolated from cow raw milk obtained from Morocco and goat raw milk as well as cottage cheese from Yugoslavia. Many strains which hybridized to the 68RCa probe, and therefore suspected to be *L. lactis* subsp. *cremoris*, surprisingly were phenotypically like *L. lactis* subsp. *lactis*. These were isolated from fresh corn, Chinese raw milk, and Yugoslavian cottage cheese and cream. Some isolates, however, were typical of *L. lactis* subsp. *cremoris*.

The phenotypical, morphological, and physiological characteristics of the newly isolated lactococcal strains generally

agreed with the standard description for the genus. They were able to grow at 10°C and at 40°C, but not at 45°C; litmus milk was acidified and completely reduced before curding, no gas production was observed in the coagulated milk. However, some of the strains of the subspecies *lactis*, *cremoris*, and *L. lactis* subsp. *lactis* biovar. *diacetylactis* had a slightly different phenotype in regard to ability to grow at 40°C, in 4% NaCl, at pH 9.2, and ability to hydrolyze arginine.

Acid producing properties of the culture collection were variable. Many of the strains were fast acid producers, most were acceptable for flavor when grown in milk; and a few were with malty flavor which is considered a defect.

The isolation of *L. lactis* subsp. *lactis* from different plant sources confirmed that plants are a natural source of this bacterium. Strains of *L. lactis* subsp. *cremoris* were isolated from raw milk and cottage cheese from Morocco and Yugoslavia, but not from plants. This suggests that subspecies *cremoris* strains occur in association with dairy environments.

INTRODUCTION

Lactococcus bacteria are employed as starter cultures for a variety of industrial dairy fermentations (34). *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* are relied upon for acid production and subsp. *lactis* biovar. *diacetylactis* for flavor in cottage cheese, cultured sour cream and cultured buttermilk.

Lactococci occur in milk (27, 33). The literature provides evidence that the natural reservoir for the lactococci, especially *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis*, is green plant material. However, the natural habitat of *L. lactis* subsp. *cremoris* is not yet confirmed. In one study of 3000 isolates from 59 samples of commercial raw milk only 4% were *L. lactis subsp. cremoris* (17). In another study by Hirch (9), 2 of 35 raw milk isolates were *L. lactis* subsp. *cremoris*. Radich (23) reported isolation of three *L. lactis* subsp. *cremoris* strains from 31 producer raw milk samples. Unfortunately, most of the reported *cremoris* isolates were either lost because they failed to survive frozen storage or produced acid too slowly for usage in dairy industry. Later investigators, who had refined differential tests for the identification of the lactococci, failed in many cases to isolate the organisms from similar sources. Attempts to isolate *L. lactis* subsp. *cremoris* from plant material have been made by several investigators with no success (6, 22, 23, 36). *Lactococcus lactis* subsp. *cremoris* strains were reported to have been isolated from frozen peas (4, 5) but subsequent work (5) showed that these cultures had been incorrectly identified and were in fact unusual Group N streptococci with

properties different from those of both subsp. *lactis* and *cremoris*. The natural habitat of *L. lactis* subsp. *cremoris* thus remains unknown. Therefore studies of their distribution in nature and relationship to other lactococci are needed.

Several scientists have suggested that *L. lactis* subsp. *cremoris* may only be isolated from nature infrequently (1, 9, 13, 14, 15, 16, 18, 23, 29, 35, 36). Lawrence and co-workers (14) also emphasized the great need that exists for more strains of *L. lactis* subsp. *cremoris* for use as starter cultures. The limited number of strains of this subspecies available for the dairy industry have been used extensively, resulting in intensified problems with bacteriophage infections. In a previous study (25) we described a 16S rRNA-targeted species-specific probe (212RLa) for *L. lactis* and a subspecies-specific probe (68RCa) for *L. lactis* subsp. *cremoris*. *Lactococcus* strains hybridized to these probes as predicted, with the exception of an atypical strain, *L. lactis* subsp. *lactis* C2, which had the same nucleotide sequence as *L. lactis* subsp. *cremoris* at the positions of the 68RCa probe target, and thus hybridized strongly. In another study (26) we described a colony hybridization method which permits the direct identification of the *L. lactis* subsp. *cremoris* rRNA genotype in a mixed environmental population. This study was undertaken to: (1) isolate these bacteria from nature with newly developed screening methods, and (2) characterize the newly isolated lactococcal strains to determine if they possess suitable acid producing, flavor, and phage resistance properties for their potential use in fermented milk product manufacture.

MATERIALS AND METHODS

Collection and preparation of samples. Twenty three different plant species were examined for the presence of lactic acid bacteria as listed in Table 1. Plant materials were collected from trees, shrubs, grass, or weeds around the Oregon State University campus and the Gibson farm. The occurrence of lactococci on vegetables from local produce markets in the Corvallis area and other types of environmental samples from the Gibson farm located at Junction City, Oregon, also were studied. The various samples examined are listed in Table 2. Weighed portions of collected material were added to stomacher bags (Lab-Blender 400, Tekmar company, Cincinnati, Ohio) containing 99 ml of sterile 11% skim milk and stomached for 1 min. The bags were then held at 21°C for 2 days to enrich for lactococci. Dilutions of the enrichments were spread on the surfaces of PMP agar plates (32) and incubated anaerobically at 21°C for 2 to 3 days.

In addition, several raw milk samples obtained from China and Yugoslavia and several other milk products from Yugoslavia were tested; these are shown in Table 3. Cow raw milk samples and colostrum were procured from the Gibson and Bricker farms. These samples were incubated for 2 days at 21°C as an enrichment for lactococci. Several other cow and goat raw milk samples were obtained from China, Morocco and Yugoslavia. Other dairy product samples such as homemade hard cheese, cottage cheese, cream, and yogurt originated from Yugoslavia. Four of the milk samples

TABLE 1. The occurrence of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* on green plant material

Plant species	Occurence of: L. l*	L. c#
Lamium purpureum (red nettle)	+	-
Sonchus oleraceus (common sow-thistle)	+	-
Rubus discolor (Himalayan blackberry)	+	-
Solanum nigrum (Black nightshade)	+	-
Lolium perenne (perennial ryegrass)	-	-
Brassica campestris (field mustard)	-	-
Anthriscus scandicina (bur chervil)	-	-
Brassica sp. (mustard)	-	-
Hypericum calycinum (Gold flower)	-	-
Prunus laurocerasus (Cherry Laurel)	-	-
prunus sp. (Plum)	-	-
Hedera helix L. (English Ivy)	-	-
Acer platanoides L. (Green lace)	-	-
Ginkgo biloba L. (Maidenhair Tree)	-	-
Abelia X grandiflora (Rhed)	-	-
Spiraea sp. (Spiraea)	-	-
Cotinus cogygria Scop. (Smoke Tree)	-	-
Brassica oleracea L. (Cabbage)	-	-
Phalaris arundinacea L. (Reed Canary-grass)	-	-
Gramineae (Grass)	-	-
Compositae	-	-
Malva neglecta (Dwarf mallow)	-	-
Amaranthus powellii (Powell's Amaranth)	-	-

* *Lactococcus lactis* subsp. *lactis*.

\# *Lactococcus lactis* subsp. *cremoris*.

TABLE 2. The occurrence of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* on vegetables from local produce and other types of environmental samples from a dairy farm location and in raw milk samples from the United States.

Source: Vegetables	Occurence of: L. l*	L. c#	Source: raw milk & others	Occurence of: L. l	L. c
Potato	+	-	colostrum-1	+	-
Cucumber	+	-	colostrum-2	+	-
Sweet peas	+	-	raw milk (6)@	-	-
Beans	+	-	cow's tail-1	+	-
Cantilope	+	-	cow's tail-2	+	-
Corn	+	-	cow's body	+	-
Broccoli	+	-	cow's saliva	-	-
Squash	-	-	cow's feces	-	-
Parsley	-	-	Alfalfa feed	-	-
Mushroom	-	-	dry feed	-	-
Tomato	-	-	salt lick	-	-
Cauliflower	-	-	salt lick box	-	-
Red cabbage	-	-	drinking water	-	-
Asparagus	-	-			

* *Lactococcus lactis* subsp. *lactis*.

\# *Lactococcus lactis* subsp. *cremoris*.

@ Six samples tested.

obtained from China (CM-1 to CM-4) came from Behai Dairy Plant, Palian Liaoning Province. Each sample was hand-milked from ten different cows. The fifth Chinese milk sample (CM-5) was provided by Dr. Paul Kindstedt, University of Vermont, College of Agriculture, Department of Microbiology. This sample was commingled milk from a large storage tank at a processing dairy plant in Harbin, China. The milk sample from Morocco was provided by Dr. Noreddine Benkerroum, Institut Agronomique et Veterinaire Hassan II, Department de Microbiologie Alimentaire et de Biotechnologie, B.P 6202 Rabat Instituts, Morocco. The sample was obtained from a farmer in a local market. All Chinese and Moroccan milk samples were naturally enriched upon arrival from China and Morocco, and were screened for lactococci by colony hybridization without further incubation. Samples from Yugoslavia were collected during the period of October, 1991 through March, 1992. Samples were either obtained from private farms or bought from local markets. All samples originated from farms around Sarajevo, Bosnia except for two, the goat raw milk-2 and cream-13 which were obtained from Fojnica and Uzice respectively (Table 3). All samples were kept frozen whenever possible and were enriched upon arrival for lactococci as described earlier in the text for raw milk and other types of samples. After enrichment, samples were frozen again until tested. However, some samples were plated and few colonies were picked at random before the samples were frozen again for further study.

For colony lifts, dilutions from each of these samples were directly spread onto large (150 X 15 mm) PMP agar plates. The large

TABLE 3. The occurrence of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* in milk and milk products from Yugoslavia China and Morocco.

Source	Locality	Occurence of: L. l*	L. c#
Cottage cheese-9	Kiseljak-market	+	+
Cottage cheese-11	Ilijas-market	+	+
Cow'sraw milk-3	Barice	+	+
Cottage cheese-7	Kobilja Glava-farm	+	-
Cottage cheese-6	Gornja Vogosca-market	+	-
Cream (Uzice)-13	Uzice	+	-
Cow's raw milk-1	Zuc	+	-
Cows raw milk-4	Petrovici	+	-
Goats raw milk-2	Fojnica	+	-
Cottage cheese-4a	Vogosca-market	+	-
Cottage cheese-8	Pazaric-farm	+	-
Cottage cheese-10	Kiseljak-market	+	-
Cottage cheese-11	Ilijas	+	-
Cream-10a	Sjerogoste	-	-
Yogurt-6a	Nahorevo-farm	-	-
hard cheese-3a	Vrelo Bosne	-	-
Cottage cheese-12	Vogosca-market	-	-
China milk-1	Behai-China, dairy plant	+	+
China milk-2	Behai-China, dairy plant	+	-
China milk-3	Behai-China, dairy plant	+	-
China milk-4	Behai-China, dairy plant	+	+
China milk-5	Harbin-China, dairy plant	+	-
Morocco	Farmer, local market	+	+

* *Lactococcus lactis* subspecies *lactis*.
\# *Lactococcus lactis* subspecies *cremoris*.

plates allowed the use of lower dilutions of samples with less crowded plates, thus facilitating return to those colonies needed for further investigation following colony hybridizations. The PMP agar plates were incubated anaerobically at 21°C for 2 to 3 days prior to colony hybridization. After colony lifting, the plates were re-incubated for an additional 2 days to allow re-growth.

RNA-DNA colony hybridization. Colonies were lifted from M17 or PMP agar plates onto glass microfibre filters (Whatman no. 934-AH). The glass fiber filters were previously soaked in poly-L-lysine (50 mg /ml in 10 mM Tris-HCl, pH 8), air dried and sprayed on the back with a thin layer of acrylic spray (Krylon 1301, Crystal Clear Acrylic) for mechanical support during subsequent treatments. Colony hybridizations were performed using a method we developed earlier (26) which is a modified combination of the Anderson and McKay (2) and Kapperud (11) methods. In the modified procedure, the glass fiber filters with the lifted colonies were placed, colony side up, on Whatman no. 1 paper soaked with 5% SDS and left for 3 to 5 min. The filters were then dried at 80°C in a vacuum oven for 10 to 15 min. After this treatment, 5-10 ml of prehybridization buffer (6x SSPE [1.08 M NaCl, 60 mM $NaPO_4$, and 60 mM EDTA, pH 7.5], 5x Denhardt's solution [0.1% Ficol, 0.1% polyvinylpyrolidone, and 0.1% BSA], and 0.1% SDS) were added to the filters in a Petri plate or HB-1 hybridizer (Techne Ltd., Duxford, Cambridge CBZ 4PZ. U. K). Prehybridization was carried out for 45 to 60 min at 65°C. The prehybridization buffer was then replaced with 5-7 ml of hybridization buffer (6x SSPE, 1x Denhardt's solution, 0.1% SDS, and

approximately 10^6 cpm of ^{32}P-labeled probe). The hybridization was carried out at room temperature overnight. Filters were washed 3 times for 20-30 min at room temperature in 6x SSPE, 0.1% SDS, then one time at the predetermined stringency temperature (45°C for 212RLa probe, 50°C for 68RCa probe and 37°C for both 1406R and 1406F probes). Filters were then exposed to X-ray film for 6 to 24 h.

Glass fiber filters with lifted colonies were hybridized two times to two different probes in the following order: 68RCa, then 212RLa. After each hybridization and X-ray film exposure, the filter was washed at 65°C for 4 to 5 h, then exposed to X-ray film to insure that all of the hybridized probe was washed off completely before performing the next hybridization.

Whole-cell dot blot hybridization. After re-growth of the lifted colonies on the PMP plates, cells which hybridized to either 212RLA or 68RCa or both probes were picked and streaked for purification on PMP plates. The pure colonies were grown in M17 broth for whole-cell dot blot hybridization to confirm their relatedness to lactococci. Whole cells were hybridized to oligonucleotide probes as described previously (25).

Phenotypic and physiologic characterization. The environmental isolates which were suspected to belong to the *Lactococcus lactis* species by genotypic testing were further tested phenotypically for arginine hydrolysis and gas production from citrate using the differential broth described by Reddy and co-

workers (24), growth at pH 9.2, growth in 4% NaCl and growth at 40°C. Arginine hydrolysis was also tested by means of Niven's broth(19). Reactions to these tests distinguish lactococci from other related microorganisms as well as differentiate between *L. lactis subsp. lactis* and *L. lactis* subsp. *cremoris*. Litmus reduction before milk curding also is considered an important diagnostic aid for lactococci. All strains were inoculated into litmus milk and observed frequently until the time of curding. Also all strains were confirmed as Gram positive cocci.

Fast or slow acid production. About 120 isolates were chosen for further analysis. These were subcultured in 5 ml of sterile 11% nonfat milk. Cultures were incubated at 21°C until they clotted the milk. The coagulated cultures were then used as inoculum at the rate of one percent into a second tube of sterile milk. These tubes were incubated at 21°C for 16-18 hours. Cultures which coagulated milk within 16-18 hours were considered to be fast acid producing strains and those which did not do so were streaked on fast-slow differential agar (FSDA II) plates (21, 27), and incubated at 30°C for 48 hours to screen for fast cells. Fast Lac^+ $Prot^+$ cells formed yellow colonies against an opaque light-blue background. Lac^+ $Prot^-$ colonies were less brightly colored and resembled yellow doughnuts. Colonies of Lac^- $Prot^+$ and Lac^- $Prot^-$ were indistinguishable from each other but were clearly differentiated from the other two types; they appeared as colorless translucent colonies. Fast colonies were tested again for coagulating 11% milk

within 16-18 hours. Cultures which coagulated milk after more than 18 hours were considered slow strains.

Organoleptic and flavor evaluation. A one percent inoculum was added to 100 ml of sterile 11% nonfat milk in a Pyrex flask and incubated at 21°C for 18 hours. Samples of the coagulated milk culture were poured into drinking cups and flavor evaluations made noting in particular any bitter, malty, or salty flavors that might be present.

RESULTS AND DISCUSSION

Representative strains of the lactococci isolated from plants, vegetables, colostrum, and other types of environmental samples from the United States are listed in Table 4. From plant material and vegetables, only *L. lactis* subsp. *lactis* was isolated. Neither *L. lactis* subsp. *cremoris* nor *L. lactis* subsp. *lactis* biovar. *diacetylactis* was found on any of the plant species or vegetables examined. Colonies were lifted onto glass fiber filters and hybridized to two probes, 68RCa and 212RLa, as described in Materials and Methods. *Lactococcus lactis* subsp. *lactis* was found on *Lamium purpureum*, *Sonchus oleraceus*, *Rubus discolor*, and *Solanum nigrum* (Table 1 and 4). It was also found on sweet peas, potato, cucumber, beans, cantaloupe, broccoli, and corn (Table 2 and 4). Most of the colonies which hybridized to the 68RCa probe and some of the colonies which hybridized only to the 212RLa probe were selected from the original agar plate after reincubation, streaked for purification, and confirmed for positive hybridization to both probes by means of whole-cell dot blot hybridization (25). All of the colonies confirmed for positive hybridization to the probes were further tested phenotypically for *L. lactis* characteristics. Table 4 lists the genotypic and phenotypic characteristics of some of the environmental strains isolated from vegetables, plants, raw milk, colostrum, and other samples from a dairy farm using the colony hybridization method. According to our results, *L. lactis* subsp. *lactis* seems to occur only on a few plants. This finding has been substantiated by other workers

TABLE 4. Genotypic and phenotypic characteristics of some of the environmental strains isolated from vegetables, plants, raw milk, colostrum, and other samples from a dairy farm (U.S) using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	*Hybridization to:		Source:	Growth at :					Hydrolysis of Arginine	#no. of isolates
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2		
FB50	-	+	*L. purpureum*	+	+	-	+	+	+	5
FB55	-	+	*L. purpureum*	+	-	-	+	+	+	1
FB58	-	+	*S. oleraceus*	+	+	-	+	+	+	1
FB61	-	+	*R. discolor*	+	+	-	+	+	+	6
SPS1	-	+	Sweet peas	+	+	-	+	+	+	16
POT53	-	+	Potato	+	+	-	+	+	+	11
CUC80	-	+	Cucumber	+	+	-	+	+	+	4
COR105	-	+	Corn-2	+	+	-	+	+	+	9
BEN121	-	+	Beans	+	+	-	+	+	+	4
Col	+	+	corn-1	+	+	-	+	+	+	6
F2	-	+	cow's tail-1	+	+	-	+	-	+	14
F8	-	+	cow's tail-1	+	+	-	+	+	+	5
F11	-	+	cow's tail-1	+	-	-	+	-	+	4
FB11	-	+	cow's tail-2	+	+	-	+	+	+	18
FB19	-	+	cow's tail-2	+	-	-	+	+	+	4
FB33	-	+	cow's tail-2	+	-	-	+	-	+	1
FB34	-	+	cow's body	+	-	-	+	+	+	3
FB35	-	+	cow's body	+	+	-	+	+	+	13
F36	-	+	colostrum-1	+	+	-	+	+	+	1
FB1	-	+	colostrum-2	+	+	-	+	+	+	9
FB9	-	+	colostrum-2	+	-	-	+	+	+	1

*Whole-cell dot blot hybridization

#Number of isolates with the same genotypic and phenotypic characteristics.

such as Stark (30) who isolated *L. lactis* subsp. *lactis* from only six plant sources, by Pinter (22) who was able to find this organism only on three different plants, and by Radich (23) who reported only six vegetables to be a source of *L. lactis* subsp. *lactis*. Although we were able to find *L. lactis* subsp. *lactis* on the above mentioned plant species and vegetables, it was only isolated from *Lamium purpureum*, *Sonchus oleraceus*, *Rubus discolor*, sweet peas, potato, cucumber, beans, and corn. These isolated strains are represented in table 4 by FB50, FB55, FB58, FB61, SPS1, POT53, CUC8, COR105, and BEN121. *Lactococcus lactis* subsp. *lactis* was also isolated from potatoes, corn, cucumber, peas, beans, and cantaloupe by Radich (23). Radich failed to isolate *L. lactis* subsp. *cremoris* or *L. lactis* subsp. *lactis* biovar. *diacetylactis* from any of the vegetable or fruit samples he tested (23). All *L. lactis* subsp. *lactis* strains that we isolated from plant material and vegetables had the same phenotype except for one strain, FB55, isolated from *Lamium purpureum*, which failed to grow at 40°C (Table 4). Six strains of *L. lactis* subsp. *lactis* isolated from a fresh corn sample and represented by Co1 hybridized to both probes (68RCa and 212RLa). These strains had the phenotypic characteristics of *L. lactis* subsp. *lactis*. They were positive for arginine dihydrolase, growth at pH 9.2, growth in 4% NaCl and growth at 40°C (Table 4). In this respect they resemble *L. lactis* subsp. *lactis* strain C2 which has the rRNA genotype of *L. lactis* subsp. *cremoris* but resembles *L. lactis* subsp. *lactis* in phenotype(26).

Three types of *L. lactis* subsp. *lactis* phenotypes were isolated from a cow's tail, and another three types from a different cow's tail. These phenotypes are represented by strains F2, F8, F11, FB11, FB19,

and FB33 (Table 4). Two different *L. lactis* subsp. *lactis* phenotypes were isolated from a cow's body. These are represented by FB34 and FB35 (Table 4). Strains F8, FB11, and FB35 represent the same phenotype; strains FB19 and FB34 represent another similar phenotype. *Lactococcus. lactis* subsp. *lactis* was also isolated from two samples of colostrum. Only one phenotype was isolated from the first sample (colostrum-1). This is represented by strain F36 (Table 4). Strains FB1 and FB9 represent two *L. lactis* subsp. *lactis* phenotypes isolated from another colostrum sample (colostrum-2, Table 4).

Table 5 lists representative strains of confirmed lactococci isolated from the milk samples obtained from China. All samples contained *L. lactis* subsp. *lactis* strains as represented by CM1-1, CM2-63, CM3-90, CM4-7, and CM5-7 (Table 5). Other isolates were phenotypically similar to *L. lactis* subsp. *lactis,* but with minor differences. These are represented by CM1-31, 33, 39, 42; CM4-7; CM5-3, and CM5-4 (Table 5). Three of the five milk samples (CM1, CM2, and CM5) contained organisms of the *L. lactis* subsp. *lactis* C2 group, similar to those found previously in the corn sample (*L. lactis* subsp. *cremoris* genotype with *L. lactis* subsp. *lactis* phenotype, Table 3). These strains are represented by CM1-54, CM2-86, and CM5-5 (Table 5).

Isolates of *L. lactis* subsp. *cremoris* (CM1-3 and CM4-27; Table 3) were found in two of the five milk samples tested. RNA was extracted from these two strains and hybridized to both 212RLa and 68RCa probes by means of dot blot hybridization (25). Both strains showed specific hybridization to both probes (data not presented).

These results, together with the phenotypic characteristics presented in Table 5, suggest that both strains are *L. lactis* subsp. *cremoris*. However when re-tested for biochemical characteristics using the differential broth after storage at -80°C (24), these strains were both no longer negative for arginine dihydrolase. However, they remained negative when Niven's broth (19) was used for testing. Seasonal variations in phage sensitivity of some *L. lactis* subsp. *cremoris* starter cultures have been reported by Heap and Lawrence (8). Their explanation for these seasonal differences was the existence of variant cells within the individual strains which differed markedly in phage sensitivity. They suggested that the proportion of these variants may change upon subculturing. It is possible, therefore, that the two strains (CM1-3 and CM4-27; Table 3) were not exactly the same cultures when subcultured in different kinds of media such as Niven's or differential broth since variant cells of different types could have emerged in one medium but not the other.

TABLE 5. Genotypic and phenotypic characteristics of some of the environmental strains isolated from five Chinese raw milk samples using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	**Hybridization to: 68RCa	212RLa	Source:	Growth at : 10°C	40°C	45°C	NaCl 4%	pH 9.2	Hydrolysis of Arginine	#No. of isolates
CM1-1	-	+	China milk-1	+	+	-	+	+	+	33
CM1-3@	+	+	China milk-1	+	-	-	-	-	-	1
CM1-31	-	+	China milk-1	+	+	-	+	+/-	+/-	1
CM1-33	-	+	China milk-1	+	+	-	+	+	+/-	1
CM1-39	-	+	China milk-1	+	+	-	+	-	+	3
CM1-42	-	+	China milk-1	+	+	-	-	+	+	1
CM1-54	+	+	China milk-1	+	+	-	+	+	+	20
CM2-63	-	+	China milk-2	+	+	-	+	+	+	3
CM2-86	+	+	China milk-2	+	+	-	+	+	+	3
CM3-90	-	+	China milk-3	+	+	-	+	+	+	3
CM4-5	-	+	China milk-4	+	+	-	+	+	+	10
CM4-7	-	+	China milk-4	+	+	-	+/-	+	+	1
CM4-27*	+	+	China milk-4	+	-	-	+/-	-	-	1
CM5-3	+	+	China milk-5	+	+	-	+/-	+/-	+	1
CM5-4	+	+	China milk-5	+	+	-	+/-	-	+	1
CM5-5	+	+	China milk-5	+	+	-	+	+	+	1
CM5-6	+	+	China milk-5	+	+	-	-	-	+	1
CM5-7	-	+	China milk-5	+	+	-	+	+	+	5

**Whole-cell dot blot hybridization

#Number of isolates with the same genotypic and phenotypic characteristics.

@ Changed into arginine hydrolase and growth at 4% NaCl positive after storage at -80oC.

*Changed into arginine hydrolase and growth at 40°C, and at pH 9.2 positive after storage at -80°C.

Strain CM1-3 also became able to grow at 4% NaCl, and strain CM4-27 developed the capacity to grow at 40°C and at pH 9.2. Instances of variability in certain pure cultures of lactic acid bacteria have been described in the literature. Substrains of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* varying in their ability to attack maltose and sucrose were described by Sheman and Hussong as cited in Hunter (10). Orla Jensen (20) encountered variable characteristics for certain lactococci. Yawger and Sherman (36) reported the isolation of variants of *L. lactis* subsp. *lactis* from milk which did not ferment lactose. In a study done by Hunter (10) it was reported that variation was observed within some strains of *L. lactis* subsp. *cremoris*, especially with regard to acid production (or viability), response to high temperature conditions, ability to produce ropiness in milk, and degree of resistance to phage attack. Variation in acid production also has been observed for some of our environmental isolates of lactococcal strains.

For the first time in years, we recently succeeded in isolating strains of the *L. lactis* subsp.*cremoris* from two raw milk samples obtained from Morocco and Yugoslavia and from two cottage cheese samples originating in Yugoslavia. Tables 6 and 7 list representative strains of the confirmed lactococi isolated from the Moroccan and Yugoslavian samples. Genotypically and phenotypically, 20 of the strains isolated from the Moroccan milk (represented by MS-22, and MS-19) were *L. lactis* subsp. *lactis*, as indicated by their reaction with the 212RLa probe and phenotypic characteristics (Table 6). Four strains, represented by MS-19, produced gas from citrate

TABLE 6. Genotypic and phenotypic characteristics of some of the environmental strains isolated from one Morocco raw milk sample using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	*Hybridization to: 68RCa	212RLa	Source:	Growth at : 10°C	40°C	45°C	NaCl 4%	pH 9.2	Hydrolysis of Arginine	#no. of isolates
MS-5@	+	+	Morocco milk	+	-	-	-	-	-	24
MS-6	-	+	Morocco milk	+	-	-	+	-	NR	15
MS-21	-	+	Morocco milk	+	-	-	-	-	NR	2
MS-19	-	+	Morocco milk	+	+	-	+	+	+(G)	4
MS-22	-	+	Morocco milk	+	+	-	+	+	+	16
MS-46	-	+	Morocco milk	+	+	-	+	+	+(S)	1
MS-70	-	+	Morocco milk	+	-	-	+	+	+	1

* Whole-cell hybridization

\# Number of isolates with the same genotypic and phenotypic characteristics.

@ *L. lactis subsp. cremoris*

(G): Gas production from citrate

(S): Slow acid production

TABLE 7. Genotypic and phenotypic characteristics of some of the environmental strains isolated from several Yugoslavian raw milk and milk product samples using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	*Hybridization to:		Source:	Growth at :					Hydrolysis	#No. of
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2	of Arginine	isolates
K1	+	+	Cottage cheese-7	+	+	-	+	-	+	3
K2	+	+	Cottage cheese-7	+	+	-	+	-	+	1
A3	+	+	Cream (Uzice)-13	+	+	-	+	+	+	7
C8	+	+	Cream (Uzice)-13	+	+	-	+	-	+	2
112	+	+	Cottage cheese-11	+	+	-	+	-	-	1
1102	+	+	Cottage cheese-11	+	+	-	+	+	+	2
1117M	+	+	Cottage cheese-11	+	-	-	-	-	-	1
AM1	-	+	Cottage cheese-9	+	-	-	+/-	-	+	2
AM4	+	+	Cottage cheese-9	+	-	-	-	+/-	-	1
AM12	+	+	Cottage cheese-9	+	+	-	-	+/-	-	1
AM13	-	+	Cottage cheese-9	+	-	-	+	+	+	1
032	+	+	Raw milk-3	+	+	-	+	+	-	1
011	-	+	Raw milk-1	+	+	-	+	+	+	2
022	-	+	Goat raw milk-2	+	+	-	+	+	+	2
023	-	+	Goat raw milk-2	+	+	-	+	+	+(G)	1

* Whole- cell dot blot hybridization.

\# Number of isolates with the same genotypic and phenotypic characteristics.

(G): Gas production from citrate.

TABLE 7 (continued). Genotypic and phenotypic characteristics of some of the environmental strains isolated from several Yugoslavian raw milk and milk product samples using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are Gram positive cocci and acidify litmus milk before coagulation.

Isolate	*Hybridization to:		Source:	Growth at :					Hydrolysis	#No. of
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2	of Arginine	isolates
41	-	+	Raw milk-4	+	+	-	+	+	+	1
61	-	+	Cottage cheese-6	+	+	-	+	+	+	1
072	-	+	Cottage cheese-7	+	+	-	+	+	+	1
073	-	+	Cottage cheese-7	+	+	-	+	-	+(G)	1
82	-	+	Cottage cheese-8	+	+	-	-	+	+	1
83	-	+	Cottage cheese-8	+	+	-	+	+	+	1
91	-	+	Cottage cheese-9	+	+	-	+	+	+	1
92	-	+	Cottage cheese-9	+	+	-	+	+	+(G)	1
102	-	+	Cottage cheese-10	+	+	-	+	+	+	4
111	-	+	Cottage cheese-11	+	+	-	+	+	+	1
1105	-	+	Cottage cheese-11	+	-	-	+	-	+	1
1113	-	+	Cottage cheese-11	+	+	-	+/-	+/-	+	1
404	-	+	Cottage cheese-4a	+	+	-	+	-	+(G)	1
408	-	+	Cottage cheese-4a	+	+	-	+	+	+	1

* Whole- cell dot blot hybridization.

\# Number of isolates with the same genotypic and phenotypic characteristics.

(G): Gas production from citrate.

in differential broth (Table 6). Also, these four strains tested positive for acetylmethylcarbinol and diacetyl production when checked by the king's test (12) and the Voges-Proskauer test (7). These are characteristics of *L. lactis* subsp. *lactis* biovar. *diacetylactis*. Another 19 strains reacted with the 212RLa probe, proving their relation to the *Lactococcus* genus, but showed variability in their phenotypic characteristics These strains are represented by MS-6, 21, 46, and 70 (Table 4). Unlike the Chinese milk sample strains, none of the strains isolated from the Moroccan milk sample belonged to the C2 group. Twenty-four isolates, represented by MS-5, hybridized to both probes (212RLa and 68RCa), and had the phenotypic characteristics of *L. lactis* subsp. *cremoris*.

Out of the seventeen samples tested from Yugoslavia, thirteen tested positive for lactococci (Table 3). Cream-10a, yogurt-6a, hard cheese-3a, and cottage cheese-12 were the four samples that did not show any hybridization to any of our probes (68RCa, 212RLa). All samples from Yugoslavia which tested positive as lactococci contained *L. lactis* subsp. *lactis* with variable phenotypes as represented by AM1 and AM13 (cottage cheese-9); 011 (raw milk-1); 022 (goat raw milk-2); 41 (raw milk-4); 61 (cottage cheese-6), 072 (cottage cheese-7); 82 and 83 (cottage cheese-8); 91 (cottage cheese-9); 102 (cottage cheese-10); 111, 1105, and 1113 (cottage cheese-11); and 408 (cottage cheese-4a). Some samples contained organisms of the *L. lactis* subsp. *lactis* C2 group. These strains are represented by K1 and K2 (cottage cheese-7); A3 and C8 (cream, Kajmak-13), and 1102 (cottage-11). All of these strains are listed in Table 7. Four strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis*

(023, 073, 92, and 404) were found in goat raw milk-2, cottage cheese-7, cottage cheese-9, and cottage cheese-4a, respectively (Table 7). So *L. lactis* subsp. *lactis* biovar. *diacetylactis* was only isolated from raw milk and cottage cheese but not from plants. Other investigators have likewise found this to be the case. For example Radich (23) was able to isolate this organism only from individual producer milk samples and Swarthing (31) obtained his strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis* from raw milk or starter cultures. The only person who isolated this bacterium from plant material was Matuszewski as cited by Briggs (3). Isolates of the subspecies *cremoris* (112, 1117, and AM4) were found in cottage cheese-11, cottage cheese-9, and raw milk-3 (Table 7). King and Koburger (10) characterized Group N streptococci isolated from meats, vegetables, dairy products, barn-trough water, and poultry feed. No *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains were isolated and *L. lactis* subsp. *cremoris* was recovered from only cottage cheese and raw milk. Unfortunately, the *L. lactis* subsp. *cremoris* strains found in milk were lost because they failed to survive freezing.

Of 120 strains chosen for further testing, 64 were fast acid producers, either when tested directly for coagulating milk within 16-18 h at 21°C, or after being streaked on FSDA II. Almost all of these strains tested acceptable when evaluated for flavor (Table 8). Only two strains of *L. lactis* susp. *lactis* from the Moroccan milk (MS-22 and 70) tested malty, and another strain of *L. lactis* susp. *cremoris* from Yugoslavia tasted bitter. These findings suggest that some of these strains may be useful to the dairy product industry. These

TABLE 8. Flavor evaluation of some newly isolated lactic acid bacteria.

Isolate	flavor	pH
L. l C2*	clean, acid flavor	4.3
L. c 205#	clean, acid flavor	4.4
L. c 107/6#	flat-low acid	4.9
L. l Co1	clean, low acid flavor	4.6
L. l Co2	clean, acid flavor	4.5
L. l Co3	clean, acid flavor	4.5
L. l Co4	clean, acid flavor	4.5
L. l Co5	clean, low acid flavor	4.7
L. l Co6	clean, acid flavor	4.5
L. c MS3	clean, acid flavor	4.2
L. c MS5	clean, acid flavor	4.15
L. c MS7	clean, acid flavor	4.15
L. d MS19 (slow)	clean, low acid flavor	4.9
L. d MS38	clean, acid flavor	4.25
L. l MS22	malty	4.25
L. l MS41	clean, acid flavor	4.15
L. l MS46 (slow)	clean, not sour	4.5
L. l MS58	clean, acid flavor	4.2
L. l MS70	malty	4.25
L. l CM1-2	clean, acid flavor	4.4
L. l CM3-90	clean, acid flavor	4.4
L. l CM3-93 (slow)	clean, acid flavor	4.4
L. c CM4-27	clean, acid flavor	4.5
L. l CM5-6	clean, acid flavor	4.45
L. l CM5-7	clean, acid flavor	4.35

* Control *Lactococcus lactis* ssp. *lactis* strain C2

\# Control *Lactococcus lactis* ssp. *cremoris* strains 107/6 and 205

TABLE 8 (continued). Flavor evaluation of some newly isolated lactic acid bacteria.

Isolate	flavor	pH
L. l MS17	clean, acid flavor	4.4
L. c CM1-3	clean, acid flavor	4.5
L. l CM2-63 (slow)	clean, acid flavor	4.4
L. l CM5-8	clean, acid flavor	4.4
L. l CM1-54	clean, acid flavor	4.4
L. l FB1	clean, acid flavor	4.4
L. l FB10	clean, low acid flavor	4.6
L. l FB12	clean, acid flavor	4.5
L. l FB33	clean, acid flavor	4.3
L. l FB34	clean, acid flavor	4.4
L. l FB35	clean, acid flavor	4.5
L. l FB58	clean, acid flavor	4.6
L. l FB61	clean, low acid flavor	4.6
L. l FB62	clean, acid flavor	4.55
L. l BEN121	clean, acid flavor	4.35
L. l MS39	clean, acid flavor	4.4
L. l MS71 (slow)	clean, low acid flavor	4.7
L. l AM5	clean, acid flavor	4.4
L. c 112	clean, acid flavor	4.4
L. l 85	slightly different.	4.4
L. l 011	clean, acid flavor	4.2

* Control *Lactococcus lactis* ssp. *lactis* strain C2

\# Control *Lactococcus lactis* ssp. *cremoris* strains 107/6 and 205

new isolates, especially *L. lactis* subsp. *cremoris*, will provide a source of natural genetic variation which can be used to provide greater protection against phage infection in cheese plants.

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CHAPTER 5

Characterization of Novel Strains of *Lactococcus lactis* Using the Biolog Carbon Source Utilization System and Phage Typing

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ABSTRACT

Two types of probes (the 212RLa, specific for *Lactococcus lactis* species and the 68RCa, specific for *Lactococcus lactis* subspecies *cremoris*) were used for the isolation of lactococcal strains from natural environments. Thirty-two new strains of *Lactococcus lactis* subspecies *cremoris* were recovered from raw milk samples from Morocco and Yugoslavia and from home style cottage cheese obtained from Yugoslavia. Dairy and vegetation samples collected from the U.S.A as well as dairy samples from Morocco, China, and Yugoslavia have yielded 346 *L. lactis* subspecies *lactis* strains. Out of the 120 new strains tested for acid production 64 were fast acid producers Almost all of these strains were judged to be acceptable when evaluated for flavor. Also, the majority of the strains were resistant to a wide range of *Lactococcus* bacteriophages.

The biochemical and physiological characteristics of these environmental isolates and their resistance to many bacteriophages encountered by the dairy industry indicate the potential usefulness of these strains as dairy starter cultures for use in making fermented milk products.

INTRODUCTION

The isolation of new *Lactococcus* strains has been achieved by using oligonucleotide probes complementary to unique sequences in ribosomal RNAs, to rapidly screen a large number of colonies from green plant surfaces, enriched raw milk samples, and other types of environmental samples. The availability of these new strains, particularly *L. lactis* subsp. *cremoris*, is highly significant to the dairy industry. The anticipation that wild-type strains might be less susceptible to phage attack, and could have improved proteolytic and flavor properties for cheese making has enhanced demand for these strains within the dairy industry. Phage problems in particular have had a negative commercial impact. Practices such as phage typing and starter culture rotation, which circumvent some of the effects of phage attack, rely on the maintenance of genetically diverse starter culture strains.

It is evident that *L. lactis* strains with novel genetic and phenotypic properties are of great interest to the dairy industry. It is also evident that significant genetic diversity exists among subspecies of *L. lactis*. Natural phylogenetic relationships among bacterial strains are an important basis for classification and commercial use. In the past few years genetic criteria have provided significant insight into the natural relationships among lactococci and have led to important changes in their taxonomy and nomenclature. However, at present, there is no phenotypic or genotypic data base available that characterizes these strains sufficiently well to

distinguish among ecotypes. Information of this type is available for some bacterial species, though much is based on allozyme variability. This investigation was conducted to study the nature of wild strains of *L. lactis* and provide genetic and phenotypic bases for identifying lactococci.

Recently, a technique to identify gram-negative or gram-positive bacteria on the basis of metabolic fingerprinting was developed by Biolog Inc. (Hayward, Calif.) (2, 3). Several Scientists have applied this system for microbiological studies (1, 4, 5, 6,). Trial tests with strains sent to Biolog indicated that *Lactococcus* strains could effectively reduce the indicator dyes used by this system, permitting the rapid screening of strains for utilization of 95 carbon sources. We consider the ability to acquire this extensive biochemical information to be an important and functional adjunct to genetic measures of relatedness.

MATERIALS AND METHODS

Bacterial strains and environmental samples. Strains used for this study are listed in Tables 1 and 2. The bacterial strains used as controls for Biolog carbon source utilization system tests (*L. lactis* subsp. *cremoris* BK5, *Lactococcus lactis* subsp. *lactis* f2d2 and C2, and *Lactococcus lactis* subsp. *lactis*. biovar *diacetylactis* 26-2.) were obtained from Oregon State University (OSU) culture collection. The environmental strains were isolated from various sources by means of a colony hybridization method (8) described in Chapter 3 . These strains were verified by phenotypic tests as lactococci. All strains were streaked on PMP medium (9) and incubated at 30°C for 24h. One colony of each strain was subcultured on M17 agar plates (9) for Biolog analysis.

Metabolic characterization of *Lactococcus* strains. The aerobic utilization of 95 carbon sources in the presence of a redox indicator (tetrazolium violet) was determined for the individual environmental isolates and reference strains using GP microtiter plates (Biolog, Inc., Hyward, Calif.). Although the manufacturer's intended application for these plates is the identification of bacteria, they were used in this study to obtain a metabolic profile for a group of environmental lactococcal strains as a measure of their relatedness. Cell suspensions of the environmental and control strains of lactococci in saline (0.85%) were used to inoculate the Biolog GP microplates (15µl per well). The optical densities of the

TABLE 1. Environmental strains isolated from different sources using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. These strains were chosen for biolog testing. Strains with a * designation coagulated milk within 16-18 h.

Isolate	Hybridization to: 68RCa	212RLa	Source	Growth at 10°C	40°C	45°C	NaCl 4%	pH 9.2	Hydrolysis of Arginine
Co1	+	+	corn	+	+	-	+	+	+
*Co3	+	+	corn	+	+	-	+	+	+
F2	-	+	cow's tail	+	+	-	+	-	+
*FB1	-	+	colostrum	+	+	-	+	+	+
*FB10	-	+	colostrum	+	+	-	+	+	+
*FB12	-	+	cow's tail	+	+	-	+	+	+
*FB33	-	+	cow's tail	+	-	-	+	-	+
*FB34	-	+	cow's body	+	-	-	+	+	+
FB51	-	+	*Lamium puppureum*	+	+	-	+	+	+
*FB58	-	+	*Sonchus oleraceus*	+	+	-	+	+	+
FB61	-	+	*Rubus discolor*	+	+	-	+	+	+
*BEN121	-	+	Beans	+	+	-	+	+	+
*CMP1-3	+	+	China milk-1	+	-	-	+	-	+
*FB62	-	+	*Rubus discolor*	+	+	-	+	+	+
CM1-39	-	+	China milk-1	+	+	-	+	-	+
CM1-42	-	+	China milk-1	+	+	-	-	+	+
CM1-54	+	+	China milk-1	+	+	-	+	+	+
CM2-63	-	+	China milk-2	+	+	-	+	+	+
CM2-64	-	+	China milk-2	+	+	-	+	+	+
*CM2-87	+	+	China milk-2	+	+	-	+	+	+
*CM3-90	-	+	China milk-3	+	+	-	+	+	+

TABLE 1 (continued). Environmental strains isolated from different sources using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. These strains were chosen for biolog testing. Strains with a * designation coagulated milk within 16-18h.

Isolate	Hybridization to: 68RCa	212RLa	Source	Growth at 10°C	40°C	45°C	NaCl 4%	pH 9.2	Hydrolysis of Arginine
CM5-4	-	+	China milk-4	+	+	-	+/-	-	+
*CM4-27	+	+	China milk-4	+	-	-	+/-	-	-
*CM5-6	+	+	China milk-5	+	+	-	-	-	+
CM5-7	-	+	China milk-5	+	+	-	+	+	+
*CM5-8	-	+	China milk-5	+	+	-	+	+	+
*MS-17	+	+	Morocco milk	+	-	-	-	-	-
*MS-39	-	+	Morocco milk	+	+	-	+	+	+(G)
*MS-22	-	+	Morocco milk	+	+	-	+	+	+
*MS-41	-	+	Morocco milk	+	+	-	+	+	+
MS-44	+	+	Morocco milk	+	-	-	-	-	-
MS-46	-	+	Morocco milk	+	+	-	+	+	+
MS-52	+	+	Morocco milk	+	-	-	-	-	-
MS-70	-	+	Morocco milk	+	-	-	+	+	+
MS-71	-	+	Morocco milk	+	+	-	+	+	+(G)
K2	+	+	Cottage cheese-7	+	+	-	+	-	+
K9	+	+	Cottage cheese-7	+	+	-	+	+	+
A3	+	+	Cream (Uzice)-13	+	+	-	+	+	+
C8	+	+	Cream (Uzice)-13	+	+	-	+	-	+
M1	+	+	Cream (Uzice)-13	+	+	-	+	+	+
1102	+	+	Cottage cheese-11	+	+	-	+	+	+

TABLE 1 (continued). Environmental strains isolated from different sources using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. These strains were chosen for biolog testing. Strains with a * designation coagulated milk within 16-18 h.

Isolate	Hybridization to: 68RCa	212RLa	Source	Growth at 10°C	40°C	45°C	NaCl 4%	pH 9.2	Hydrolysis of Arginine
1117M	+	+	Cottage cheese-11	+	-	-	-	-	-
AM4	+	+	Cottage cheese-9	+	-	-	-	+/-	-
AM5	-	+	Cottage cheese-9	+	-	-	-	-	-
AM12	+	+	Cottage cheese-9	+	+	-	-	+/-	-
032	-	+	Raw milk-3	+	+	-	+	+	-
*011	-	+	Raw milk-1	+	+	-	+	+	+
*022	-	+	Goat's raw milk-2	+	+	-	+	+	+
*023	-	+	Goat's raw milk-2	+	+	-	+	+	+(G)
41	-	+	Raw milk-4	+	+	-	+	+	+
*61	-	+	Cottage cheese-6	+	+	-	+	+	+
073	-	+	Cottage cheese-7	+	+	-	+	-	+(G)
*82	-	+	Cottage cheese-8	+	+	-	-	+	+
*83	-	+	Cottage cheese-8	+	+	-	+	+	+
91	-	+	Cottage cheese-9	+	+	-	+	+	+
92	-	+	Cottage cheese-9	+	+	-	+	+	+(G)
102	-	+	Cottage cheese-10	+	+	-	+	+	+
*111	-	+	Cottage cheese	+	+	-	+	+	+
1105	-	+	Cottage cheese	+	-	-	+	-	+(G)
404	-	+	Cottage cheese-4a	+	+	-	+	-	+
408	-	+	Cottage cheese-4a	+	+	-	+	+	+

TABLE 2. Environmental strains isolated from different sources using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are fast acid producers and were chosen for phage testing.

Isolate	Hybridization to:		Source	Growth at					Hydrolysis of Arginine
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2	
Co3	+	+	corn	+	+	-	+	+	+
FB1	-	+	colostrum	+	+	-	+	+	+
FB12	-	+	cow's tail	+	+	-	+	+	+
FB62	-	+	*Rubus discolor*	+	+	-	+	+	+
BEN121	-	+	Beans	+	+	-	+	+	+
CM1-3	+	+	China milk-1	+	-	-	+	-	+
CM4-27	+	+	China milk-4	+	+	-	-	+	+
CM5-6	+	+	China milk-5	+	+	-	-	-	+
MS-5	+	+	Morocco milk	+	-	-	-	-	-
MS-11	+	+	Morocco milk	+	-	-	-	-	-
MS-17	+	+	Morocco milk	+	-	-	-	-	-
MS-22	-	+	Morocco milk	+	+	-	+	+	+
MS-39	-	+	Morocco milk	+	+	-	+	+	+(G)

(G): Gas production from citrate

TABLE 2 (continued). Environmental strains isolated from different sources using the colony hybridization procedure with ^{32}P-labeled 16S rRNA probes. All strains are fast acid producers and were chosen for phage testing.

Isolate	Hybridization to:		Source	Growth at					Hydrolysis
	68RCa	212RLa		10°C	40°C	45°C	NaCl 4%	pH 9.2	of Arginine
MS-52	+	+	Morocco milk	+	-	-	-	-	-
MS-70	-	+	Morocco milk	+	-	-	+	+	+
AM4	+	+	Cottage cheese-9	+	-	-	-	+/-	-
011	-	+	Raw milk-1	+	+	-	+	+	+
022	-	+	Goat's raw milk-2	+	+	-	+	+	+
023	-	+	Goat's raw milk-2	+	+	-	+	+	+(G)
61	-	+	Cottage cheese-6	+	+	-	+	+	+
82	-	+	Cottage cheese-8	+	+	-	-	+	+
83	-	+	Cottage cheese-8	+	+	-	+	+	+
111	-	+	Cottage cheese-11	+	+	-	+	+	+
408	-	+	Cottage cheese-4a	+	+	-	+	+	+

(G): gas production from citrate

cell suspensions were 0.4 +/- 0.05 at A=600 nm. A computer controlled Thermo Max Microplate Reader (Molecular Devices) (λ= 590 nm) was used to measure the reduced form of tetrazolium dye. The results were stored automatically in a computer file to be evaluated for metabolic profiles. Data compiled from the analysis of the metabolic fingerprint were evaluated with microlog ML2 software, which performs a cluster analysis based on principles of numerical taxonomy.

Phage typing. The 23 environmental isolates listed in Table 2 were tested for sensitivity to a variety of phages from three industry phage banks. These tests were performed by The Bioproducts Group of Quest International, Sarasota, Florida, The Cultsure Division of Galloway West Company, Millville, Utah and Marschal Division of Rhone-Poulenc, Madison, Wisconsin. Each company maintains independent phage banks isolated from whey samples obtained from commercial Cheddar cheese making factories. Quest International and Galloway West followed the following procedure: Milk cultures of the strains were prepared. Cultures were then transferred to M17 lactose broth and after overnight growth at 32°C were tested with phages. The individual isolates were overlaid on M17 lactose agar plates as M17 semi-solid agar infusion. The semi-solid agar contained 0.2% $CaCl_2$ (9). Different phage preparations were spotted on the overlaid plates with a multiple inoculation device. Plates were incubated overnight and examined for clearing. Phage samples used by Quest International were: Composite Quest phage Lab sample 1990-1992, three fresh buttermilk whey samples 1993, and

composite cheese plant phages from Canada (7). Galloway West Co. used 32 phage preparations from their own bank of phages. Marschall Products performed a different and more complete phage testing. A total of 72 positive whey samples for phage, representing one month of testing (whey samples 1-72), and 30 negative whey samples, representing two weeks, were tested (whey samples 73-102), along with 38 of single plaque isolates (SPI 1-38) from their phage bank. The cells were grown in M17 broth overnight at 32°C. Put into 7 ml overlay tubes of M17 top agar were 0.1 ml of the overnight culture and 50 µl of 1.0 M $CaCl_2$. The tubes were then gently vortexed and poured onto large, square, grid plates (100 mm^2) of M17 bottom agar. The plates were allowed to dry. The whey samples were spun down and the supernatant was transferred to a sterile Eppendorf tube. The purified phage was filter sterilized. Spots were made on the dry plates with 3 µl of either the whey sample supernatant or the purified phage stock. Plates were allowed to absorb the spot. They were then incubated at 37°C for one hour followed by 32°C ovrnight. The plates were read at approximately 24 hours. The phage test reading was based on a scale of 0 (no lysis) to 3 (total lysis) based on spot test evaluation. Only those strains with readings of 1 or greater were reported.

RESULTS AND DISCUSSION

Metabolic characterization of *Lactococcus* strains. A total of 61 environmental strains (Table 1) and 4 reference strains were tested for carbon-source utilization. Carbon sources utilized by all of the lactococcal strains tested were N-acetyl mannosamine, D-fructose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannitol, and D-mannose. Carbon sources utilized by none of the lactococci tested were inulin, arabitol, L-fucose, m-inositol, D-mannitol, D-melibiose, D-raffinose, L-mamnose, sedoheptulosan, stachyoseacetic acid, xylitol, ß-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketovaleric acid, propunic acid, succinamic acid, succinic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putresiene, thymidine-5'-monophosphate, and D-L-a-glycerol phosphate. The following sugars were utilized only by a limited number of lactococcal strains. Mannan was only utilized by strain CM-5-4, D-melezitose was utilized only by strain 61, methyl succinate was utilized only by strain 073, N-acetyl L-glutamic acid was utilized only by MS71, 2,3-butanediol was only utilized by strain K9, glucose-1-phosphate and glucose-6-phosphate were utilized only by strain 1102, D-ribose was utilized by only strains BEN 121 and CM5-7, D-sorbitol was utilized only by strains BEN121 and 61, tween 80 was utilized only by strains FB62, CM1-2, and CM5-4, α-ketovaleric acid was utilized only by strains FB62, CM2-87, and CM2-

Fig. 1. Dendogram Showing relationship between lactococcal strains as recovered from the evaluation of carbon-source utilization using the Biolog GP microtiter plate test system. L. c: *Lactococcus lactis* subsp. *cremoris*. L. l: *Lactococcus lactis* subsp. *lactis*. L. d: *Lactococcus lactis* subsp. *lactis*. biovar. *diacetylactis*. L. l*: *Lactococcus lactis* subsp. *lactis*. which hybridized to the 68RCa probe (specific for *Lactococcus lactis* subsp. *cremoris*). I, II, and III: cluster groups.

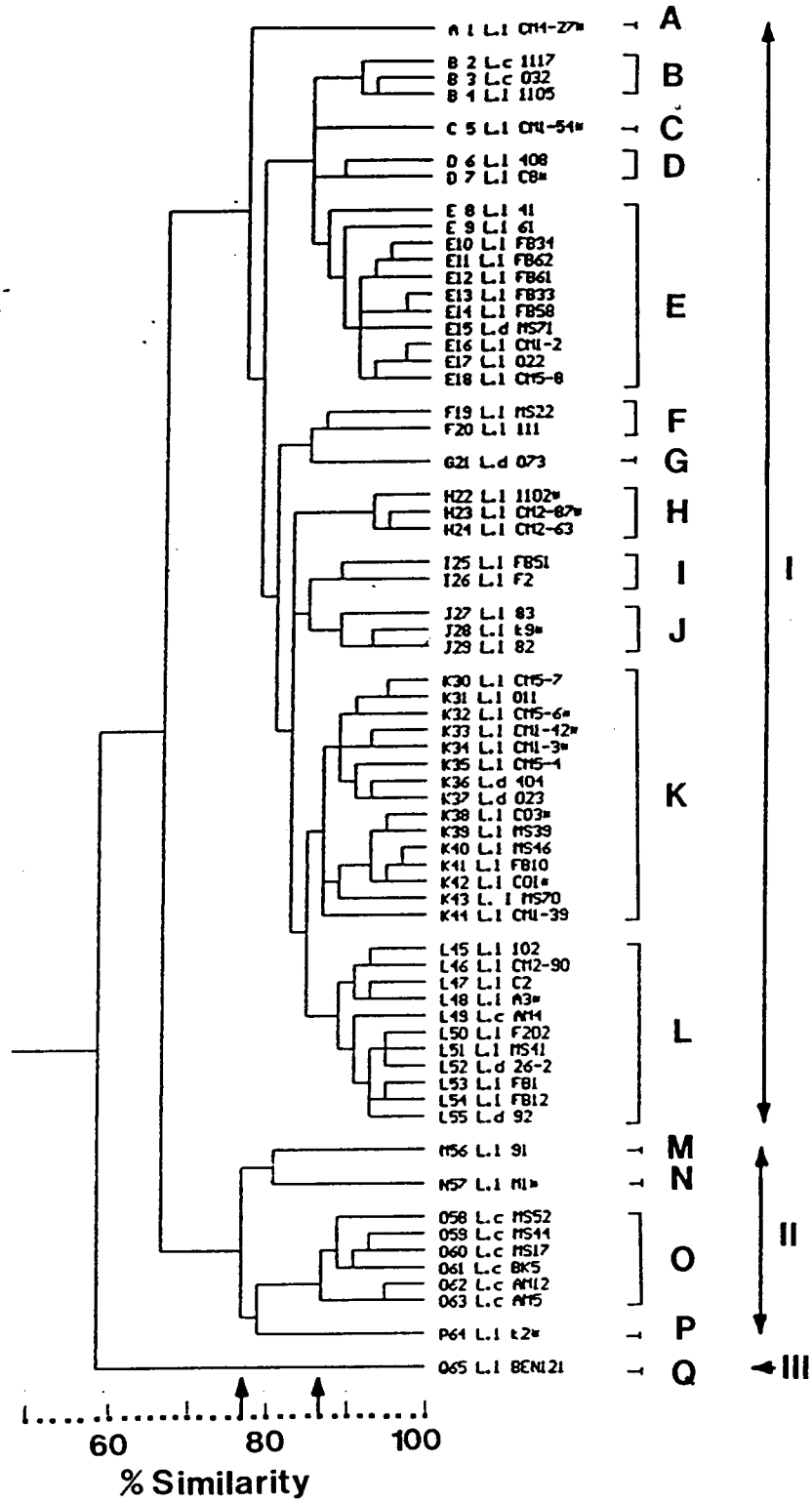

Figure 1 (Cont.)

63, and fructose-6-phoshate was utilized only by strains C8, 41, CM2-87,1102, and CM2-63. The following sugars were utilized by greater than 97% of the lactococcal strains; dextrin except for strains M1 and AM5, cellobiose with the exception of strains AM12 and AM5, D-galactose except for strain MS22, D-psicose except for strain CO1, salicin except for strain K2, adinosine except for strain MS52, deoxy adinosine except for strains MS52, and AM5, innosine except for MS17 and MS52, and N-acetyl mannosamine with the exception of strains CM1-39, CM2-87, CM4-27, CM5-6, MS70, AM5, and K2. The rest of the reactions were variable.

The data compiled from the evaluation of metabolic test battery were used to generate dendograms which showed metabolic relatedness of the lactococcal strains. A dendogram derived from the carbon-source utilization analysis is shown in Fig. 1. A total of 10 clusters (containing two or more strains) and 7 unclustered strains were obtained at similarity levels above 88%. They were given the alphabetical ordering from A to Q. Three cluster groups (I, II, and III) could be defined at similarity levels above 78%. Cluster group I (clusters A to L) contained all of the *L. lactis* subsp. *lactis* reference strains f2d2 and C2 and the biovar. *diacetylactis* 26-2. It also contained most of the environmental strains identified as *L. lactis* subsp. *lactis*. Also, all of the environmental strains identified as *L. lactis* subsp. *lactis*, which hybridized to the probe specific to the subspecies cremoris (68RCa probe), came in this cluster group. One of these strains (L. 1 A3, Fig. 1) clustered near the *L. lactis* subsp. *lactis* C2 reference strain, which also hybridized to the 68RCa probe. Only two of the *L. lactis* subsp. *cremoris* strains occurred in this

cluster group close to the top (Fig. 1). One of these strains (L. c 032) was positive for growth at 40°C and pH 9.2, which are characteristics of *L. lactis* subsp. *lactis*, but not of *L. lactis* subsp. *cremoris*. However, this strain was negative for arginine deiminase and growth at 4% NaCl, and hybridized to the 68 RCa probe, characteristics of *L. lactis* subsp. *cremoris*. The other strain (L. c 1117, Fig.1) was typical *L. lactis* subsp. *cremoris* both phenotypically and genotypically. Cluster group II (clusters M to P) contained the reference *L. lactis* subsp. *cremoris* strain BK5 together with most of the other environmental *L. lactis* subsp. *cremoris* strains. Only three of the environmental *L. lactis* subsp. *lactis* strains occurred in this cluster group (L. l 91, L. l M1, and L. l K2; Fig.1). Both L. l M1 and L. l K2 shared some characteristics with *L. lactis* subsp. *cremoris*, such as hybridization to the 68RCa probe and inability to grow at pH 9.2. Also, strain M1 failed to grow at 40°C. However, strain L. l 91 had typical *L. lactis* subsp. *lactis* characteristics. Cluster III contained only one strain (BEN 121, Fig. 1).

The differential utilization of certain carbon sources (Table 3) correlated with the clustering of the lactococcal strains into the three group clusters (I, II, and III; Fig.1). Strains of cluster group I were mostly capable of utilizing α-cyclodextrin, ß-cyclodextin, amygdalin, arbutin, gentobiose, maltose, palatinose, turanose, pyruvic acid, and glycerol. Some strains of this cluster group were capable of utilizing adinosine monophosphate an uridine monophosphate. However, strains of cluster group II were not able to utilize any of the above mentioned carbon sources with the exception of few strains which were able to utilize gentobiose or maltotriose. This may be utilized

TABLE 3. Characteristics useful for differentiating *L. lactis* strains for cluster groups.

Carbon source	Test for cluster group		
	I	II	III
α-Cyclodextrin	+/-	-	+
ß-Cyclodextrin	+/-	-	+
Amygdalin	+/-	-	+
Gentobiose	+	-/+	+
Mallotriose	+	-/+	+
Palatinose	+/-	-	+
Turanose	+/-	-	+/-
Pyruvic acid	+/-	-	+
Glycerol	+/-	-	+
Adenosine monophosphate	-/+	-	+
Uridine monophosphate	-/+	-	+

+/-: Most strains can utilize the carbon source indicated
-/+: Most strains can not utilize the carbon source indicated
+ : All strains can utilize the carbon source indicated.
- : All strains can not utilize the carbon source indicated.

as a biochemical characterization test to differentiate between for *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The only strain (L. l BEN 121) in cluster group III was capable of utilizing all of the carbon sources listed in table 3 with the exception of glycerol.

Phage typing. When the 23 environmental lactococcal strains were challenged by phages from Quest International, strains 011, CM5-6, and 111 exhibited very faint clearing. Strain CO3 showed turbid clearing. Strains 011, CM5-6, and 111 were affected by the same sample, which was one of the Quest 1993 Buttermilk whey samples. Strain CM1-3 was affected by another of Quest fresh buttermilk whey samples. CO3 was hit by the Canadian composite. All the affected strains were inoculated into 10 ml of M17 lactose broth containing calcium and 0.5 ml inoculum of the representative whey samples were added to the tubes. After 6 h at 32°C, the tubes were centrifuged, and filter-sterilized. The suspected phage lysates and the original phage suspensions were spotted in 5 microliter volumes on agar overlays containing the supposedly susceptible strains. After incubation, only CO3 showed similar turbid clearing . Other strains were unaffected. It was concluded that only strain CO3 is susceptible to phages in the Canadian preparation. The other observations were probably artifacts or caused by low amounts of lysin. Because the effect was not generalized (lysis reactions are usually non-specific) they were most likely artifacts.

Table 4 summarizes the results of phage sensitivities of some environmental strains when challenged against a bank of 32 phages from Galloway West Company. Out of the 23 strains, the following

TABLE 4. Challenging environmental lactococcal strains with Galloway West Company phages. Twenty-three strains were tested against 32 phage preparations which were numbered from 1 through 32.

Phage	Lactococcal Strains showing sensitivity					
	023	BEN121	MS70	C03	FB6	CM4-27
3			CL	CL		
5			CL	CL		
6			CL	CL		
7			TL	TL		
8	TL		CL	CL	TL	
9			CL	CL	TL	
10	CL		CL	CL	TL	TL
11	TL		CL	CL	TL	
12	TL		CL	CL	TL	
13			CL	CL		
14			CL	CL		
15			CL	CL		
16			CL	CL		
17			TL	SC		
19		TL				
20		TL		SC	TL	
21		TL			TL	
22					TL	
23		TL			TL	
24		TL		SC	TL	
25					TL	
26		TL				
31					TL	

TL: Turbid Lysis CL: Clear Lysis SC: Slight lysis

Strains FB1, CMP1-3, MS-11, MS-39, AM-4, 83, FB1, CM5-6, 022, 82, 408, MS17, MS-5, 011, 61, and 111 were resistant to all 32 phage peparations. All strains were resistant to phages 1, 2, 4, 18, 27, 28, 29, 30, and 32.

showed some sensitivity. Strain 023 showed turbid lysis (TL) to 3 phages and clear lysis (CL) to a fourth phage. Strain BEN121 exhibited TL to 6 phages. Strain FB62 gave TL with 12 phages while strain CM4-27 showed TL to only one phage. Strains MS70 and CO3 were the most sensitive of all strains. Both strains exhibited CL to 12 phages. Also, strain MS70 gave TL with an additional phage and strain CO3 showed slight sensitivity to another three phages. The rest of the strains (FB1, CM1-3, MS11, MS39, AM4, 83, FB1, CM5-6, 022, 82, 408, MS17, MS5, 011, 61, and 111) were insensitive to all of the 32 phages. Also, all 23 strains were resistant to phages 1, 2, 4, 18, 27, 28, 29, 30, and 32.

Results of phage testing done by Marschall products are shown in Table 5. Only positive results were reported. The majority of the strains wetre resistant to all of the whey or phage samples they were exposed to. Of those strains which showed sensitivity, strain CO3 was most sensitive; it showed total lysis by 8 different phage preparations (Table 5). Strain 023 showed total lysis by 2 phage preparations and strain FB62 by only one phage preparation. Strains MS39, 022, MS70, CM4-27, FB62, 023, FB1, CO3, FB12, and 408 were partially sensitive to 1 or more phage preparations.

The biochemical and physiological characteristics of these environmental lactococcal strains and their apparent resistance of to this many bacteriophages encountered by the dairy industry indicate the potential usefulness of these strains as dairy starter cultures.

TABLE 5. Challenging the environmental lactococcal strains with Marschall products Company phages. Twenty-three strains were tested against 72 positive whey samples for phage (whey samples 1-72), 30 negative whey samples (whey samles 73-102), and 38 single plaque isolates (SPI 1-38). The phage test reading was based on a scale of 0 (no lysis) to 3 (total lysis) based on spot test evaluation. Only those strains with readings of 1 or greater were reported.

Whey sample No.	Lactococcal Strains showing sensitivity					
	CM4-27	FB62	023	FB1	CO3	FB12
1		1				
10					3	
11		1			2	
12					2	
13		1				
14		1				
18					2	
21	1					
26					2	
27	1		2	2	3	2
28					2	
29					2	
31	1	1				
38			1			
39	1	1	1	1	3	
4	1					
40				1	3	
41					1	
44		2				
45					2	
46					2	

TABLE 5 (continued). Challenging the environmental lactococcal strains with Marschall products Company phages. Twenty-three strains were tested against 72 positive whey samples for phage (whey samples 1-72), 30 negative whey samples (whey samples 73-102), and 38 single plaque isolates (SPI 1-38). The phage test reading was based on a scale of 0 (no lysis) to 3 (total lysis) based on spot test evaluation. Only those strains with readings of 1 or greater were reported.

Whey sample No.	Lactococcal Strains showing sensitivity							
	MS39	022	MS70	FB62	023	FB1	C03	408
47							3	
49							2	
51							1	
52							1	
55							1	
57							2	
6				3	2			1
60					1		1	
63							1	
64							3	
68							3	
7				1				
SPI 11		1						
SPI 13	1			1	1			
SPI 18		1			3		1	
SPI 23				1				
SPI 24			1			1	3	
SPI 34				1			2	
SPI 38							1	
SPI 17					3			

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