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A method for rapid isolation of plasmid DNA from group N streptococci was developed. Chief advantages of the method were simplicity, the utilization of microliter quantities of reagents, and the obtainment of preparations highly enriched for covalently closed circular plasmid DNA. The method was also applied to over 15 Leuconostoc strains, and recovery of plasmids in the 1-to-76 megadalton mass range was demonstrated. Similarly to lactic streptococci, leuconostocs (other than L. oenos) contained at least one, and usually more, plasmid species. Plasmid DNA could not be demonstrated in four L. oenos strains examined. Thirty-eight strains of lactic streptococci were challenged with all major classes of antimicrobials, in the Bauer-Kirby disc test. Minimal inhibitory concentrations of some antimicrobials were determined for the less susceptible strains. Presence of

high-level resistance factors could not be detected, except in the case of nisin. Streptococcus lactis ATCC 7962 was resistant to >40-fold higher concentrations of this bacteriocin (>64 ug/ml) than most other strains tested. This strain was a potent nisin producer. Accidental contamination of one S. cremoris culture with Leuconostoc led to the discovery that most leuconostocs are insensitive to the antibiotic vancomycin. Tests performed with one such strain showed that resistance did not depend on drug inactivation. Derivative strains of S. lactis NCDO 1404 (a reference nisin producer which contained seven plasmid species) were obtained by protoplast- or temperature-induced plasmid curing. Curing also occurred spontaneously, during growth in broth. Comparison of derivatives and the parental strain provided evidence for plasmid linkage of proteinase and lactose fermentation in NCDO 1404. The four possible combinations of proteinase (+/-) and lactose fermentation (+/-) phenotypes could be differentiated on buffered, milk-based agar media containing pH indicators.

Antimicrobial Resistance and Plasmid Properties of *Leuconostoc* and Group N *Streptococcus*

by

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To Judith

A Scientist Doubts

The practice of science is the selfless pursue of the truth, which one frequently finds to be diametrically opposed to his or hers preconceived notions. At the heart of scientific training must reside the capacity to seek the truth rather than evidence to support one's preformed ideas. In practical terms, this means that before a hypothesis can be accepted as corresponding to reality, it must have withstood every conceivable attempt to disprove it.

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INTRODUCTION

Leuconostoc and group N *Streptococcus* are lactic acid bacteria, which are important in the manufacture of fermented foods and beverages. These microorganisms are indispensable in the production of many varieties of cheeses and other dairy products. Malo-lactic fermentation, carried out by *L. oenos*, is employed to reduce the acidity of certain wines, thus improving their quality (1,5,21,22,30,31).

The genus *Leuconostoc* comprises two distinct groups of organisms: those isolated from wine (*L. oenos*), and five species of difficult differentiation (*L. mesenteroides*, *L. dextranicum*, *L. paramesenteroides*, *L. lactis*, and *L. cremoris*), which exist in milk, milk products, or plant material (11,12). These habitats are shared (29) by dairy streptococci of the group N, which is made up by *S. cremoris*, *S. lactis*, and *S. lactis* subspecies *diacetylactis*. Garvie and Farrow recently proposed that *S. cremoris* also be considered a subspecies of *S. lactis* (13). Both lactic streptococci and leuconostocs are members of the family *Streptococcaceae* (9,11).

Because of their economic importance, lactic acid bacteria have for many years been intensively studied in numerous laboratories around the world, both from the genetic and the

physiological standpoints. In 1983 an entire issue of the Dutch microbiological journal, Antonie van Leeuwenhoek, was dedicated to the lactic acid bacteria, following a meeting in Waageningen, Holland, where over 200 scientists from around the world participated (5,14,16,19,20,23).

Since the early seventies, one of the most fertile areas of investigation on lactic acid bacteria has been the genetics of group N streptococci, especially with regard to their plasmids. The major contributions to this field of study have originated from the laboratories of C. Daly (University College, Cork, Ireland), M. J. Gasson (National Institute for Research in Dairying, Reading, England), T. R. Klaenhammer (North Carolina State University, Raleigh), D. J. LeBlanc (National Institute for Dental Research, Bethesda, MD), L. L. McKay (University of Minnesota, Minneapolis), L. E. Pearce (New Zealand Dairy Research Insitute, Palmerston North), W. E. Sandine (Oregon State University, Corvallis), M. Teuber (Federal Dairy Research Center, Kiel, West Germany), and others. The driving force behind this research has been the discovery that several of the properties of lactic streptococci which are essential for their industrial usefulness depend on the presence of plasmid-carried genes (4,6,14,17,20,23,25-28,31).

All naturally occurring strains of lactic streptococci examined to date have been found to carry plasmid DNA. The number of distinct plasmid species harbored by individual strains varies

from two to eleven, most commonly from four to seven (6,14,23,25,27). The first important consequence of the abundance of plasmid DNA in group N streptococci is the fact that plasmid profiles are extremely valuable in strain identification (7,27). Since variations in number and molecular mass of plasmid species make possible a very large number of combinations, unrelated strains can readily be distinguished, on the basis of their unique plasmid complements.

In their overwhelming majority, lactic streptococcal plasmids are still cryptic, i.e., any capacities which they may confer on their hosts have not been identified (6,23). Nevertheless, research carried out in the last decade has clearly demonstrated plasmid linkage of lactose fermentation, proteinase, citrate utilization, and bacteriocin production (6,14,17,20,23,25-27,31). Less extensive studies have indicated that utilization of galactose and other sugars, as well as restriction of bacteriophage infection, are also plasmid-mediated in strains of lactic streptococci (23,28). Most of these properties greatly influence the suitability of a given strain for use in dairy fermentations: first, fast lactose fermentation and proteinase are both required for satisfactory growth in milk during cheese manufacture. Second, production of the aroma compound diacetyl by S. lactis subsp. diacetylactis depends on the breakdown of citrate. Third, bacteriocin production must clearly be given careful consideration when composing mixed-strain fermentation

starters (31). Finally, in spite of especially designed medium formulations and a host of other protective measures, bacteriophage infection is still a major cause of failure of dairy fermentations (5,30).

Because of the great practical importance of these plasmid-determined traits, the development of genetic exchange techniques for use with lactic streptococci has for the major part focused on plasmid transfer methods. The methodologies for transfer of plasmid DNA by conjugation, transduction, or protoplast transformation or fusion have succesfully been applied to group N streptococci (6,14,18,24-26). However, genetic studies of these bacteria, in particular the development of plasmid cloning vehicles, have been hampered by the scarcity of known selectable markers endogenous to the lactic streptococci (23). In spite of their large plasmid complements, and in spite of the common association of plasmid DNA with drug resistance markers (2,8,10), such markers have not been readily found in these bacteria (23), even though they are common in streptococci outside the N antigenic group (3,4,15). Hence, the search for antimicrobial resistance markers has been recently identified by McKay as a priority area for studies of lactic streptococcal genetics (23).

Although group N streptococci are clearly more important than leuconostocs in the dairy fermentation industry, the latter bacteria do have an indispensable role as aroma producers in foods

such as cottage, cream, or Gouda cheeses, and cultured cream (5,21,22,31). Moreover, as mentioned above, L. oenos strains are employed in the improvement of the quality of certain wines. Therefore, it is somewhat surprising that in spite of a wealth of literature on lactic streptococcal genetics, but a single meeting abstract (T. O'Sullivan and C. Daly, Irish J. Food Sci. Technol. 6: 206, 1982) can be found on the presence of plasmid DNA in Leuconostoc species. This is even more surprising in view of the fact that the differentiation between non-wine Leuconostoc species is often blurred and, to a large extent, based on characteristics (such as carbohydrate fermentation) which show a high degree of intra-species variability and which could conceivably be plasmid-mediated (11,12).

The project whose results are described here is part of a search for lactic streptococcal antimicrobial resistance markers, and their possible correlation with the presence of plasmid DNA. The major contributions of this work to the genetics of lactic acid bacteria are summarized below.

The development of a method for rapid isolation of plasmid DNA from lactic streptococci is described in Chapter 1. Given the large plasmid complements of these bacteria, one of the principal advantages of this method is the obtainment of preparations highly enriched for covalently closed circular plasmid DNA, which greatly facilitates the interpretation of plasmid profiles (6,7). Other merits of the method include the use of micro quantities of

reagents, and the fact that sample analysis by gel electrophoresis does not require a previous ethanol precipitation step.

Chapter 2 describes the systematic examination of 38 strains of lactic streptococci for the presence of factors for resistance to all major classes of antimicrobial agents and one bacteriocin (nisin). Resistance to nisin was identified as a feasible selectable marker endogenous to the lactic streptococci. Also, a valuable addition was made to knowledge on S. lactis ATCC 7962, a strain whose physiology has been thoroughly studied: this strain was shown to produce nisin.

Chapter 3 is the result of an accidental discovery. One of the cultures of S. cremoris examined for antimicrobial resistance happened to harbor a contaminant bacterium, which was resistant to high levels of vancomycin. Characterization of this bacterium suggested that it was a member of the Leuconostoc genus. Attempting to disprove this hypothesis, I tested 18 other leuconostocs for susceptibility to vancomycin. This resulted in the interesting and potentially useful finding that almost all leuconostocs are insensitive to this antibiotic, which is in sharp contrast with the high susceptibility exhibited by Streptococcus. In the same investigation, I showed that the plasmid isolation method described in Chapter 1 could successfully be applied to the Leuconostoc genus. Chapter 3 contains the first complete description of a Leuconostoc plasmid isolation method, and the first photographs ever published on Leuconostoc plasmid profiles.

In Chapter 4 I describe the examination of S. lactis NCDO 1404, with regard to plasmid DNA content and its correlation with important phenotypic properties. This strain is a reference nisin producer, and it was obtained from the culture collection of the National Institute for Research in Dairying, Reading, England. As previously reported for other S. lactis strains, my results indicated plasmid linkage of lactose fermentation and proteinase in NCDO 1404. Another important contribution was the differentiation, on milk-based agar media, of the four possible combinations of the lactose fermentation (+/-) and proteinase (+/-) phenotypes. This was the first instance in which the four colony types could be distinguished, and this development should prove useful in future studies of plasmid-linked properties in the lactic streptococci. I also observed the previously unreported loss of the nisin production phenotype with retention of the sucrose fermentation phenotype.

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Chapter 1

Microscale Method for Rapid Isolation of Covalently Closed Circular Plasmid DNA from Group N Streptococci

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ABSTRACT

A method for rapid purification of plasmid DNA from lactic streptococci, utilizing microliter quantities of reagents, was developed by combination of a short lysozyme-mutanolysin cell wall digestion with a modification of the Escherichia coli plasmid isolation procedure of McMaster et al. (Anal. Biochem. 109: 47-54, 1980). The preparations obtained were highly enriched for covalently closed circular DNA, and the method was applicable to plasmids of at least 40 megadaltons. Centrifugation in CsCl-ethidium bromide density gradients was not required.

INTRODUCTION

Group N streptococci have for centuries played an essential role in the production of fermented dairy foods. Some of the most important metabolic properties of the lactic streptococci, such as the fermentation of lactose or the degradation of milk proteins, are often determined by plasmid-carried genes (5,9). The study of plasmids has therefore become essential for the understanding of the genetics and physiology of group N streptococci, as well as being a valuable aid in strain identification (10). Most known strains of these bacteria carry at least two, and frequently more, plasmid species, which makes the interpretation of the band patterns obtained by agarose gel electrophoresis (26) considerably difficult if a preparation contains significant amounts of DNA which is in other than the covalently closed circular (CCC) state. This paper describes a simple procedure for rapid isolation of plasmid DNA from lactic streptococci, which yields preparations highly enriched for CCC DNA and utilizes micro quantities of reagents. The method was developed by combining previously available knowledge regarding the proper conditions for lysis of streptococcal cells (4,10,16,20,25,27,32) with a modification of an Escherichia coli plasmid isolation procedure (24).

MATERIALS AND METHODS

Bacterial strains. Streptococcus cremoris Wg2-2 was a gift from M. Teuber (Federal Dairy Research Center, Kiel, West Germany), and S. lactis MG 1261 was kindly provided by M. J. Gasson (National Institute for Research in Dairying, Shinfield, Reading, England). Other strains were from the culture collection maintained in this laboratory.

Chemicals. Sodium triisopropylnaphthalenesulfonate (TNS; 24) was from Eastman Kodak Co. Phenol (Mallinckrodt) was redistilled, washed three times with 0.5 M NaCl, and stored at 4°C after addition of 0.1% 8-hydroxyquinoline.

Enzymes. Lysozyme was dissolved daily (20 mg/ml) in sterile 25% sucrose solution. Mutanolysin (17,27,32) was dissolved at 5,000 units/ml in 0.1 M potassium phosphate buffer, pH 6.2, and stored at -25°C until used. RNase A was prepared as a 10 mg/ml solution in sterile water, heated at 70°C for 30 min, and stored at -25°C. All enzymes were from Sigma Chemical Co.

Culture growth. Streptococcal lysis medium was an M17 formulation (30) containing 0.5% yeast extract and 20 mM D,L-threonine (4) (and 0.5% glucose instead of lactose, when used with lactose-negative strains). Sixteen microliters of late exponential phase culture in M17 broth were used to inoculate 8.0 ml of streptococcus lysis medium, and the culture incubated at 20°C (25°C for S.cremoris Wg2-2) for ca. 12 h (absorbance at 660

nm with a 1 cm light path, 0.70; late log phase).

Cell lysis and isolation of plasmid DNA. A Beckman model 11 microcentrifuge was used in all centrifugation steps after cell harvesting. The horizontal (vertical slides) rotor is here designated rotor I, and the model 13.2 fixed-angle rotor is here designated rotor II.

Cells were harvested by centrifugation at 4°C, resuspended in 1.2 ml of sterile distilled water, transferred to a 1.5 ml microcentrifuge tube, and centrifuged in the cold at 2,390 x g for 5 min (rotor I). The supernatant was discarded, and the tube placed on ice. (The volume of the cell pellet at this point was ca. 30 ul.) Lysozyme solution (100 ul) was added, the cells thoroughly resuspended by vortexing, and the preparation returned to ice. After addition of 20 ul of mutanolysin solution and vortexing, the tubes were transferred to a 37°C water bath, incubated for three minutes, and 210 ul of 0.079 M Tris-HCl in 25% sucrose solution (pH 8.1) was added. The tubes were rapidly vortexed, incubated for another 3.5 min at 37°C (16), and chilled on ice for two min. With preparations still on ice, 60 ul of 10% TNS in 250 mM Na_2EDTA, pH 8.0, was added and the samples given four gentle inversions, quickly followed by the addition of 25 ul of 3.0 N NaOH, and four more gentle inversions. Tubes were then transferred to a water bath at 30°C and incubated for seven minutes, while mixing by gentle inversions. The pH at this point was 12.0–12.2, and a clear solution was obtained.

Neutralization was effected by the addition of 30 ul of 1.0 M sodium acetate (pH 5.0), followed by five gentle inversions. Phenol reagent (600 ul) was immediately added, and the samples were thoroughly mixed by two rounds of four gentle inversions. Addition of 150 ul of chloroform was rapidly followed by six gentle inversions, and the preparations were immediately centrifuged at 11,900 x g at room temperature for 12 min (rotor II). A pipettor with large bore tips (15) was used to collect 320 ul of the clear aqueous phase (top), while carefully avoiding the white, disc-shaped interface which separated it from the yellow organic phase (bottom). Failure to avoid this interface resulted in contamination with chromosomal (chr) or open circular (OC) DNA (8,24). The aqueous phase was transferred to a fresh 1.5 ml tube, 6.0 ul of RNase solution was added, and the preparations were mixed by inversion and incubated in a 37°C water bath for 20 min. Thirty five microliters of 5.0 M NaCl was added, followed by gentle mixing, the addition of 265 ul of 30% polyethylene glycol (molecular weight, 6,000) solution, and more gentle mixing. Samples were then incubated in a salt-ice bath at -11°C for 1.5 h. Centrifugation in the cold at 11,200 x g for 15 min (rotor I) produced a pellet containing precipitated DNA and polyethylene glycol. The supernatant was discarded, 500 ul of 50 mM Tris-HCl, 5 mM Na_2EDTA, pH 8.0 solution were added to the pellet, and five gentle inversions were given to the tubes. Dissolution of the pellet was completed with the addition of 900 ul of chloroform and

gentle but very thorough mixing, which was continued for another 10 min. Samples were then chilled on ice for 10 min before centrifugation in the cold at 11,900 x g for 12 min (rotor II). The preparations were returned to ice and 300 ul of the clear aqueous phase (top) was collected. (Low temperature helped to prevent resolubilization of the white interface into the aqueous phase.) In a fresh tube, 600 ul of cold, 100% ethanol was added to the aqueous phase, the tubes were inverted five times, and incubated at -25°C for 1.5 h. The precipitated plasmid DNA was collected by centrifugation in the cold at 11,200 x g (rotor I) for 20 min. The supernatant was discarded and the tubes were thoroughly dried (inverted for 15 min in a laboratory hood) before addition of 24 ul of loading buffer (10% glycerol, 0.025% bromphenol blue, 50 mM Tris-HCl, 50 mM NaCl, and 10 mM Na_2EDTA, pH 8.0; refs. 11,23). The DNA was allowed to dissolve overnight at 4°C (7), and to warm up to room temperature before electrophoresis. A total of 6.0 ul was loaded into a 10-mm gel slot.

When sample purity was not important, e.g. when screening multiple isolates, the procedure needed to be carried only as far as the phenol extraction: plasmid DNA could be visualized by direct loading of ca. 8.0 ul of the dense, sucrose-containing aqueous phase into a 5-mm gel slot. Obvious advantages were: processing more isolates in a shorter time, and same-day electrophoresis. (The complete procedure was applied to the

samples shown in Fig. 1.1.)

Agarose gel electrophoresis. Electrophoresis buffer was 40 mM Tris base, 20 mM sodium acetate, and 2 mM EDTA, pH adjusted to 8.1 with glacial acetic acid (10). The final concentration of acetate in this system was ca. 38 mM. Horizontal electrophoresis in 0.70% agarose gels was carried out in submarine mode with buffer recirculation (14), for 4.0 h. A low voltage gradient (3.0 V/cm) was employed to optimize the separation of large plasmids (11,22,23). After electrophoresis, the gel was stained with ethidium bromide (0.5 ug/ml) for 30 min, destained in water for 20 min, and photographed through an orange filter on a model C-63 Ultraviolet Products transilluminator with Polaroid type 47 film. Plasmid mobilities were measured directly on photographic prints. Plasmid molecular masses were estimated from a logarithmic plot of mass vs. relative mobility (26,31), constructed on the basis of E. coli V517 standards (21).

RESULTS AND DISCUSSION

The plasmid profiles of representative strains of lactic streptococci are shown in Fig. 1.1, lanes A-E. Information previously published on these or closely related strains, gathered by use of centrifugation in CsCl-ethidium bromide density gradients, electron microscopy, or agarose gel electrophoresis (1,13,16,19,28), is in good agreement with the data shown here. (S. lactis C2 carried in this laboratory is somewhat different from that held by McKay's group, even though both strains are derived from S. lactis NCDO 712; this was previously shown by Davies et al., ref. 10.) It is evident that the present procedure is effective in reducing chr and OC DNA contamination, while recovering plasmids in the 1-to-40 megadalton range. The amount of DNA loaded in each lane of Fig. 1 is equivalent to that obtained from 2.0 ml of culture. High enrichment with CCC DNA greatly facilitates the interpretation of plasmid profiles (10), especially for strains containing multiple plasmid species.

Culture growth before lysis at 20°C for 12 h was employed because, at least for some plasmids, the amount of plasmid DNA relative to the total DNA in the cell increases at lower growth rates (6,12,18,29). Under these conditions, use of a small inoculum (0.2%, more or less, depending on the strain) permitted combining the convenience of overnight incubation with the obtainment of cultures in late exponential phase of growth, which

are more amenable to lysis than stationary phase cells (16).

The quantity of cells used was important for the success of the procedure. An excess of cells frequently led to poor lysis, because of insufficient digestion of the cell wall by lysozyme and mutanolysin, in the short (16) incubation period. A scarcity of cells was also undesireable, because of the recovery of inadequate amounts of plasmid DNA. A 30-ul cell pellet, obtained after washing in a microcentrifuge tube (see Materials and Methods), was an appropriate quantity for lysis by the present method.

Lysozyme was added to washed cells in a 25% unbuffered sucrose solution, with Tris-HCl added later, according to the findings of Metcalf and Deibel (25), which were also explored by Davies et al. (10). Use of a small amount of mutanolysin (17,27,32) in the presence of a high concentration of lysozyme was extremely helpful in promoting lysis of streptococcal cells.

Lysis under highly alkaline conditions allows little time for the action of endogenous nucleases (3,24), and eliminates the necessity of reducing the viscosity of the lysate through shearing (3,8). Vortexing of lysed cells was eliminated to avoid contamination with chr DNA fragments.

Alkali denaturation followed by renaturation and phenol extraction in high salt (8,24) were the most critical steps in the procedure, regarding the elimination of chr and OC DNA. It was important that neutralization be immediately followed by the addition of phenol. Presumably, only CCC plasmid molecules could

renature properly in this short interval, and thus escape precipitation caused by the addition of phenol.

Extreme care had to be employed with regard to the exposure time and pH during the alkali denaturation step. Excesses in either or both lead, upon neutralization, to the irreversible denaturation of CCC plasmid molecules into collapsed circles (14,24). This results in artifacts of sometimes difficult detection, since only one plasmid species in a multiple-plasmid strain may be affected. Examples of such artifacts are shown in Fig. 1.1, lanes F and G. In these preparations, cell lysates were exposed to pH 12.4 for 12 min, the conditions originally employed by McMaster et al. (24). Use of a less severe denaturation treatment, as described here, permitted elimination of the artifact bands.

Phenol extraction was carried out in the presence of the 25% sucrose solution used to stabilize spheroplasts before lysis. The density of the phenolic phase was smaller than that of the aqueous phase. This would be very inconvenient when collecting the latter after centrifugation, because of heavy losses and contamination with phenol and with precipitated chr and OC DNA. This problem was solved by the addition of chloroform after the phenol extraction, followed by brief mixing and centrifugation. The density of the organic phase was sufficiently increased by the added chloroform, so that reversal of phases no longer occurred. Avoidance of the white interface produced after centrifugation was

essential to minimize contamination with chr and OC DNA. Reagent volumes were increased by 50%, relatively to those used by McMaster et al.(24), so percent losses at interfaces could be diminished.

Recently, two new rapid screening procedures for isolation of plasmid DNA from lactic streptococci appeared (2,33). The present method has an advantage over one of these procedures (2), in that contamination with chr DNA is virtually eliminated.

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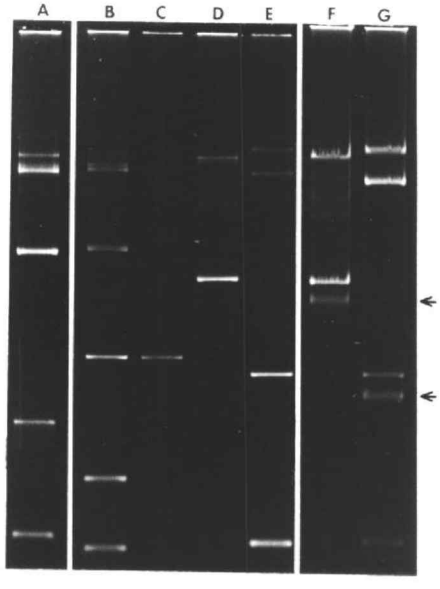

Fig. 1.1. Plasmid profiles of lactic streptococci. Apparent molecular masses (megadaltons) are indicated in parentheses, after the identity of each strain. Lane A, S. cremoris Wg2-2 (1.5, 3.4, 13, 32, 40), a derivative of S. cremoris Wg2 (ref. 28). Lane B, S. lactis C2 (1.4, 2.3, 5.6, 13, 33, 35). Lane C, S. lactis MG 1261 (5.6); both of these strains are derivatives of S. lactis NCDO 712 (ref. 10, 13). Lane D, S. cremoris B1 (11, 40; ref. 1, 16). Lane E, S. lactis C10 (1.5, 5.0, 32, 45; ref. 16, 19). The next two lanes show artifact bands (arrows) produced in S. cremoris B1 (lane F) and in S. lactis C10 (lane G) by excessive denaturation in alkali (see text). Lanes F and G were run in a separate gel.

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Chapter 2

Survey of Antimicrobial Resistance in Lactic Streptococci

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ABSTRACT

Twenty-six strains of Streptococcus cremoris and 12 strains of S. lactis were challenged with 18 antimicrobials and with nisin, in the Bauer-Kirby disc susceptibility test. All strains were susceptible to ampicillin, bacitracin, cephalothin, chloramphenicol, chlortetracycline, erythromycin, penicillin G, tetracycline, and vancomycin. All strains were resistant to trimethoprim, and almost all strains were resistant to sulfathiazole. Variability in resistance to gentamycin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifampin, and streptomycin was observed. MICs of these substances for the less susceptible strains were determined, and high-level resistance factors could not be detected, except in the case of nisin. S. lactis ATCC 7962 was resistant to at least 40-fold higher concentrations of nisin (>64 ug/ml) than most other strains tested. This strain was a potent nisin producer.

INTRODUCTION

Group N streptococci are essential for the production of fermented dairy foods. Our knowledge of the genetics of these bacteria, especially as related to their plasmids, has progressed steadily in recent years (23). All naturally occurring lactic streptococcal strains examined to date contain one or more plasmid species; plasmid-carried genes confer on these bacteria some of their most important physiological properties, such as fast lactose fermentation or milk protein hydrolysis. However, in spite of extensive studies on lactic streptococcal plasmids, reports on the presence of antimicrobial resistance determinants in these bacteria are extremely scarce (11,23). As a result, few endogenous selectable markers are presently available for genetic studies of group N streptococci, in particular for the construction of plasmid cloning vehicles.

Lactic streptococci are not ordinarily exposed to antimicrobials, except for the presence, in milk, of residues of antibiotics used in mastitis therapy. However, given the large plasmid complements of these bacteria, and given the fact that known, previous exposure to antimicrobials is not a requisite for the presence of resistance determinants (14,25,29), it would be reasonable to expect that such determinants might be found in group N streptococci.

In this paper we report the examination of 38 randomly

selected lactic streptococcal strains for their susceptibility or resistance to all major classes of antimicrobials and one bacteriocin (nisin). As also recently shown by McKay and Baldwin (24), we found resistance to nisin to be a feasible selectable marker, which is endogenous to these bacteria. High-level resistance, characterisitic of the presence of specific determinants, could not be found for any of the antimicrobials examined, with the possible exceptions of sulfathiazole and trimethoprim. The widely used strain, S. lactis ATCC 7962, was shown to produce nisin.

MATERIALS AND METHODS

Bacterial strains. Streptococcus lactis ATCC 11454 was a gift from D. J. LeBlanc, National Institute for Dental Research, Bethesda, MD. S. cremoris ATCC 14365 was kindly provided by C. F. Gonzales, Microlife Genetics, Sarasota, FL. S. lactis strains NCDO 497 and NCDO 1403 were obtained from the National Institute for Research in Dairying, Shinfield, Reading, England. Other lactic streptococci were from the culture collection maintained in this laboratory. The following strains were used in disc susceptibility tests: S. cremoris B1, C13, HP, M26, M45, R6, SK11G, SK11G-C, U134, 31N, 32, 104, 108, 108 M, 205, 224, 226, 227, 283, 284, 287, 289-C, 290 PC, 291, 292, 378; S. lactis 01, BA1, BA2, C2, C3, C10, C27, F2D2, ML8, ML8-R4, SLE, and ATCC 7962. All strains were challenged with all antimicrobials, in the disc test. Culture maintenance was in M17 broth (31). (All M17 formulations used in this work contained 0.5% yeast extract.)

Disc susceptibility tests. Disc tests were done by the Bauer-Kirby method (3), according to the recommendations of Acar (1) and Barry (2). Cultures were grown in M17 broth for 4.5 h at 30°C (ca. 5.0×10^7 to 2.0×10^8 CFU/ml) and used for inoculation of agar plates. After disc application, plates were incubated at 30°C for 20 h, and inhibition zone diameters measured. Chloramphenicol and rifampin discs were from BBL Microbiology Systems. Other commercially obtained discs were from Difco

Laboratories. Sulfathiazole or trimethoprim discs contained 300 ug or 5 ug, respectively. Other disc potencies are given in Figs. 2.1 and 2.2. Purified nisin (ca. 40,000 units/mg) was purchased from Aplin & Barrett Ltd., Beaminster, Dorset, England. Nisin-containing discs were prepared (27) by spotting 20 ul of nisin solution (6.4 mg/ml in sterile distilled water) onto sterile filter paper discs of ca. 6.5 mm diameter (Difco). Bacitracin, chlortetracycline, sulfathiazole, trimethoprim, and vancomycin disc tests were on Mueller-Hinton agar (Difco); all other disc tests were on M17 agar.

MIC determinations (2, 33). A fresh overnight culture in M17 broth was diluted with normal saline (1:10), and ca. 1 ul of this suspension was used to inoculate the surface of M17 agar containing the test antimicrobial. Presence or absence of growth was recorded after 24 h incubation at 30°C. With the exception of nisin, all antimicrobials were from Sigma Chemical Co.

Bacteriocin production tests. The method of Kékessy and Piguet (19) and glucose-M17 agar plates were used. Plates were inoculated with ca. 1 ul of a fresh culture of the strain to be tested as producer, incubated at 30°C for 20 h, inverted, and inoculated with the indicator strain. Results were read after a second 20-h period at 30°C.

Tests of identity between the bacteriocin produced by S. lactis ATCC 7962 and nisin (produced by S. lactis NCDO 497) were performed essentially as described by Gasson (15). These two

strains, as well as S. cremoris B1 (indicator strain and negative control for bacteriocin production), were grown in M17 broth (with glucose instead of lactose, since 7962 ferments lactose slowly; 9). Cultures were prepared from 0.2% inocula and incubated at 30°C for 10 h. Cells were removed by centrifugation, and the supernatants were sterilized by membrane filtration (0.2 um pore size). Filtrates were divided in three parts: A (untreated), B (pH adjusted to 2.0 with HCl), and C (pH adjusted to 8.0 with NaOH). Fractions B and C were boiled for 30 min, cooled, and their pHs adjusted to 5.8. Four-hour-old cultures (ca. 7.0×10^7 CFU/ml) of all three strains were used to prepare lawns on glucose-M17 agar. After drying, the lawns were spotted with ca. 1 ul of each fraction (A, B, or C). All three strains were tested both as bacteriocin producers and as indicators. Presence or absence of inhibition were recorded after 20 h incubation at 30°C. The same protocol was used for testing bacteriocin sensitivity to proteolytic digestion. Culture filtrates had their pHs adjusted to 7.5, and ∝-chymotrypsin or trypsin (both from Sigma Chemical Co.) were added (5.0 mg/ml final concentration). After 2 h incubation at 25°C, the filtrates were spotted onto lawns.

RESULTS

Disc susceptibility tests. The distributions of inhibition zone diameters observed with the strains tested are shown in Figs. 2.1 and 2.2. In both Figs., the vertical arrows indicate the maximum inhibition zone diameter exhibited by bacteria considered clinically resistant to the given antimicrobial. (This information, when available, was compiled from ref. 3, or from materials published by BBL Microbiology Systems.) Clearly, two types of situations were observed. The first type was represented by the distributions shown in Fig. 2.1: all strains tested had inhibition zones significantly larger than the maximum accepted as compatible with resistance. No further tests were conducted with the antimicrobials grouped in Fig. 2.1.

A second type of situation, displayed in Fig. 2.2, was discernible: the more resistant strains showed inhibition zone diameters equal to, or smaller than, the maximum diameter observed with clinically resistant strains (arrows). In other words, as a first approximation, the disc test indicated the presence of strains resistant to one or more of these substances. These strains were then subjected to the more stringent test of MIC determination (see below).

Susceptibility tests were also performed with discs containing sulfathiazole or trimethoprim. In both instances, Mueller-Hinton agar was used as the test medium, since it is

practically free of interfering substances. Only three strains (S. lactis BA1, BA2, and C10) showed a zone of complete inhibition around a disc with sulfathiazole. None of the strains was inhibited by trimethoprim.

MIC determinations. Strains showing inhibition zone diameters indicative of resistance to one or more antimicrobials were used in MIC determinations. The following strains were tested in this manner: S. cremoris B1, HP, R6, SK11G, U134, 31N, 108, 224, 226, 290 PC, and 291; S. lactis BA2, C2, C10, C27, ML8, SLE, and ATCC 7962. The results of these determinations are shown in Table 2.1. This Table includes, when available, the MIC values of the same substances for strains of Streptococcus known to carry resistance factors. Comparison of the highest experimental MICs with the modal experimental MICs, or with these values from the literature, indicated that the stains examined did not carry determinants for high-level resistance to any of these antimicrobials, with the exception of nisin. S. lactis ATCC 7962 was resistant to at least 40-fold higher concentrations of nisin than most other strains tested.

Bacteriocin production tests. Since S. lactis ATCC 7962 was clearly much more resistant to nisin than any of the other strains, it was logical to test whether it might be a nisin producer. Bacteriocin production by S. lactis 7962 and by three reference nisin producers was tested against five indicator strains, including 7962 itself. The results, shown in Table 2.2,

confirmed that 7962 was resistant to nisin, and demonstrated that this strain was a potent bacteriocin producer. The sensitivities of the indicator strains to this bacteriocin paralleled their sensitivities to nisin. For both bacteriocins, S. lactis C10 showed the smallest inhibition zones, while S. cremoris U134 showed the largest.

We tested whether the 7962 bacteriocin could be distinguished from nisin on the basis of sensitivity to boiling at pH 2.0 or at pH 8.0. S. lactis NCDO 497 (a reference nisin producer) and S. cremoris B1 (a nisin-sensitive organism) were used as controls. The capacity of 7962 or 497 culture filtrates to inhibit S. cremoris B1 was retained after boiling at pH 2.0, but destroyed by boiling at pH 8.0, as expected for nisin (17). None of the three filtrates from S. cremoris B1 inhibited any of the three strains. Nisin-containing 497 filtrates inhibited none of the two S. lactis strains; the same was true for the 7962 filtrates, showing that S. lactis NCDO 497 was insensitive to the 7962 bacteriocin. Like nisin, this bacteriocin was inactivated by α-chymotrypsin, but not by trypsin. These results indicated that S. lactis ATCC 7962 produced nisin.

DISCUSSION

In his recent review (23), McKay pointed out the scarcity of known selectable markers in the lactic streptococci, and addressed the need for studies directed at uncovering such markers, for use in genetic manipulations of these bacteria. The present work directly meets that need.

In this study, at least one representative of every major class of antimicrobial was used to challenge 38 randomly selected strains of lactic streptococci. More than one antimicrobial of a given structural class were included, in case different mechanisms of inactivation are known to exist (e.g., kanamycin and neomycin, which might be inactivated by different enzyme specifities).

Although the probability of finding antimicrobial resistance determinants in lactic streptococci is clearly much lower than in isolates from clinical or animal sources (32), it certainly cannot a priori be considered vanishingly small. The lactic streptococci carry, as a rule, large plasmid complements (6,23); all of the strains tested in this work contained at least two plasmid species (results not shown). Furthermore, resistant determinants have often been found in bacteria not previously exposed to man-disseminated antimicrobials (14,25,29).

In view of these premises, it is somewhat surprising that resistance factors have not been readily found in lactic streptococci (23). The results of the present work support the

hypothesis that such factors might indeed be rare in these strains, with the possible exception of determinants for resistance to sulfathiazole or trimethoprim. Our findings on the scarcity of resistance factors in group N streptococci are corroborated by previous work by Cogan (7) and Lipinska (21), who found no evidence of antimicrobial resistance among lactic acid bacteria.

In a large survey which included 15 lactic streptococcal strains, Reinbold and Reddy (26) reported sensitivity of these strains to almost all of 30 antimicrobials, with the notable exceptions of colistin, nalidixic acid, polymixin B, and sulfonamides. Evidence of resistance was from disc tests only, and confirmatory MIC determinations were not performed. Similarly, Sozzi and Smiley (30) reported antimicrobial resistance in yogurt bacteria, but the level of resistance was not assessed by means of MIC tests.

The work of Dobrzanski et at. (11) presently stands as the sole finding of plasmid-mediated resistance to antimicrobials (bacteriocins excluded) in mesophilic lactic streptococci. These authors reported transformation of Bacillus subtilis to kanamycin resistance, by plasmid DNA from S. lactis (kanamycin MIC, 50 ug/ml). Such a low-level marker would seem inadequate for genetic experiments with lactic streptococci. Antibiotics of the aminoglycoside-aminocyclitol class are not effectively uptaken by streptococci because of their non-oxidative metabolism

(4,10,14,28). In fact, media containing these substances are routinely used for the isolation of streptococci from clinical specimens (12). These bacteria can be resistant to levels of aminoglycoside-aminocylitols of the order of 250 ug/ml, in the absence of specific resistance factors (5). Our results with gentamycin, kanamycin, neomycin, and streptomycin are in complete agreement with these notions.

Low susceptibility of lactic streptococci to sulfonamides was reported by Reinbold and Reddy (26). Sozzi and Smiley (30) found evidence of resistance to sulfonamides and trimethoprim in S. thermophilus. Our results support and extend these findings. Clearly, more work is needed to determine whether factors for resistance to these antimicrobials are present in these bacteria.

As shown by McKay and Baldwin (24) and by our results, resistance to nisin is a suitable selectable marker for use in genetic studies of the lactic streptococci. Plasmid linkage of this marker has been strongly suggested by the results of many workers (15,17,23).

Bacteriocin production is common among lactic streptococci (16). Our results show that S. lactis ATCC 7962 produces nisin, a denomination that actually applies to several closely related polypeptides (17). As is the case with most nisin producers (15,17), this strain ferments sucrose (13). The information presented here on nisin production by S. lactis ATCC 7962 should be a valuable addition to the knowledge on what is, from a

physiology standpoint, the most thoroughly studied lactic streptococcal strain (9,13,18,22).

ACKNOWLEDGMENTS

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TABLE 2.1. MIC of selected antimicrobials for strains of Streptococcus[a]

Substance	Experimental MIC (ug/ml) Mode(s)	Highest	MIC for R-factor-carrying strains Value (ug/ml)	Ref.
Gentamycin	4.0	80	>2,000	5, 8
Kanamycin	8.0, 16	160	>2,000	5, 8
Lincomycin	1.0	8.0	>1,000	20
Nafcillin	1.0	2.0	--	--
Neomycin	16, 64	160	>2,000	5
Nisin	1.6[b]	>64[c]	--	--
Rifampicin	32	64	--	--
Streptomycin	8.0, 64	160	>2,000	5

[a] Comparison of MIC values determined in this work with those reported for strains known to carry resistance factors (R factors).
[b] Lowest concentration tested.
[c] Poor solubility prevented testing of higher concentrations.

TABLE 2.2. Bacteriocin production by *S. lactis* strains[a]

Producer[b]	Indicator strains				
	S. lactis		*S. cremoris*		
	7962	C10	14365	B1	U134
S. lactis ATCC 7962	0	13	16	15	21
S. lactis ATCC 11454	0	8	11	10	17
S. lactis NCDO 497	0	6	10	10	14
S. lactis NCDO 1403	0	8	10	10	13

[a] Kékessy-Piguet method (19); figures denote inhibition zone diameters in mm.
[b] All five indicators were also tested as producers; among these five strains, *S. lactis* ATCC 7962 was the only one to inhibit any other strain.

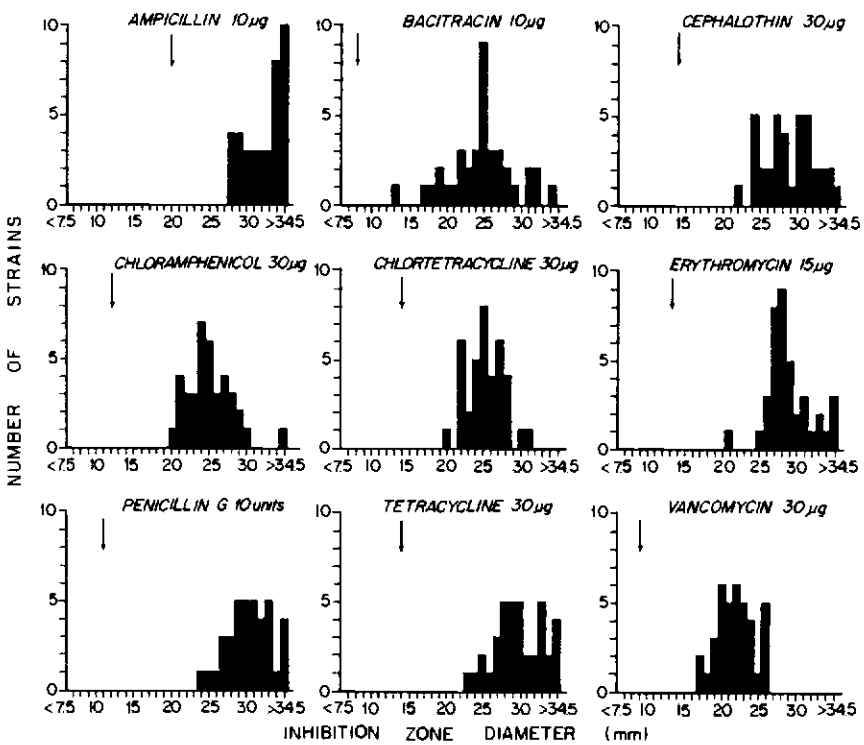

Fig. 2.1. Distributions of inhibition zone diameters observed in disc tests. Grouped in this Fig. are those antimicrobials for which all strains had inhibition zones larger than the maximum exhibited by clinically resistant bacteria (vertical arrows).

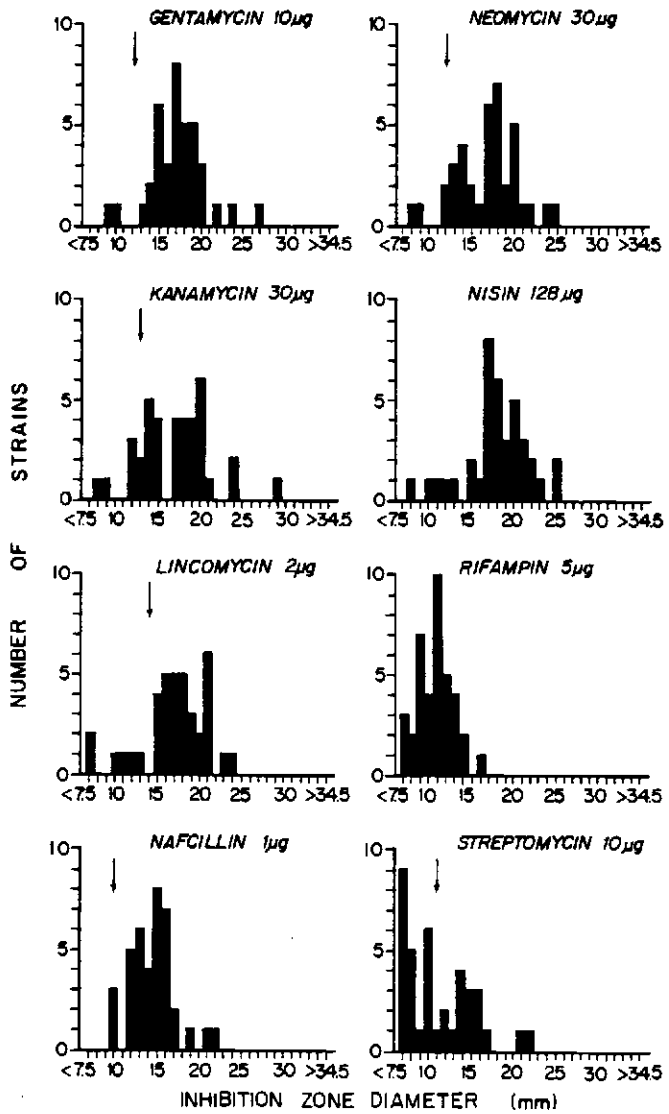

Fig. 2.2. Distributions of inhibition zone diameters observed in disc tests. Grouped in this Fig. are those antimicrobials for which some strains had inhibition zones smaller than the maximum exhibited by clinically resistant bacteria (vertical arrows; values were not available for rifampin or for nisin).

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Chapter 3

Common Occurrence of Plasmid DNA and Vancomycin Resistance in *Leuconostoc*

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ABSTRACT

Resistance to vancomycin permitted detection, in a culture of Streptococcus cremoris 290 PC, of a contaminant Gram-positive coccus. Morphological and physiological characteristics indicated that this bacterium was a strain of Leuconostoc, designated PO 184. This strain contained four plasmid species which were distinct from those harbored by S. cremoris 290 PC. Antibiotic disc susceptibility tests indicated that Leuconostoc PO 184 was also resistant to sulfathiazole and trimethoprim, and sensitive to 17 other antimicrobials. MIC of vancomycin for this strain was > 2,000 ug/ml, and resistance did not depend on drug inactivation. Leuconostoc PO 184 produced a substance which was inhibitory to S. cremoris U134, but not to S. lactis ATCC 11454. Five other leuconostocs produced substances with antibacterial activity. Out of 18 strains of Leuconostoc, 14 were resistant to at least 500 ug/ml vancomycin, including four L. oenos strains which harbored no plasmid DNA in the 1-76 megadalton (Md) range. Twelve Leuconostoc strains contained at least one plasmid species in this mass range. These findings are discussed from the physiological, taxonomical, and ecological standpoints, as well as their potential applications.

INTRODUCTION

Leuconostoc is a genus of fastidious, Gram-positive bacteria of the family Streptococcaceae, found in fermenting vegetables, dairy products, wine, and in sugar refineries (7). Their capacities to produce C_4 compounds (diacetyl and acetoin) and to carry out malo-lactic fermentation are important to the dairy and wine industries, respectively. Leuconostocs are taxonomically and ecologically related to group N streptococci.

Vancomycin, a product of Streptomyces orientalis, is a complex glycopeptide antibiotic which acts by interfering with the biosynthesis of the bacterial cell wall peptidoglycan. It is highly potent against Gram-positive bacteria, but not against Gram-negative organisms. Mycobacteria, molds, and yeasts are insensitive to vancomycin (10,17). Among the susceptible bacterial species, resistant mutants are very rare or nonexistent (4,13).

This paper reports on the occurrence of high-level resistance to vancomycin as a common trait of Leuconostoc strains. We have also applied a recently described, streptococcal plasmid isolation method (16) to these bacteria, to extend the original observation of O'Sullivan and Daly (T. O'Sullivan and C. Daly, abstr., Irish J. Food Sci. Technol. 6: 206, 1982) on the presence of plasmid DNA in Leuconostoc.

MATERIALS AND METHODS

Bacterial strains. The identity and origin of the strains used in this study are listed in Table 3.1.

Culture media. For L. oenos strains, culture maintenance, cell propagation before lysis, and MIC determinations were carried out in modified Rogosa medium (18), from which liver extract was omitted, and to which fructose (3.0 g/1) and L-malic acid (2.0 g/1) were added. For other Leuconostoc strains, the medium employed was MRS broth (3) containing only 0.4% glucose, and in some instances fortified with filtered V-8 juice (Campbell Soup Co.) at 200 ml/1. (Siegel et al. observed that cells of Streptococcus grown in media containing higher glucose concentrations were refractory to lysis; ref. 20). This modified MRS broth was also employed in the determination of vancomycin MICs for lactic streptococci. Escherichia coli V517 (14) was propagated in M17 broth (21).

Carbohydrate fermentation test broth contained, in g/1: beef extract, 3.0; proteose-peptone no.3, 10.0; yeast extract, 3.0; bromocresol purple, 0.015; $MnSO_4 \cdot 2H_2O$, 0.055; $MgSO_4$, 0.24; and the indicated carbohydrate, 10.0. The pH was 6.65. Acid production was detected as color change to yellow, after 24 h incubation at 24°C (0.1% inoculum). Sucrose indicator agar (15) was fortified with 3.0 g/1 Polypeptone™ (BBL Microbiology Systems) and 0.12 g/1 $MgSO_4$.

Mueller-Hinton agar, brain-heart infusion, MRS, Pennassay, and Todd-Hewitt broths were from Difco Laboratories.

Chemicals. Vancomycin hydrochloride was purchased from Sigma Chemical Co. Stock solutions were prepared in sterile distilled water, immediately before use.

Determination of antibiotic susceptibility. Disc tests were performed on Mueller-Hinton agar (2), using commercially obtained antibiotic discs (Difco or BBL). MIC determinations in the media indicated above were performed at 24°C. For L. oenos strains, 5% inoculum and up to 44 h incubation were used; for all other strains, these parameters were 1% and 22 h, respectively.

Test for bacteriocin production. The method of Kékessy and Piguet (12) was followed. The medium employed was MRS agar containing V-8 juice, as described above. Each plate was incubated anaerobically for 23 h at 29°C, before inoculation of the reverse side of the agar with an indicator strain. Results were read after a second incubation period, under the same conditions.

Cell lysis, isolation of plasmid DNA, and agarose gel electrophoresis. A recently described method (16) was used, with the following modifications: 8-ml Leuconostoc cultures from a 10% inoculum were incubated at 24°C until their absorbance at 660 nm (1.0 cm path) reached at least 0.50 (but not more than 0.70). For most strains, including L. oenos Ey2d, 10 h were sufficient; other L. oenos strains (23, Er1a, ML34, and MLT-k1i) required up

to 48 h incubation. Cells were harvested, washed, and frozen overnight at -72°C before lysis. Tris-sucrose buffer (16) was added after 6.5 min incubation with lysozyme and mutanolysin at 37°C, and the samples immediately chilled for 2 min, before addition of triisopropylnaphthalenesulfonic acid (sodium salt), followed by NaOH. In this protocol, the strains examined lysed to transparency, with the exception of L. oenos Ey2d and the new isolate described in this paper (Leuconostoc PO 184). The latter strain nevertheless yielded plasmid DNA in quantities comparable to those obtained from other leuconostocs. After RNase treatment (15 min), the samples were extracted with 200 ul chloroform : isoamyl alcohol (24:1), and 10 ul of the aqueous phase used directly in electrophoresis. Molecular masses were estimated from a double-logarithmic plot based on E. coli V517 standards (14).

RESULTS

Isolation and characterization of a vancomycin-resistant, Gram-positive coccus. During an investigation of antimicrobial resistance factors in mesophilic lactic streptococci (this thesis, Chapter 2), we observed that a culture of Streptococcus cremoris 290 PC (and only this culture, out of 48 tested) exhibited heterogeneity with regard to sensitivity to vancomycin. In the Bauer-Kirby disc susceptibility test (2), numerous resistant clones grew inside the zone of inhibition around a disc containing this antibiotic (30 ug). Presence of a contaminant was further indicated by the fact that heterogeneity could not be observed after culture reisolation, with a zone of complete inhibition by vancomycin occurring instead. Moreover, while the original culture displayed heavy bottom growth and very light growth throughout M17 broth (21), after 24 h incubation at 24°C, only heavy bottom growth was seen with the reisolated culture. In M17 broth containing vancomycin (15 ug/ml), the original culture still displayed very light growth throughout the medium, while heavy bottom growth was abolished; the reisolated culture produced no growth in this medium. When this test was repeated with doubling concentrations of vancomycin, even 480 ug/ml did not prevent light, diffuse growth in M17 broth, if the original culture (but not the reisolated one) was used as inoculum. Cells from such an M17 broth culture, containing 60 ug/ml vancomycin, were isolated

on glucose-M17 agar with the same concentration of the antibiotic.

This vancomycin-resistant isolate displayed the following characteristics: round or slightly elongated Gram-positive cocci, mostly in chains of 4 to 10 cells, but also in pairs or small clusters; catalase-negative. In MRS broth (3), growth was observed at 7.5 °C and at 30°C, but not at 37°C. At 30°C, this strain grew poorly in brain-heart infusion, Pennassay broth, or Todd-Hewitt broth, and only slightly better in M17 broth containing either glucose or lactose. In contrast, abundant growth was observed in MRS broth and in tomato-juice broth (1). On blood agar, this isolate produced very small colonies (less than one mm diameter) and no hemolysis, after 48 h of aerobic incubation at 30°C. Large (2.0 mm) mucoid colonies, suggestive of dextran production, were observed after 24 h of anaerobic incubation at 30°C on sucrose-indicator agar (15). The results of a series of carbohydrate fermentation and litmus milk tests performed with this strain and, for comparison, with two strains of Leuconostoc, are shown in Table 3.2. None of these three cultures fermented dulcitol, erythritol, glycerol, inositol, inulin, rhamnose, or sorbose, in complete agreement with what is accepted for the genus Leuconostoc (7). Taken together, these results indicated that the vancomycin-resistant isolate was a strain of Leuconostoc. This bacterium is here designated Leuconostoc PO 184.

Confirmation of non-identity between Leuconostoc PO 184 and

the culture which originally harbored it, S. cremoris 290 PC, came from the comparison of the plasmid profiles of the two strains, which are obviously different (Fig. 3.1). Great dissimilarity was also observed upon microscopic examination of the two cultures: S. cremoris 290 PC formed very long chains (>50 cells in length), while Leuconostoc PO 184 did not.

Clearly, it was of interest to examine whether Leuconostoc PO 184 exhibited resistance to other antimicrobial agents. Discs containing the following substances were employed in the Bauer-Kirby susceptibility test (2): amikacin, ampicillin, bacitracin, cephalothin, chloramphenicol, chlortetracycline (aureomycin), erythromycin, gentamycin, kanamycin, lincomycin, nafcillin, neomycin, penicillin G, rifampicin, streptomycin, sulfathiazole, tetracycline, tobramycin, trimethoprim, and vancomycin. This organism was susceptible to all of these antimicrobials, with the exceptions of sulfathiazole, trimethoprim, and vancomycin, which caused no detectable inhibition. Low sensitivity to sulfathiazole and to trimethoprim has also been observed in our studies of the lactic streptococci (this thesis, Chapter 2).

Vancomycin resistance and plasmid DNA in strains of Leuconostoc. Eighteen strains of Leuconostoc were randomly selected and examined for susceptibility to vancomycin and for plasmid DNA content. The strains and their respective vancomycin MICs are listed in Table 3.1, and their plasmid profiles shown in

Figs. 3.1 and 3.2. Four strains of L. oenos examined contained no plasmid DNA in the range of 1-76 Md; lysis of a fifth strain (L. oenos Ey2d) was not achieved. All of the other strains examined did contain plasmid DNA, varying in molecular mass from ca. 1.9 to ca. 76 Md. Fourteen of the strains were resistant to at least 500 ug/ml vancomycin, while three others, as well as five lactic streptococcal strains tested under identical conditions, were inhibited by the smallest concentration tested (10 ug/ml).

Lack of vancomycin inactivation and production of an antibacterial substance by Leuconostoc PO 184. An experiment was performed to examine whether the high-level resistance to vancomycin exhibited by Leuconostoc PO 184 was due to inactivation of the drug in the culture medium. Three tubes were prepared, each containing MRS broth (4 ml). To tubes A and B vancomycin was added to a final concentration of 1,000 ug/ml; tube C received no antibiotic. Tubes A and C were inoculated with 50 ul of a fresh culture of Leuconostoc PO 184; tube B was not inoculated. All three tubes were incubated at 30°C for 15 h. Cultures A and C were then neutralized with 10% NaOH, to the pH of uninoculated broth (6.4), and the cells were removed by centrifugation, followed by filtration through a Millipore membrane (0.45 um pore size). Each solution (20 ul of A, B, or C) was spotted onto 6.5 mm sterile filter paper discs (Difco), which were allowed to dry briefly, and used in the Bauer-Kirby susceptibility test (2). (It can easily be calculated that such discs contained no more than 20

ug of vancomycin.) Four strains of vancomycin-sensitive, lactic streptococci were used as indicator organisms. A commercially obtained disc with 30 ug vancomycin (Difco) was included as a control. Inhibition zone diameters were measured after 24 h incubation at 30°C.

The results, shown in Table 3.3, indicated that this bacterium produced little or no alteration in the concentration of vancomycin in the medium. It was evident that a rather high concentration of active antibiotic remained in the broth, after growth of Leuconostoc PO 184. A clear zone of inhibition of S. cremoris U134 was produced by the filtrate of a culture to which vancomycin had not been added, showing that Leuconostoc PO 184 produces a substance or substances with antibacterial activity.

Production of antibacterial substances by strains of Leuconostoc. With the exception L. oenos strains, all of the remaining leuconostocs listed in Table 1 were employed, both as producers and as indicators, in the Kékessy-Piguet (12) test for bacteriocin production. Indicator strains also included S. lactis ATCC 11454 and NCDO 1404. The following strains caused inhibition of Leuconostoc or S. lactis strains: LDF, 29, 39, 180, and 181.

DISCUSSION

In 1974 Reinbold and Reddy (19) observed that two strains of L. dextranicum were resistant to vancomycin, as determined by a disc susceptibility test. These authors did not examine this matter in further detail.

Typical MICs of vancomycin for Gram-positive bacteria, such as Streptococcus or Staphylococcus, are of the order of 1.0 ug/ml (10). Approximately 500-fold higher concentrations of this antibiotic could not inhibit most of the Leuconostoc strains examined in this study. To our knowledge, this is the first detailed account of the existence of non-acid-fast, Gram-positive bacteria which are naturally resistant to high levels of vancomycin. To a certain extent, it is not surprising that the insensitivity of Leuconostoc strains to this antibiotic remained undetected for so long after its discovery, given the non-pathogenicity and lack of involvement of this genus in any aspect of medical microbiology. It should prove interesting to investigate whether other medically unimportant bacteria related to Leuconostoc, such as Pediococcus, might also be insensitive to vancomycin.

The antibacterial action of vancomycin is due to complexation with the pentapeptide precursor of peptidoglycan biosynthesis. Binding of the antibiotic requires that the peptide precursor have five aminoacids, and that the terminal pair be D-alanyl-D-alanine

(4,6,17). Our results show that in the case of one strain at least, resistance to vancomycin does not depend on its inactivation. Although Harney et al. concluded that there was little difference between the chemical compositions of the Leuconostoc cell wall and of the Streptococcus cell wall (9), our observations suggest that one or more steps in the respective biosynthetic pathways are dissimilar enough to account for an enormous difference in susceptibilities to this antibiotic. The fact that not all of the Leuconostoc strains examined were insensitive to vancomycin (or, for that matter, equally susceptible to the lysis protocol employed) might reflect the findings of Kandler, that several types of murein exist within this genus (11).

The high susceptibility to vancomycin found in lactic streptococci, which are closely related to Leuconostoc from the standpoint of ecology, suggests that the resistance exhibited by the latter genus might be coincidental, rather than a specific adaptation to the possible presence of antibiotic-producing Streptomyces in their habitat. An obvious application of our observations would be the use of vancomycin in selective media for the isolation of wild strains of Leuconostoc, or for the enumeration of these bacteria in mixed-strain, lactic starter cultures. Media containing sodium azide (J. V. Mayeux, W. E. Sandine, and P. R. Elliker, abstr. M47, J. Dairy Sci. 45: 655, 1962), salt (5), or low concentrations of tetracycline (F. E.

McDonough, R. E. Hargrove, and R. P. Tittsler, abstr. M48, J. Dairy Sci. 45: 656, 1962) have been employed for these purposes.

The production of inhibitory substances by Leuconostoc strains, as exemplified by our results, must clearly be taken into consideration when composing mixed-strain fermentation starters.

We have also demonstrated the application of a recently described, lactic streptococcal plasmid isolation method (16) to the genus Leuconostoc. O'Sullivan and Daly first reported on the presence of plasmid DNA in these bacteria (T. O'Sullivan and C. Daly, abstr., Irish J. Food Sci. Technol. 6: 206, 1982). All of ten strains examined by these authors contained at least one plasmid species, in the 2.5 to 40 Md range; wine leuconostocs were not examined.

In her description of the genus Leuconostoc, Garvie (7) emphasizes the clear distinction between L. oenos and the other species in this genus. The results obtained with the strains of L. oenos examined in this study suggest that the presence of plasmid DNA may be less frequent in this species than in other leuconostocs; if true, this would be yet another characteristic separating the two groups of microorganisms. On the other hand, their unity as parts of the same genus is supported by the shared trait of resistance to vancomycin.

Garvie has also stated (8) that L. dextranicum and L. cremoris may be subspecies of L. mesenteroides, and that further work on the relationship of these bacteria is needed. It seems

reasonable to suppose, with basis on what is known for the lactic streptococci, that some of the traits for which Leuconostoc bacteria exhibit a high degree of variability, such as the fermentation of different carbohydrates, might depend on plasmid-carried genes. This hypothesis is supported by the concurrent loss of plasmid DNA and of the capacity to utilize lactose or citrate, observed in leuconostocs by O'Sullivan and Daly (abstr., Irish J. Food Sci. Technol. 6: 206, 1982).

The complexity of Leuconostoc plasmid profiles makes these bacteria similar to the lactic streptococci, in yet another aspect. It is tempting to speculate that future genetic manipulations of cultures used in the dairy and wine industries might involve transfer of genetic material between these two groups of microorganisms.

ACKNOWLEDGMENTS

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TABLE 3.1. Bacterial strains

Identity	Source	Vm MIC (ug/ml)[a]	Plasmid profile (Fig. & lane)
Escherichia coli V517	E. Lederberg (PRC)[b]	Not tested	3.1-A, 3.2-A
Leuconostoc oenos ML 34	R. Kunkee (UCD)	> 1,000[c]	3.1-E, d
L. oenos MLT-kli	A. Gryczka (MLT)	> 1,000[c]	d
L. oenos 23	T. Sozzi (NESTEC)	1,000	d
L. oenos Er1a	Oregon wine[e]	1,000	d
L. oenos Ey2d	"	> 1,000	Not examined
L. mesenteroides 98	This laboratory	> 1,600	3.2-E
L. cremoris 91404	"	1,000	3.1-F
Leuconostoc sp. 16-9	"	> 1,000	3.2-F
L. cremoris CAF 7	"	> 1,000	3.2-B
Leuconostoc sp. P325	"	1,000	3.2-J
L. dextranicum LDF	"	> 1,000[c]	3.2-I
Leuconostoc sp. 14 AM	"	< 10	3.2-M
Leuconostoc sp. 39	"	< 10	3.2-L
Leuconostoc sp. 29	"	> 1,000	3.2-H
L. dextranicum 180	"	> 1,000	3.2-C
L. dextranicum 181	"	> 1,000[c]	3.2-D
L. cremoris 225	"	500	Not examined
L. dextranicum 688	"	< 5	3.1-D, 3.2-K
Leuconostoc PO 184	This study	> 2,000	3.1-C, 3.2-G

TABLE 3.1. Bacterial strains (cont.)

Identity	Source	Vm MIC (ug/ml)[a]	Plasmid profile (Fig. & lane)
Streptococcus lactis MG 1363	M. Gasson (NIRD)	< 10	Not examined
S. lactis NCDO 1403	NIRD	Not tested	"
S. lactis NCDO 1404	NIRD	< 10	"
S. lactis ATCC 11454	D. LeBlanc (NIDR)	Not tested	"
S. lactis ATCC 7962	This laboratory	< 10	"
S. lactis C10	"	Not tested	"
S. cremoris 290 PC	"	< 10	3.1-B
S. cremoris U134	"	< 10	Not examined

[a] Vm = vancomycin; concentrations tested were 0, 10, 30, 100, 500, and 1,000 ug/ml, unless otherwise noted.

[b] Individual affiliations: PRC = Plasmid Reference Center, Stanford University School of Medicine; UCD = Dept. of Viticulture and Enology, University of California, Davis; MLT = Microlife Technics, Sarasota, Florida; NESTEC = Nestlé Products Technical Assistance Co. Ltd., La Tour du Peilz, Switzerland; NIRD = National Institute for Research in Dairying, Shinfield, Reading, England; NIDR = National Institute for Dental Research, Bethesda, Maryland.

[c] Growth was impaired, but not completely inhibited, at this concentration.

[d] This strain contained no plasmids in the 1-76 megadalton mass range; see Fig. 3.1 legend.

[e] Y. S. Izuagbe et al., submitted for publication.

TABLE 3.2. Carbohydrate fermentation and litmus milk tests

	L. mesenteroides 98	*L. dextranicum* 688	Vm^R isolate[a]
Acid from:			
arabinose	+	+	+
cellobiose	+	+	-
dextrin	+	+	-
glucose	+	+	+
lactose	-	+	+
mannitol	-	-	-
melibiose	+	-	+
ribose	+	+	-
salicin	+	+	+
sucrose	+	+	+
trehalose	+	+	-
xylose	+	-	+
Litmus milk[b]			
clot	-	-	-
reduction	-	-	-
gas	+[c]	+[c]	+[c]
Supplemented litmus milk[d]			
clot	-	+	+
reduction	+	+	+
gas	+	+	+

[a] Vm^R = vancomycin-resistant.
[b] Results were read after 36 h at 24°C.
[c] Minute amounts.
[d] Glucose (1.0%) and yeast extract (0.5%) added.

TABLE 3.3. Activity of vancomycin added to Leuconostoc PO 184 culture medium

	Diameter of inhibition zone (mm)[a]			
	Std. disc[b]	Disc with filtrate (20 ul) from		
		A-Culture with 1,000 ug/ml Vm[c]	B-Uninoculated control w/ 1,000 ug/ml Vm	C-Culture without Vm
Indicator				
S. lactis C10	21	21	19	7.5 [d]
S. lactis ATCC 11454	20	20	20	0
S. lactis NCDO 1403	18	18	18	7.5 [d]
S. cremoris U134	22	22	22	10

[a] Two discs from each filtrate and one standard disc were employed per indicator strain. Results from filtrates represent averages.
[b] Commercially obtained disc with 30 ug vancomycin (Difco).
[c] Vm = vancomycin.
[d] A very narrow zone of inhibition was visible around disc (disc diameter = 6.5 mm).

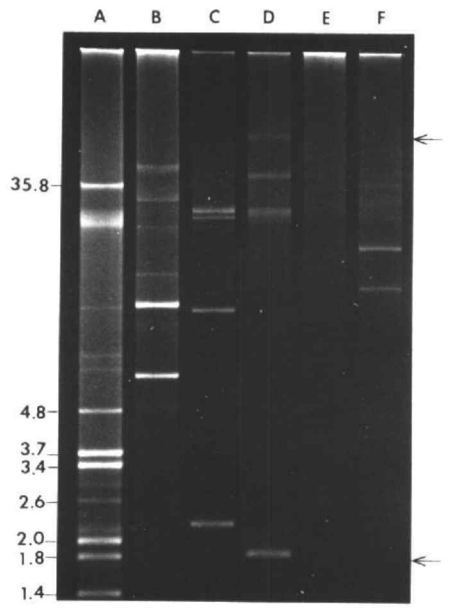

Fig. 3.1. Bacterial plasmid profiles. Lane A, E. coli V517 ; values on the left denote molecular masses in Md (ref. 14). Lane B, S. cremoris 290 PC (6.5, 11, 33, 49 Md). Lane C, Leuconostoc PO 184 (2.4, 11, 25, 28 Md); this strain was originally harbored, as a contaminant, by the culture shown in lane B. Lane D, L. dextranicum 688 (1.9, 47, 76 Md); among the leuconostocs examined, this bacterium carried both the smallest and largest plasmid species (arrows). Lane E, L. oenos ML 34, showing absence of plasmid DNA in this size range; similar results were obtained with L. oenos strains 23, Erla, and MLT-kli. Lane F, L. cremoris 91404 (13, 19, 35 Md).

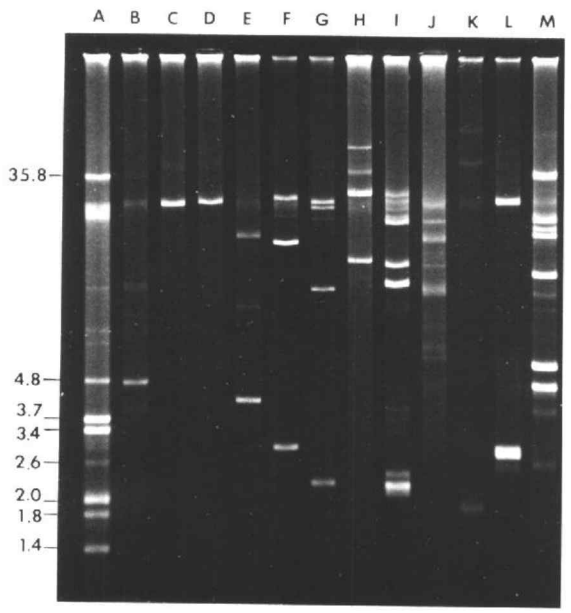

Fig. 3.2. Bacterial plasmid profiles. Lane A, E. coli V517. Lanes B-M, Leuconostoc strains identified in Table 3.1. The apparent molecular masses (Md) of the plasmid species observed are given after the letter which identifies the lane: B (4.8); C (27); D (27); E (4.3, 18); F (2.9, 17, 29); G (2.4, 11, 25, 28); H (14, 32, 40, 65); I (2.2, 2.3, 11, 13, 21, 26, 31); J (6.2, 6.5, 10, 18, 22, 26); K (1.9, 47, 76); L (2.7, 2.9, 28); and M (4.9, 5.5, 12, 19, 20, 22, 41).

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Chapter 4

Plasmid Linkage of Proteinase and Lactose Fermentation in *Streptococcus lactis* NCDO 1404

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ABSTRACT

Streptococcus lactis NCDO 1404, a nisin producer, contained seven plasmid species, with masses ranging from ca. 1.8 to ca. 45 megadaltons (Md). Transfer in M17 broth resulted in loss of the 45-Md plasmid, with concurrent impairment of growth in milk or milk-based agar media, in spite of fast lactose fermentation. Growth at 41°C or protoplast regeneration were used for plasmid curing. Among seven partly-cured, lactose-negative derivatives, one retained the 45-Md plasmid and formed significantly larger colonies on milk-based agar media. These data indicated linkage of proteinase (Prt^+) to the 45-Md plasmid. A 35-Md species was the only plasmid absent in all lactose-negative derivatives; this plasmid was present in NCDO 1404 and in two lactose-positive derivatives. These data indicated linkage of lactose fermentation (Lac^+) to the 35-Md plasmid. NCDO 1404 and its derivatives produced four colony types on buffered, milk-based agar media containing pH indicators; the colony types corresponded to the four possible combinations of the Prt (+/-) and Lac (+/-) phenotypes. All strains were capable of fast sucrose fermentation, including a nisin-negative derivative. Assignment of nisin production (Nis^+) to a certain plasmid species could not be made.

INTRODUCTION

The study of plasmids has become one of the most important areas of research on the lactic streptococci. In many strains of these bacteria, the association of key phenotypic properties (such as lactose fermentation, proteinase, citrate utilization, or bacteriophage resistance) with plasmid DNA has been demonstrated (17). Although in some instances there seems to be a remarkable uniformity among strains, with regard to the size of plasmids linked to a given phenotype (e.g., citrate utilization depends on the presence of a 5.5 Md plasmid in many strains of S. lactis subsp. diacetylactis; ref. 17), in other cases variability seems to be the rule. Proteinase activity has been reported to depend on plasmids of very different sizes, ranging from 8.5 to 33 Md. The larger Prt^+-associated plasmids may be linked to lactose fermentation as well (6,12,17,22).

We have studied the nisin-producer, S. lactis NCDO 1404, with regard to plasmid content and its relationship to phenotypic properties. Plasmid-curing methods lacking high mutagenic potential (growth at high temperature or regeneration of whole cells from protoplasts) were used to obtain derivatives of NCDO 1404. The analysis of these strains, described in this paper, showed plasmid-dependence of the Lac^+ and Prt^+ phenotypes. In particular, we demonstrated the previously unreported linkage of Prt^+ to a large, 45-Md plasmid which is not associated with the

Lac^+ phenotype. We also observed loss of the Nis^+ phenotype without concurrent loss of the capacity to ferment sucrose, even though a close association of these two markers has been indicated by the work of other researchers (7,10,14).

MATERIALS AND METHODS

Bacterial strains. Escherichia coli V517 (15) was obtained from E. Lederberg, Plasmid Reference Center, Stanford University School of Medicine. S. lactis NCDO 1404 was obtained from the National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, Reading, England. The origin and properties of partly-cured derivatives of this strain are shown in Table 4.1. S. cremoris B1 was from the culture collection maintained in this laboratory. Unless otherwise noted, routine culture maintenance was in M17 broth (24) containing glucose or lactose, as appropriate. In all instances, M17 formulations contained 0.5% yeast extract.

Carbohydrate fermentation tests. Lactose- or sucrose-indicator agars (19) were fortified with 3.0 g/l Polypeptone™ (BBL Microbiology Systems) and 0.12 g/l $MgSO_4$. Plates were incubated at 30°C for 24 h. Strains capable of fermenting the given carbohydrate produced white colonies on these media; non- or slow-fermenters produced red colonies.

Carbohydrate fermentation test broth contained, in g/l: beef extract, 3.0; proteose-peptone no. 3, 10.0; yeast extract, 3.0; bromocresol purple, 0.015; $MnSO_4.2H_2O$, 0.055; $MgSO_4$, 0.24; and lactose or sucrose, 10.0. The initial pH was 6.65. A fresh overnight culture in M17 both was used as inoculum (2.5%). Incubation was at 24°C. In this protocol, fast fermenters changed

the broth color to yellow within three hours; slow fermenters required ca. eight hours.

S. lactis NCDO 1404 and all its derivative strains were tested by both of these methods, and in every case the results were in complete agreement.

Temperature-induced plasmid curing. A culture of S. lactis NCDO 1404 in glucose-M17 broth was incubated at 41°C for 24 h, transferred (2.0%) into fresh broth, and incubated at 41°C for another 24 h. This culture was then intensely vortexed (ca. 3 min at highest setting) and streaked on lactose-M17 agar. Vortexing was done with the purpose of disrupting cell associations, thus increasing the probability of isolation of cured cells (13). Plates were incubated at 41°C for 48 h, colonies were selected on the basis of smaller-than-average size, and transferred to lactose- and sucrose-indicator agars. Out of 18 such colonies tested, seven were Lac^-; none was Suc^-.

Protoplast-induced plasmid curing (6,20). Culture growth and preparation for cell wall digestion were as previously described (21). Incubation with lysozyme and mutanolysin was for 2 h and 45 min at 37°C; this was followed by addition of 250 ul of sucrose-maleate-Pennassay buffer (2). This buffer was modified by an increase in sucrose concentration to 0.7 M (9), by omission of $MgCl_2$, and by addition of filter-sterilized bovine serum albumin (0.1% final concentration; refs. 4,9). After 30 min additional incubation at 37°C, the preparation was stored overnight (17 h) at

4°C. Protoplasts were then pelleted at 1,430 x g (cold room, six minutes), resuspended in sucrose-maleate-Pennassay buffer, pelleted and resuspended a second time. Serial dilutions in the same buffer were spread onto glucose-M17 solid medium containing 0.5 M sucrose (5), 0.4% gelatin (4), and 2.1% agar (8,26). Protoplasts were also plated onto glucose-M17 agar of normal tonicity, for assessment of osmotic stability. Less than 0.2% of the treated cells were able to grow on the latter medium. Plates were incubated anaerobically (GasPak™, BBL) at 30°C for 72 h, although regenerant colonies could be seen after as little as 20 h. Colonies were randomly selected and transferred to lactose- and sucrose-indicator agars. Out of ca. 100 such colonies tested, two were Lac^-; none was Suc^-.

Growth in litmus milk. A fresh, overnight culture in M17 broth was used to inoculate (at 1.0%) sterile nonfat milk containing 0.075% litmus (Difco Laboratories). Incubation was at 24°C, for 48 h at most.

Fast/slow differential agars (FSDA). Two types of buffered, milk-based agar media were used which permitted differentiation of four colony types, corresponding to the possible combinations of the Lac (+/-) and Prt (+/-) phenotypes. FSDA-I (A. R. Huggins and W. E. Sandine, J. Dairy Sci., in press) contained 1.5% agar, 1.9% sodium glycerophosphate (Ruger Chemical Co., Princeton, NJ), 0.1% litmus (Difco), and 10.0% nonfat milk powder. The latter was autoclaved separately from the other components, and added before

pouring plates. FSDA-II (D. L. Wilrett, Ph.D. thesis, Oregon State University, 1982) contained 1.5% agar, 0.5% trimagnesium phosphate (Stauffer Chemical Co., St. Louis, MO), 0.005% bromocresol purple, and 10.0% nonfat milk powder. This medium is prepared as three separate parts (agar plus indicator; insoluble buffer; and milk), which are mixed immediately before pouring plates. Both FSDA-I and FSDA-II are described in detail elsewhere (W. E. Sandine, The streptococci: milk products, in S. Gilliland, ed., Bacterial starter cultures for foods, CRC Press, Boca Raton, FL, in press). Plates were inoculated by streaking with a 10-fold, normal saline dilution of a fresh litmus milk culture (15 h, 24°C), incubated anaerobically (GasPak™, BBL) at 30°C for 48 h, and photographed.

Test for nisin production and sensitivity. The method of Kekessy and Piguet (11) and glucose-M17 agar plates were used. Plates were inoculated with ca. one microliter of a fresh culture of the strain to be tested as producer, incubated at 30°C for 20 h, inverted, and inoculated with the indicator strain. Transfer of the agar to the plate lid was greatly facilitated by use of a 1.7 cm-wide, sterile spatula, with rapid inversion of the plate, instead of tapping against the bench surface. Results were read after a second 20-h period at 30°C.

Cell lysis, isolation of plasmid DNA, and agarose gel electrophoresis. A recently described method (21) was used, with the following modifications: tris-sucrose buffer was added after

6.5 min incubation with lysozyme and mutanolysin at 37°C, and the samples immediately chilled for 2 min, before addition of triisopropylnaphthalenesulfonic acid (sodium salt), followed by NaOH. After RNase treatment (15 min), the samples were extracted with 200 ul chloroform : isoamyl alcohol (24:1), and 18 ul of the aqueous phase used directly in electrophoresis (4 h 20 min). Molecular masses were estimated from a double-logarithmic plot based on E. coli V517 standards (15).

RESULTS

Plasmid linkage of Prt$^+$. S. lactis NCDO 1404 contained seven plasmid species, with molecular masses of ca. 1.8, 2.3, 4.7, 15, 30, 35, and 45 Md (Figs. 4.1 and 4.2). We observed that after serial transfer in lactose-M17 broth, this strain spontaneously lost its 45-Md plasmid, producing the derivative strain PO 384. Two other characteristics distinguished PO 384 from the parental strain: a significant delay in the coagulation of litmus milk (Table 4.1), and visibly impaired growth on both types of FSDA (Figs. 4.3 and 4.4, and Table 4.2). Since PO 384 could ferment lactose as fast as NCDO 1404 (Table 4.1), these results suggested that the 45-Md plasmid coded for Prt$^+$, which is required for optimum growth in milk (12,17), and which is conceivably dispensable in the aminoacid- and peptide-rich M17 medium (24).

Further support for the hypothesis of association of Prt$^+$ and the 45-Md plasmid came from the examination of seven Lac$^-$ derivatives of NCDO 1404 (Table 4.1). Although these strains could not be distinguished on the basis of their growth in litmus milk, one of them (PO 254) grew outstandingly better than the other six, on both types of FSDA (Figs. 4.3 and 4.4, and Table 4.2). PO 254 was the only Lac$^-$ strain which contained the 45-Md plasmid.

Plasmid linkage of Lac$^+$. The seven Lac$^-$ derivatives of S. lactis NCDO 1404 described in this study were obtained by

plasmid-curing methods of low mutagenic potential. All seven strains were missing a 35-Md plasmid species (Figs. 4.1 and 4.2); there was no other plasmid band which was absent in all seven derivatives. All three Lac^+ strains described here (NCDO 1404, PO 384, and PO 2551) did contain the 35-Md plasmid. Moreover, a comparison of the plasmid profiles of strains PO 384 (Lac^+), PO 316 (Lac^-), and PO 317 (Lac^-) revealed that the only difference between them was that the 35-Md plasmid band was present in the former, and absent in the latter two strains (Figs. 4.1 and 4.2). These results strongly suggested an association between this plasmid and the Lac^+ phenotype.

Three other Lac^+ isolates, obtained after growth at 41°C, had plasmid profiles identical to those of PO 316 and PO 317, and were not characterized further.

Selection of a Lac^+ Prt^+ variant in milk. Derivative strain PO 255, although Lac^- (as determined by growth on lactose-indicator agar and in lactose fermentation test broth), was initially found to coagulate litmus milk after ca. 24 h at 24°C. This result was in disagreement with those observed with all other Lac^- strains examined here; this suggested that PO 255, although picked as an isolated colony after protoplastization and whole-cell regeneration, was in fact a mixture of strains. Re-examination of a plate of lactose-indicator agar, onto which PO 255 had been streaked for confirmation of its Lac^- phenotype, revealed that it did contain ca. 2.5% Lac^+ colonies. After growth

in litmus milk and streaking on lactose-indicator agar, this figure increased to ca. 75%, clearly demonstrating the selection of the Lac^+ variant, and explaining the late milk coagulation. Reisolation of the Lac^+ variant (from the original plate containing only ca. 2.5% such colonies) produced strain PO 2551. This strain, except for the loss of the 15-Md plasmid (Figs. 4.1 and 4.2), was indistinguishable from NCDO 1404. Lac^- strain PO 2550 was concurrently isolated, for comparison; it had also lost the 15-Md plasmid, which is present in PO 255 (Fig. 4.1).

Differentiation of the four possible Lac / Prt phenotype combinations on agar media. Figs. 4.3 and 4.4 clearly show four colony types produced by S. lactis NCDO 1404 and its derivative strains, on both types of FSDA. The descriptions given in Table 4.2 complement this information. Although differentiation of two, and even three colony types on these media have been reported (A. R. Huggins and W. E. Sandine, J. Dairy Sci., in press; W. E. Sandine, The streptococci: milk products, in S. Gilliland, ed., Bacterial starter cultures for foods, CRC Press, Boca Raton, FL, in press), to our knowledge this is the first instance when all four possible phenotype combinations could be distinguished on a solid medium.

Conversion to Nis^- phenotype with retention of Suc^+ phenotype. All isolates tested after growth at 41°C or protoplast regeneration were Suc^+, including all Lac^- strains described here. S. lactis NCDO 1404, its derivative strains, and S. cremoris B1

were tested for nisin production and sensitivity. S. cremoris B1 did not produce nisin, and was sensitive to it. With the exception of PO 315, all other strains produced nisin, and were not inhibited by it, in the Kékessy-Piguet test. Strain PO 315 was inhibited by all 1404-related strains except itself; it did not inhibit any of the other strains, including S. cremoris B1 (Fig. 4.5). Thus, strain PO 315 underwent conversion to the Nis^- phenotype, while retaining the Suc^+ phenotype.

DISCUSSION

Plasmid linkage of proteinase and lactose fermentation in the lactic streptococci are well-known phenomena (12,17). Our results indicate that in S. lactis NCDO 1404 the Lac^+ phenotype depends on the presence of a 35-Md plasmid; this is well in agreement with the 30-40 Md range of most of the Lac^+-associated plasmids, characterized in other strains (6,16,17). Prt^+ activity is known to be determined by plasmids ranging from 8.5 to 33 Md; the larger type of Prt^+-associated plasmid might also confer the Lac^+ phenotype (6,12,17,22). Our results indicate association of Prt^+ with a large, 45-Md plasmid that is dispensable in a Lac^+ strain (PO 384). The spontaneous loss of this plasmid by S. lactis NCDO after serial transfer in broth shows that milk-based media, rather than broth, should be preferred for maintenance of lactic streptococcal starter cultures.

FSDA media clearly have great potential for use in genetic studies with the lactic streptococci, as well as in practical applications such as monitoring of starter cultures. This has been shown by Huggins and Sandine (J. Dairy Sci., in press) and by Thunell et al. (25), and is also demonstrated in the present study.

Many workers have proposed plasmid linkage of the Nis^+ phenotype (3,7,10,14,17,18), with basis on results with numerous S. lactis strains, including NCDO 1404 (7). Strain PO 315,

described here, was converted to Nis^- by growth at 41°C. A comparison of the profiles shown in Fig. 4.1 indicates that none of the plasmids in the 1.8–45 Md range, present in S. lactis NCDO 1404, can be associated with the Nis^+ phenotype: no plasmid band present in all other (Nis^+) strains is also absent in PO 315. Scherwitz et al. (23) have suggested that the inability to assign Nis^+ to a certain plasmid species might result from poor recovery of very large plasmids during cell lysis. Perhaps methods known to be adequate for large plasmid isolation (1) could be successful in demonstrating a Nis^+-associated plasmid.

Previous experiments with Nis^+ strains have indicated association of this phenotype with Suc^+ (7,10,14,17); the existence of a "sucrose-nisin plasmid" has recently been proposed (7). Strain PO 315, described in this paper, was isolated without selection for the loss of either marker, and showed conversion to Nis^- with retention of Suc^+. While this is certainly a preliminary observation, there is clearly a need for further studies on the relationship of the two markers in strains of S. lactis.

ACKNOWLEDGMENTS

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TABLE 4.1. Origin and properties of *S. lactis* strains

Strain	Plasmid-curing method	Time for milk coagulation (h)[a]	Phenotype[b] Lac	Prt
NCDO 1404	none	16	+	+
PO 384	spontaneous	24	+	-
PO 254	protoplast	c	-	+
PO 255	"	c	-	-
PO 2551	" d	16	+	+
PO 2550	" d	c	-	-
PO 313	growth at 41°C	c	-	-
PO 315	"	c	-	-
PO 316	"	c	-	-
PO 317	"	c	-	-

[a] Incubation at 24°C, with 1.0% inoculum.
[b] As determined by lactose fermentation tests and colony morphology on FSDA-I and FSDA-II.
[c] This strain did not coagulate milk, even after 48 h at 24°C.
[d] Further information on the origin of this strain is given in the Results section.

TABLE 4.2. Colony morphologies on differential agars[a]

Phenotype	Type strain	FSDA-I	FDSA-II
Lac^+ Prt^+	NCDO 1404[b]	1.5–2.0 mm, convex, yellow center	1.5–2.0 mm, slightly convex, yellow center
Lac^+ Prt^-	PO 384	1.0 mm, flat, whitish center	1.0–1.5 mm, slightly convex, colorless
Lac^- Prt^+	PO 254	1.0 mm, convex, whitish center	1.0–1.5 mm, slightly convex, whitish center
Lac^- Prt^-	PO 315[c]	<1.0 mm, flat, colorless, barely visible	1.0 mm, flat, colorless, barely visible

[a] Figures denote colony diameters.
[b] This description also applies to strain PO 2551.
[c] This description applies to all Lac^- strains, with the exception of PO 254.

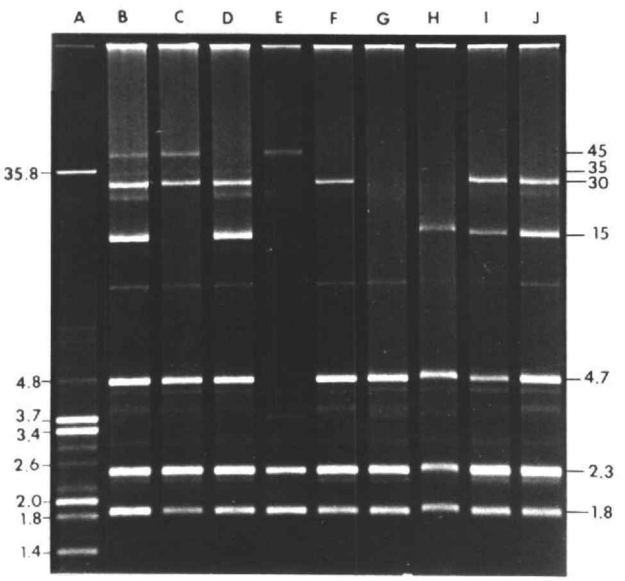

Fig. 4.1. Bacterial plasmid profiles. Figures denote molecular masses (Md). Lane A, E. coli V517 (ref. 15). Lane B, S.lactis NCDO 1404 (Lac^+Prt^+). Lanes C-J, derivative strains PO 2551 (Lac^+Prt^+), PO 384 (Lac^+Prt^-), PO 254 (Lac^-Prt^+), PO 315 (Lac^-Prt^-), PO 2550 (Lac^-Prt^-), PO 255 (Lac^-Prt^-), PO 316 (Lac^-Prt^-), and PO 317 (Lac^-Prt^-), respectively. Strain PO 313 (Lac^-Prt^-), not shown, had the same profile as those of PO 316 and PO 317. The 35-Md plasmid present only in Lac^+ strains (lanes B-D) is more clearly shown in Fig. 4.2.

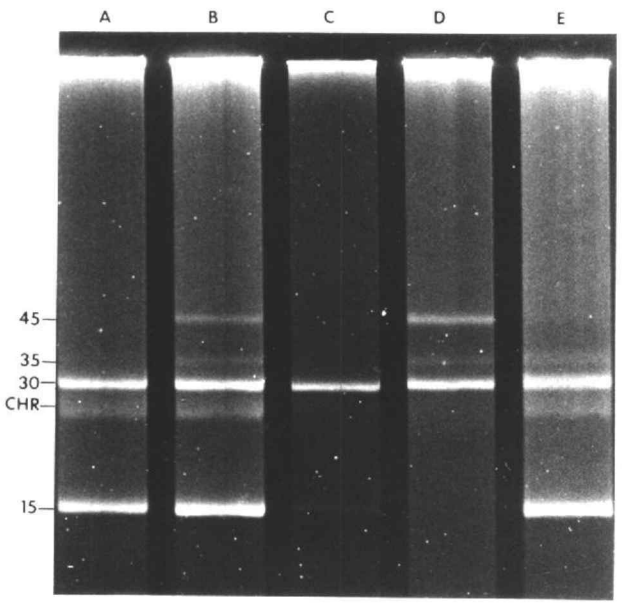

Fig. 4.2. Close-up photograph of agarose gel showing large plasmids in S. lactis NCDO 1404 and derivative strains. Figures denote molecular masses (Md); CHR indicates chromosomal DNA fragments. Shown in lanes A-E are the following strains: PO 317 (Lac^-Prt^-), NCDO 1404 (Lac^+Prt^+), PO 315 (Lac^-Prt^-), PO 2551 (Lac^+Prt^+), and PO 384 (Lac^+Prt^-), respectively. A 35-Md plasmid is present only in the Lac^+ strains (lanes B, D, and E).

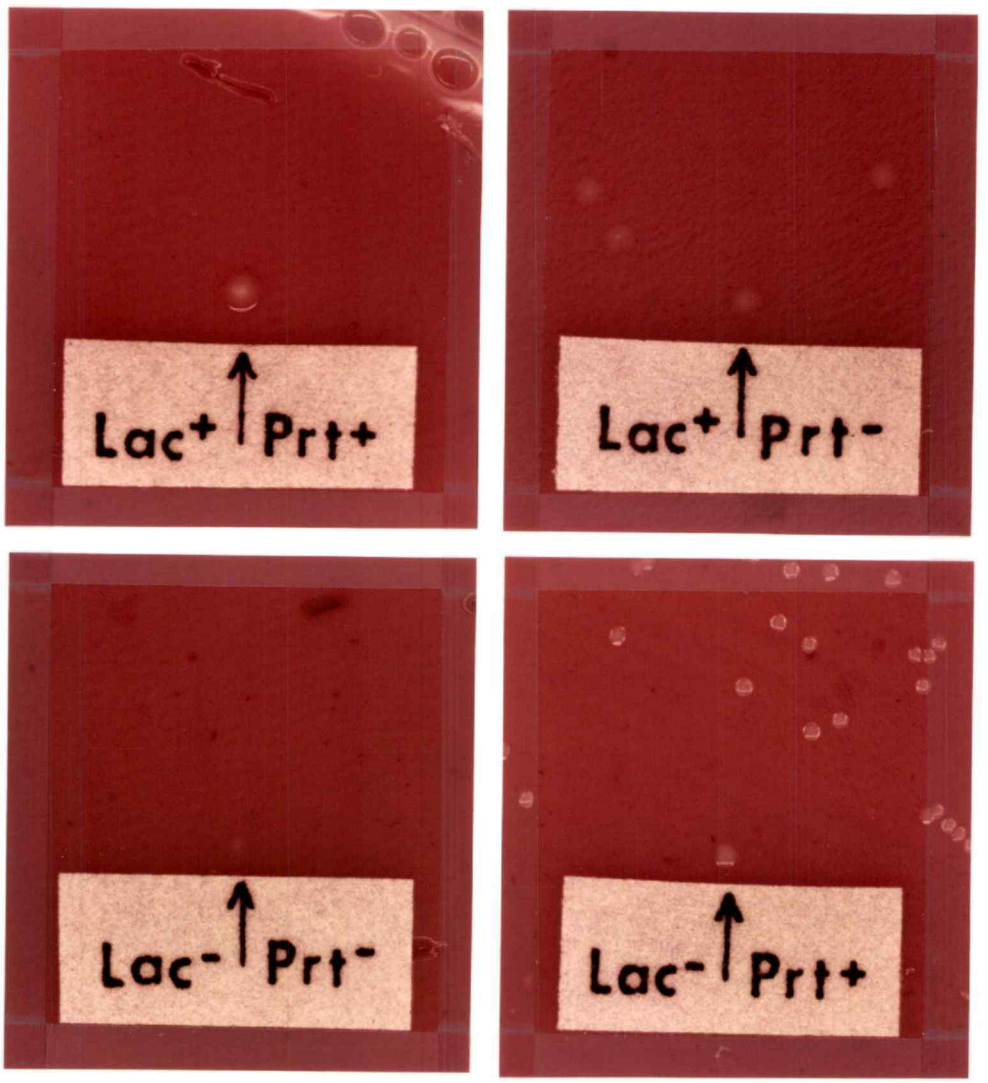

Fig. 4.3. Colony types produced by S. lactis strains on FSDA-I. Clockwise from top left: NCDO 1404, PO 384, PO 254, and PO 315.

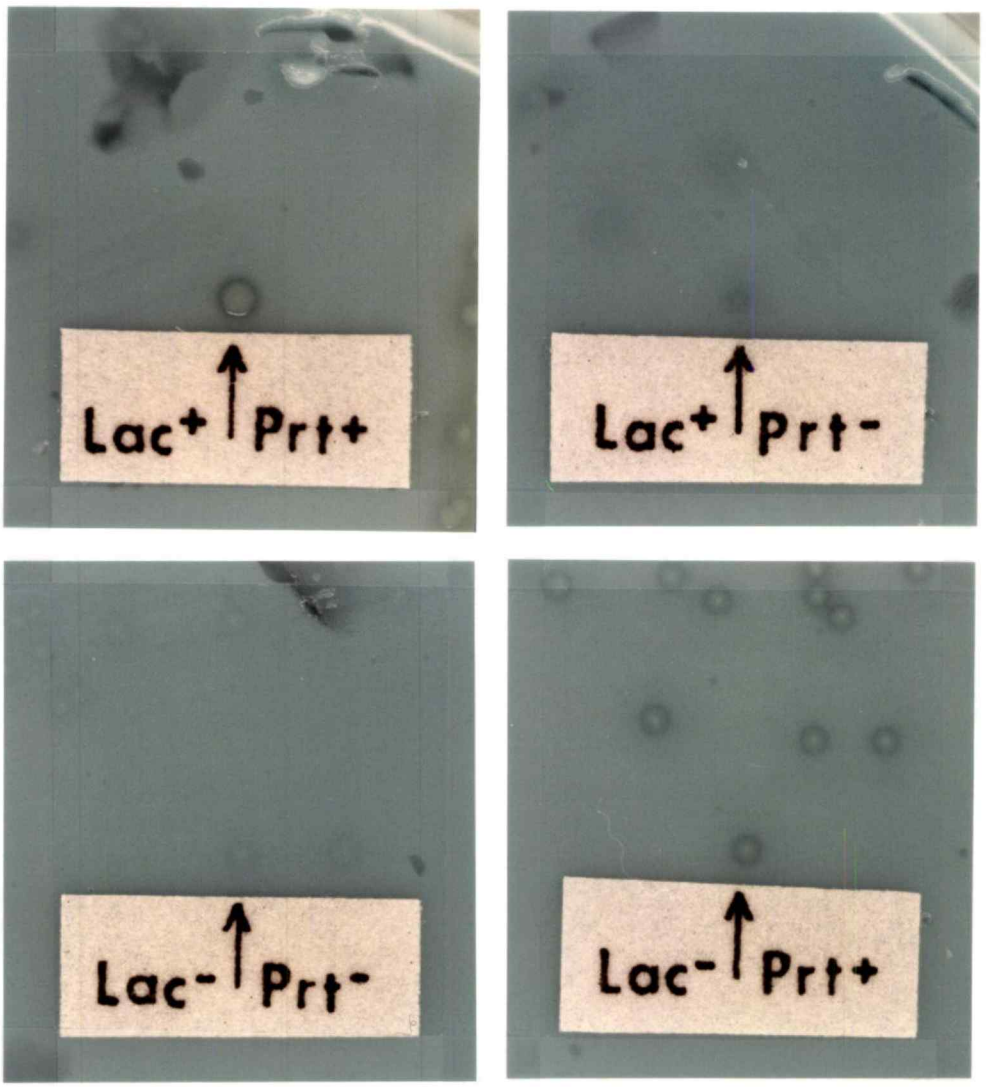

Fig. 4.4. Colony types produced by S. lactis strains on FSDA-II. Clockwise from top left: NCDO 1404, PO 384, PO 254, and PO 315.

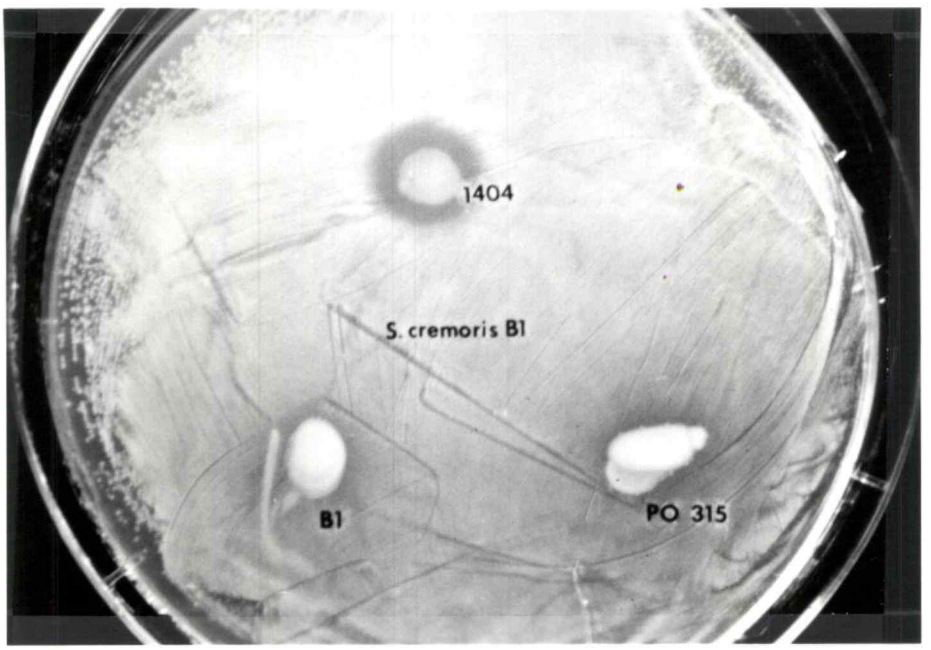

Fig. 4.5. Kékessy-Piguet test plate showing nisin production by S. lactis NCDO 1404 (top), but not by S. cremoris B1 (lower left) or S. lactis PO 315 (lower right). S. cremoris B1 was used as the indicator organism (lawn).

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CONCLUDING REMARKS

It seems appropriate to close this report with a realistic assessment of the value of studies such as described here. R. C. Lawrence, a well-known microbiologist at the New Zealand Dairy Research Institute, has expressed skepticism of the possibility that significant strain improvements could actually result from work on the genetics of lactic acid bacteria (W. E. Sandine, personnal communication). A recent report by Anderson and McKay (1) would seem to strengthen Lawrence's position. Those authors reported that an increase in the copy number (from 10 to 20 per cell) of plasmid-carried *lac* genes, following an *in vivo* recombination event, did not confer on the host bacterium any detectable advantage for growth in milk.

A position of skepticism seems less tenable, however, upon closer inspection of the literature. The paper by McKay and Baldwin (3) is a prime example of strain improvement in the lactic streptococci through the application of genetic techniques. These authors reported the chromosomal integration of *lac* genes following transduction into a cured, Lac^- derivative of *S. lactis* C2. Stabilization of lactose metabolism was clearly demonstrated by the fact that these Lac^+ transductants showed no conversion to Lac^- in continuous culture, whereas under the same conditions the original strain (with plasmid-carried *lac* genes) exhibited 96% conversion to Lac^-.

Improving the capacity of bacterial strains to resist bacteriophage infection seems to fall within the realm of what could realistically be expected from genetic studies of lactic acid bacteria. A patent recently filed in Great Britain (GB 2 126 237, 1 September 1983) by investigators from Eli Lilly & Co. (Indianapolis, IN) could be cited in support of this notion. Researchers C. L. Hersberger and P. R. Rosteck reportedly were able to protect bacteria from phage infection by introduction of plasmid vectors containing genes for restriction and modification (Biotechnology Newswatch 4: 5, 21 May 1984; McGraw-Hill, Inc., New York).

Even if one endorses the view that the capacity of dairy bacteria to ferment milk has already evolved to its optimum, and cannot be significantly improved by artificial means (as suggested by the work of Anderson and McKay, mentioned above), one can still think of several other reasons why genetic studies of lactic microorganisms should continue. At least that seems to be the position held by the ever-growing biotechnology industry. In recent months, a leading European genetic engineering concern and a major U.S. food processing company have contacted this laboratory, in search of a specialist in the genetics of lactic acid bacteria. In the 1983 Oregon Dairy Industries Meeting in Corvallis, L. L. McKay suggested that in the future lactic streptococci could become important in industrial applications of genetic engineering, mainly because of their lack of

pathogenicity. The growing interest of biotechnology companies would seem to support this hypothesis.

There are yet other areas which can benefit from studies such as described here. One example is the discovery of the insensitivity of leuconostocs to vancomycin, for we now have a highly effective means for the selective enumeration of these bacteria, when in mixed culture with lactic streptococci. Another example is the the study of strain interactions, especially with regard to bacteriocin production and bacteriophage cross-infection.

The remarkable wealth of plasmid DNA in lactic streptococci and leuconostocs, and the fact that the overwhelming majority of these plasmids remain cryptic (2), should in themselves arouse much curiosity, in all but the least inquisitive of scientific minds. Why do so many of these strains often carry so numerous plasmids, obviously at great physiological expense?

Finally, and most importantly, we should constantly remind ourselves that some of the scientific discoveries of greatest impact resulted from basic research which could hardly be linked, at the outset, to clearly discernible practical benefits. I quote from Cesar Milstein (4):

> "At that time we were not thinking about monoclonal antibodies...At that point a lucky circumstance led us to the hybrid-myeloma technique...It is always hard to define the boundary between basic and applied research, but to experience personally the transition from one to the other has made a deep impression on me. I cannot think that if my research aim five or six years ago had

been the production of monoclonal antibodies, I would ever had stumbled on the idea of attempting simultaneously to derive mutant antibody-secreting cells in one corner of the laboratory and to fuse two myeloma cells in another corner. Yet that was the combination that led to the initial production of monoclonal antibodies against sheep red blood cells."

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