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Involvement of a Plasmid in Production of Ropiness (Mucoidness) in Milk Cultures by *Streptococcus cremoris* MS

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Curing and genetic transfer experiments showed that lactose-fermenting ability (Lac^+) and the ability to produce mucoidness in milk cultures (Muc^+) in *Streptococcus cremoris* MS were coded on plasmids. The Lac^+ phenotype was associated with a 75.8-megadalton plasmid, pSRQ2201. The Muc^+ phenotype was associated with a 18.5-megadalton plasmid, pSRQ2202. The Lac plasmid, pSRQ2201, was first conjugatively transferred from *S. cremoris* MS to Lac^- *S. lactis* ML-3/2.2. Later, the Muc plasmid, pSRQ2202, was conjugatively transferred from Lac^- Muc^+ *S. cremoris* MS04 to Lac^+ nonmucoid *S. lactis* transconjugant ML-3/2.201. Subsequently, pSRQ2201 and pSRQ2202 were cotransferred from Lac^+ Muc^+ *S. lactis* transconjugant ML-3/2.202 to Lac^-, nonmucoid, malty *S. lactis* 4/4.2 and *S. lactis* subsp. *diacetylactis* SLA3.25. Transconjugants showing pSRQ2201 were Lac^+; those containing pSRQ2202 were Muc^+. With the transfer of pSRQ2202, the transconjugants *S. lactis* ML-3/2.202 and *S. lactis* subsp. *diacetylactis* SLA3.2501 not only acquired the Muc^+ phenotype but also resistance to bacteriophages, which were lytic to the respective parent strains *S. lactis* ML-3/2.201 and *S. lactis* subsp. *diacetylactis* SLA3.25.

The occurrence of lactic streptococci that produce a mucoid, ropy texture in milk is well documented (8). Such ropy lactic streptococci are used in Scandinavian fermented milks called taette (5, 19), Swedish lang mjolk (2, 11), and Finnish villii (20). Forsen (4) isolated mucoid strains of all three lactic streptococci, namely, *Streptococcus cremoris*, *Streptococcus lactis*, and *Streptococcus lactis* subsp. *diacetylactis*, from Finnish villii.

The instability of the mucoid characteristic in lactic streptococci has been observed by several investigators (5, 8, 11). Foster et al. (5) reported that mucoid lactic streptococci gained or lost the slime-producing property "capriciously." Macura and Townsley (11) found that ropy lactic streptococci lost the mucoid property after 10 to 12 serial transfers; some strains became nonmucoid even after six transfers. Brooker (3), working with a pure milk culture of a ropy *S. cremoris* strain, observed considerable variations in the proportion of cells producing extracellular capsular material. Traditionally, in the production of Scandinavian ropy milks, low-temperature incubation between 13 and 18°C is preferred, because incubation at temperatures higher than 27 to 30°C results in considerable reduction or loss of the desirable high viscosity and mucoidness (2, 11).

The association of several metabolic functions in lactic streptococci with plasmid DNA is now well recognized (15). On the basis of the observed instability of the ropy characteristic in lactic streptococci, Macura and Townsley (11) and McKay (15) suggested that plasmid DNA may be involved in the expression of the mucoid phenotype (Muc^+).

In this paper, we report evidence for the involvement of plasmid DNA (Muc plasmid) in the expression of the Muc^+ phenotype in *S. cremoris* MS. Additionally, data are presented which demonstrate the conjugal transfer of the Muc plasmid from a ropy *S. cremoris* to a nonmucoid (Muc^-) *S. lactis* and from the resultant mucoid *S. lactis* transconjugant to a malty variant of *S. lactis* (formerly *S. lactis* subsp. *maltigenes* [5]) and a strain of *S. lactis* subsp. *diacetylactis*. The expression of the Muc^+ phenotype was observed in all the transconjugants containing the Muc plasmid.

MATERIALS AND METHODS

Cultures and bacteriophages. Bacterial strains used in this study are listed in Table 1. Phage c2 which lyses *S. lactis* C_2 and *S. lactis* ML-3 was obtained from T. R. Klaenhammer, North Carolina State University, Raleigh. Phage 643 which is lytic for *S. lactis* ML-3 and a lytic phage (designated phage 18-16) which infects *S. lactis* subsp. *diacetylactis* 18-16 were obtained from W. E. Sandine, Oregon State University, Corvallis.

Culture media and propagation. Cultures that fermented lactose (Lac^+) were routinely propagated in sterile 10% reconstituted nonfat dry milk (NFM) at 24°C for 14 to 16 h. Strains that were lactose negative (Lac^-) were grown in sterile NFM fortified with 0.5% glucose and 0.2% yeast extract (FNFM).

Stock cultures grown in NFM or FNFM containing 10% sterile glycerol as a cryoprotectant were dispensed into cryogenic vials and stored in liquid nitrogen. In curing experiments to eliminate the Muc^+ phenotype, either NFM or FNFM was initially used. Later, BMG broth (7) was used, and for the selection and purification of Lac^- colonies, BML agar (BMLA) described by Gonzalez and Kunka (7) was used.

Milk indicator agar (MIA) was used for the selection and purification of phenotypes expressing variations of Lac and Muc ($Lac^{\pm}$ $Muc^{\pm}$) in curing and mating experiments. MIA was made up in two separate parts which after sterilization and tempering at 60°C were mixed together. The first part consisted of distilled water at half the final volume of the medium (final volume, 1 liter), containing the required amount of nonfat milk solids to give a final concentration of 5%; the second part consisted of the remaining half of distilled water containing the required amount of agar to obtain 1.5% in the final medium and 10.0 ml of 0.8% aqueous solution of bromocresol purple.

Whey broth was made in two parts which were sterilized

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TABLE 1. Bacterial strains

Streptococcus strains	Chromosomal phenotype[a]	Plasmids (MDa)	Description and source[a,b]
S. cremoris			
MS	None	105.6, 75.8, 35.8, 18.5, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	Wild type; this study; Lac^+ Muc^+[c]
MS01	None	105.6, 75.8, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	This study; Lac^+ Muc^-
MS02	None	105.6, 75.8, 35.8, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	This study; Lac^+ Muc^-
MS03	None	105.6, 75.8, 18.5, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	This study; Lac^+ Muc^+
MS04	None	105.6, 18.5, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	This study; Lac^- Muc^+
MS0401	None	105.6, 75.8, 18.5, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	MS04 transconjugant; this study; Lac^+ Muc^+
MS05	None	105.6, 6.3, 5.8, 4.7, 3.0, 2.7, 1.5	This study; Lac^- Muc^-
S. lactis			
ML-3/2.2	Str^r Fus^r	5.2, 2.2, 1.5	This study; Lac^- derivative of ML-3, Str^r Fus^r
ML-3/2.201	Str^r Fus^r	75.8, 5.8, 5.2, 2.2, 1.5	This study; Lac^+ transconjugant of ML-3/2.2
ML-3/2.202	Str^r Fus^r	75.8, 18.5, 5.8, 5.2, 4.7, 3.0, 2.2, 1.5	This study; Lac^+ Muc^+ transconjugant of ML-3/2.201
4/4.2 (malty)	Rif^r Fus^r	None	Rif^r Fus^r plasmid-cured derivative of malty *S. lactis* 4; this study[d]
4/4.201 (malty)	Rif^r Fus^r	75.8, 18.5, 5.8	Transconjugant of malty 4/4.2; Lac^+ Muc^+; this study
S. lactis subsp. *diacetylactis*			
SLA3.25	Rif^r	25.8, 5.5, 4.7, 3.4, 3.2	Lac^- Rif^r derivative of strain 18-16[e]
SLA3.2501	Rif^r	75.8, 25.8, 18.5, 5.8, 5.5, 4.7, 3.4, 3.2	Transconjugant of SLA3.25; Lac^+ Muc^+; this study

[a] Fus, Fusidic acid; Rif, rifampin; Str, streptomycin; r, resistant.
[b] Lac^+, Lactose fermenting; Lac^-, lactose negative; Muc^+, mucoid; Muc^-, nonmucoid.
[c] Isolated from a ropy milk culture in the Microlife Technics culture collection.
[d] Parent malty *S. lactis* 4 (Morgan) from W. E. Sandine, Oregon State University, Corvallis.
[e] Strain obtained from C. F. Gonzalez, Microlife Technics, Sarasota, Fl.

separately and, after cooling, mixed together. To make up 1 liter of whey broth, 70.0 g of sweet whey powder (Pallio Dairy Products Corp., Campbell, N.Y.) was dissolved in 500 ml of distilled water and centrifuged to remove undissolved residue. Sodium β-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) (19.0 g) was added to the clear supernatant, mixed well, and sterilized at 121°C for 15 min. The second portion of the medium consisted of 5.0 g of yeast extract, 10.0 g of tryptone, 5.0 g of gelatin, 0.5 g of sodium acetate, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.2 g of $CaCl_2 \cdot 2H_2O$ dissolved in 500 ml of distilled water. After sterilization at 121°C for 15 min, the two parts were mixed together after cooling to 50°C. Whey-glucose broth was made up by including 5.0 g of glucose in the formulation for whey broth. Matings were performed on 5% milk-glucose agar (MGA) plates as described by McKay et al. (17).

Curing. For temperature-curing experiments, *S. cremoris* strains were incubated between 38 and 39°C; *S. lactis* and *S. lactis* subsp. *diacetylactis* strains were incubated between 41 and 42°C. For elimination of the Lac^+ phenotype, cultures inoculated in BMG broth (0.05% [vol/vol] inoculum) were incubated overnight at elevated temperatures and plated at suitable dilutions onto BMLA plates. Presumptive white Lac^- colonies were confirmed for inability to ferment lactose and purified by single-colony isolation on BMLA. For elimination of the Muc^+ phenotype, cultures inoculated in NFM or FNFM or BMG broth (0.05% [vol/vol] inoculum) were incubated at elevated temperatures overnight, and suitable dilutions were plated on MIA plates. Individual colonies were picked into either NFM (Lac^+) or FNFM (Lac^-) and incubated at 24°C until coagulation or thickening of the milk occurred. The cultures were then tested for mucoidness with 1.0-ml graduated serological pipettes. Resistance to easy flow and formation of stringiness (long ropy strands) during free fall from the pipette tip were used as test criteria to screen for mucoidness.

Mating. Donor and recipient cultures for mating were grown to the logarithmic phase (6 to 8 h at 24°C) in either whey broth (Lac^+ strains) or whey-glucose broth (Lac^- strains). Mating mixtures and respective donor and recipient controls spread on MGA plates were incubated overnight at 24°C in a Gas-Pak anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.).

Cells from the surface of MGA plates were harvested with sterile BM broth (7), using 1.0 ml of BM broth per plate. Washings from each set of five plates containing one experimental variable (i.e., plates with donor cells or recipient cells or mating mixture) were pooled together, centrifuged, and resuspended in 1.0 ml of BM broth. The entire suspension was then plated onto five BMLA plates (0.2 ml per plate) containing appropriate concentrations of selective drugs. Streptomycin was added to obtain a final concentration of 1,000 μg/ml; fusidic acid was added to a final concentration of 20 μg/ml; and rifampin was added to a final concentration of 300 μg/ml. Plates were incubated at 24°C for 72 h and examined. All Lac^+ colonies were transferred to NFM to test for mucoidness. Mucoid isolates were purified by single-colony isolation on MIA and subjected to confirmatory tests.

In mating experiments in which a specific lytic phage was used as a selective agent, pelleted cells harvested from MGA plates were suspended in 2.0 ml of phage lysate. A 0.1-ml portion of 0.2 M $CaCl_2$ was added to each tube, mixed gently, and allowed to stand for 15 to 20 min at room temperature for phage adsorption. The contents of each tube were then spread on 10 BMLA plates (0.2 ml per plate).

For donor input counts, dilutions were plated on BMLA (Lac^+) or BMG plus agar (BMGA) (Lac^-). Platings for

counts were made immediately before mating mixtures and controls were spread on MGA. Transfer frequencies were calculated as the number of mucoid colonies per donor CFU. Mating experiments were repeated at least once. To exclude transduction as a possible mode of genetic transfer, in parallel mating mixtures, donor culture samples were replaced with an equal amount of cell-free filtrates (Millipore filter, 0.45-μm pore size; Millipore Corp., Bedford, Mass.) of the donor. To exclude transformation we added DNase at a final concentration of 100 μg/ml to the mating mixture before plating and to the MGA used. Additionally, in parallel mating mixtures, donor culture samples were replaced with heat-killed (boiling water bath for 10 min) donor culture portions.

Confirmatory tests. Confirmatory testing was done for arginine hydrolysis (18), diacetyl-acetoin production from citrate in milk (9), and susceptibility to specific phages by spot test on seeded agar-overlay plates. When necessary, resistance or sensitivity to additional drug markers not selected for in the mating protocols was determined. Maltiness in milk cultures was confirmed by the method of Langeveld (10).

Cell lysis and electrophoresis. For rapid screening of strains for plasmids, the method described by Anderson and McKay (1) was used. Procedures described by Gonzalez and Kunka (7) were used for routine examination of plasmid DNA and for preparing purified plasmid DNA with cesium chloride-ethidium bromide gradients.

Viscosity measurements. Viscosity measurements of mucoid and nonmucoid cultures were made with FNFM-grown cultures with the Boekel Viscosimeter (Zahn cup type) according to the operating instructions of the manufacturer (Boekel Industries Inc., Philadelphia, Pa.). Zahn cup no. 4 readings taken at 25°C were converted to viscosity in centipoises by using the conversion chart provided by the manufacturer.

RESULTS

Curing of Muc$^+$ phenotype. Initial experiments with *S. cremoris* MS showed that mucoidness (or ropiness) in this strain could be easily eliminated by incubating inoculated NFM tubes at 38°C for 14 to 16 h. In three separate trials, an average of 30% of the colonies isolated from MIA plates spread with *S. cremoris* MS which had been incubated at elevated temperatures in NFM were nonmucoid. A total of 20 mucoid and 20 nonmucoid, milk-coagulating (in 18 to 24 h at 24°C) isolates were purified and examined for plasmid DNA content by agarose gel electrophoresis. All mucoid types tested with one exception, namely, strain MS03, showed plasmid profiles similar to that of the wild-type mucoid *S. cremoris* MS (Fig. 1). In *S. cremoris* MS03 the 35.8-megadalton (MDa) plasmid was absent. All the nonmucoid isolates with one exception showed plasmid profiles similar to that of *S. cremoris* MS01 (Fig. 1). In these strains, two plasmids, the 35.8- and 18.5-MDa plasmids, were missing. In the single nonmucoid isolate, *S. cremoris* MS02, only the 18.5-MDa plasmid was absent (Fig. 1). By examining the plasmid profiles of *S. cremoris* MS, MS01, MS02, and MS03, only the 18.5-MDa plasmid (pSRQ2202) could possibly be associated with the Muc$^+$ phenotype because strain MS03 retained the Muc$^+$ phenotype even with the loss of 35.8-MDa plasmid. On the other hand, strain MS02, which possessed the larger 35.8-MDa plasmid, became nonmucoid with the elimination of pSRQ2202.

Curing of Lac$^+$ phenotype. It is now well established that the lactose-fermenting ability in lactic streptococci is plasmidborne (14). To determine whether the Lac plasmid in Muc$^+$ *S. cremoris* MS03 could be cured without the loss of the mucoid or ropy characteristic, the culture was incubated at 38°C for 16 h and plated on BMLA. Of a total of 80 colonies appearing on the BMLA plates, 15% were Lac$^-$. All the Lac$^-$ colonies were transferred into FNFM and incubated at 24°C until thickening or coagulation occurred. The cultures were checked for mucoidness at that stage. With the exception of two isolates, all others were nonmucoid. *S. cremoris* MS04 represented the Lac$^-$ Muc$^+$ phenotype (Fig. 1). Analysis of the plasmid profiles of Lac-cured derivatives suggested that the loss of lactose-fermenting ability was associated with the elimination of a 75.8-MDa plasmid (pSRQ2201). The Lac$^-$ Muc$^-$ phenotype is represented by *S. cremoris* MS05.

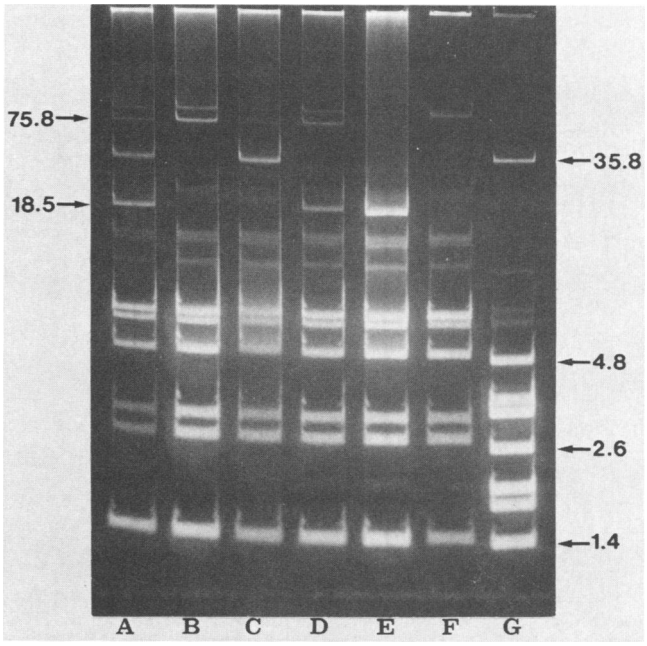

FIG. 1. Agarose gel electrophoresis of plasmid DNA from *S. cremoris* MS and its cured derivatives. (A) Parent strain MS; (B) MS01; (C) MS02; (D) MS03; (E) MS04; (F) MS05; (G) reference plasmid DNA for molecular sizing from *Escherichia coli* V517. Numbers show size in megadaltons.

Because conjugative transfer of plasmids in lactic streptococci is well documented (6, 16, 17), we decided to examine whether the Lac plasmid in mucoid strains could be used as a metabolic marker to detect cotransfer of the Muc plasmid in mating experiments.

Conjugative transfer of Lac plasmid. In a mating experiment, the Lac$^+$ phenotype was transferred from Lac$^+$, mucoid *S. cremoris* MS to a nonmucoid, Lac-cured strain, *S. lactis* ML-3/2.2. The transconjugant *S. lactis* ML-3/2.201 was Lac$^+$ but nonmucoid and had acquired a 75.8-MDa plasmid from *S. cremoris* MS. In addition to the Lac plasmid, a cryptic 5.8-MDa plasmid was also tranferred from the donor to the recipient (Table 1; Fig. 2). Additionally, the transconjugant was positive for the arginine hydrolysis test and susceptible to phage c2 and 643. The expression of the Lac$^+$ phenotype by *S. lactis* ML-3/2.201 as a result of the acquisition of the 75.8-MDa plasmid, pSRQ2201, from the donor and the curing data obtained with *S. cremoris* MS and its derivatives indicated that pSRQ2201 coded for lactose utilization in *S. cremoris* MS.

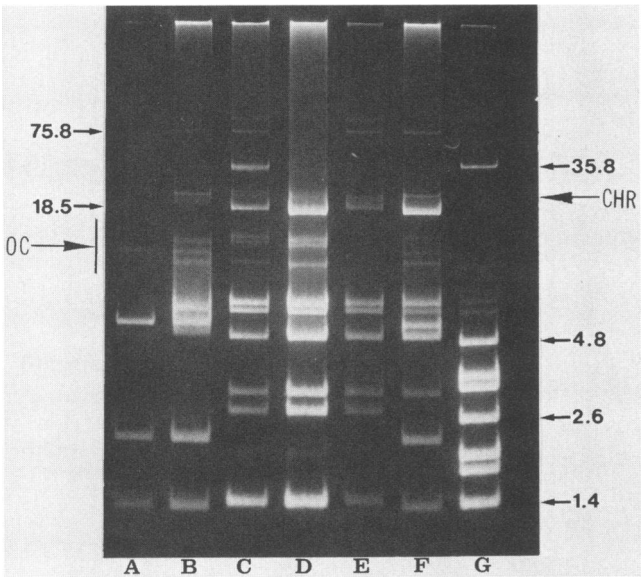

FIG. 2. Agarose gel electrophoresis of plasmid DNA from (A) *S. lactis* ML-3/2.2; (B) *S. lactis* ML-3/2.201; (C) *S. cremoris* MS; (D) *S. cremoris* MS04; (E) *S. cremoris* MS0401; (F) *S. lactis* ML-3/2.202; (G) reference plasmid DNA for molecular sizing from *E. coli* V517. OC, open circular forms; CHR, chromosome. Numbers show size in megadaltons.

Conjugative transfer of Lac and Muc plasmids. A Lac^+ transconjugant, *S. lactis* ML-3/2.201, was used as donor in a mating with *S. cremoris* MS04 to determine whether the Lac plasmid pSRQ2201 observed in the transconjugant *S. lactis* ML-3/2.201 could be transferred back to *S. cremoris* MS04, a Lac^- Muc^+ derivative of the wild type. The selective agent for this mating was phage c2 (at a titer of 10^4 PFU/ml), which lysed the donor, *S. lactis* ML-3/2.201. The phage does not infect the recipient *S. cremoris* MS04. Because of the low titer of phage used for selecting against donor cells, control donor plates showed several Lac^+ survivor colonies per plate. Plates containing the mating mixtures, however, had at least twice the number of Lac^+ colonies per plate as in the control donor plates. All acid-producing colonies from BMLA plates containing the mating mixtures were transferred into NFM and, after incubation, checked for mucoidness. Some 200 randomly chosen nonmucoid isolates were purified and checked for resistance to streptomycin (1,000 μg/ml) on BMLA. All were resistant; additionally, they gave positive tests for liberation of NH_3 from arginine, indicating that they were donor types. Six isolates that were mucoid were purified on MIA and checked for streptomycin sensitivity or resistance and for arginine hydrolysis. Two of the mucoid isolates were negative for arginine hydrolysis, and the other four were positive. Repurified isolates of the six mucoid cultures showed the same arginine hydrolysis characteristics as in the first testing. The two mucoid isolates that failed to liberate NH_3 from arginine were also sensitive to streptomycin (1,000 μg/ml) and fusidic acid (20 μg/ml), indicating that they were recipient-type Lac^+ transconjugants. The remaining four mucoid cultures that were positive for arginine hydrolysis were resistant to the same levels of streptomycin and fusidic acid as the donor, *S. lactis* ML-3/2.201. All six mucoid isolates were resistant to phages c2 and 643. Agarose gel electrophoresis of plasmid DNA from the six isolates showed that two types of transconjugants were obtained. The two arginine-negative, streptomycin-sensitive, Lac^+ Muc^+ isolates were recipient *S. cremoris* MS04 types that had acquired the Lac plasmid (and the Lac^+ phenotype) from the donor (exemplified by *S. cremoris* MS0401 [Fig. 2]). The four arginine-positive, Lac^+ Muc^+ isolates were donor *S. lactis* ML-3/2.201-type transconjugants which showed the 18.5-MDa Muc plasmid from *S. cremoris* MS04 (exemplified by *S. lactis* ML-3/2.202 [Fig. 2]). Phage resistance of Muc^+ transconjugants of donor *S. lactis* ML-3/2.201 type suggested that the acquisition of the 18.5-MDa plasmid pSRQ2202 and the Muc^+ phenotype conferred virus resistance to the ML-3/2.202 transconjugant, although the parent strain ML-3/2.201 (Lac^+, nonmucoid) was susceptible to the same phage. Based on these initial observations, the mating was repeated with *S. cremoris* MS04 as the donor and with a high-titer c2 phage lysate (3.0×10^9 PFU/ml) to select effectively against Lac^+, phage-sensitive, recipient *S. lactis* ML-3/2.201 cells. Colony-free recipient control plates were obtained. Because the use of high-titer phage lysate provided effective selection against nonmucoid, Lac^+, phage-sensitive recipient cells, Lac^+ colonies appearing on mating plates probably were transconjugants of donor *S. cremoris* MS04 type that had acquired the Lac plasmid from *S. lactis* ML-3/2.201 or were Muc^+, phage-resistant transconjugants of ML-3/2.201 type that had acquired the Muc plasmid from *S. cremoris* MS04. Based on that premise, all Lac^+ colonies from BMLA plates containing that mating mixtures were transferred into NFM and tested for mucoidness. All mucoid isolates were purified and subjected to a confirmatory arginine hydrolysis test. With the use of high-titer phage lysate, the transfer of the Muc^+ phenotype was observed at a frequency of 3.0×10^{-4}. In this mating, other cryptic plasmids were also transferred along with the Muc plasmid from the donor to the recipient (Table 1; Fig. 2).

Incubation of the Lac^+ Muc^+ transconjugant *S. lactis* ML-3/2.202 at 41 to 42°C allowed the selection of all possible combinations of $Lac^\pm$ $Muc^\pm$ phenotypes (data not shown). Agarose gel electrophoresis profiles of plasmid DNA from such derivatives confirmed that the Lac^+ phenotype was expressed when the 75.8-MDa pSRQ2201 plasmid was present. In the absence of pSRQ2201 the bacteria were Lac^-. Similarly, the presence and absence of the 18.5-MDa pSRQ2202 plasmid was directly associated with the expression of the Muc^+ and Muc^- phenotypes, respectively.

Cotransfer of Muc plasmid with Lac plasmid. To determine whether the Muc phenotype could be transferred to other *S. lactis* strains, *S. lactis* ML-3/2.202 was mated with a plasmid-free, nonmucoid, malty strain, *S. lactis* 4/4.2 (obtained by curing two resident plasmids from the wild-type malty strain, *S. lactis* 4). Plating of the mating mixtures on BMLA containing the selective antibiotic allowed the selection of Lac^+ transconjugants. Lactose-positive purified isolates screened for sensitivity to streptomycin were tested for the Muc^+ phenotype. Presumptive Lac^+ Muc^+ transconjugants were confirmed by testing for the production of a malty odor in milk containing the sodium salt of 4-methyl-2-oxopentanoic acid (10). Mobilization of other cryptic plasmids in addition to the Muc plasmid was observed with the transfer of the Lac plasmid from *S. lactis* ML-3/2.202 to *S. lactis* 4/4.2 (Fig. 3).

Similarly, we were able to transfer pSRQ2201 by itself and pSRQ2201 and pSRQ2202 together to a Lac-cured, nonmucoid strain of *S. lactis* subsp. *diacetylactis*. The recipient, *S. lactis* subsp. *diacetylacis* SLA3.25, and the Lac^+ transconjugants were sensitive to phage 18-16. The Lac^+ Muc^+ transconjugant *S. lactis* subsp. *diacetylactis*

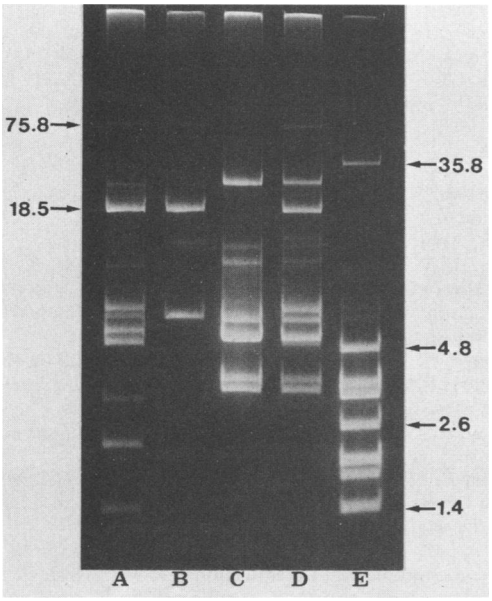

FIG. 3. Agarose gel electrophoresis of plasmid DNA from (A) *S. lactis* ML-3/2.202; (B) malty *S. lactis* 4/4.201; (C) *S. lactis* subsp. *diacetylactis* SLA3.25; (D) *S. lactis* subsp. *diacetylactis* SLA3.2501; (E) reference plasmid DNA for molecular sizing from *E. coli* V517. Numbers show size in megadaltons.

SLA3.2501, however, was resistant to phage 18-16. By incubating the Lac$^+$ Muc$^+$ transconjugant SLA3.2501 at 41 to 42°C, all possible combinations of Lac and Muc phenotypes (Lac$^\pm$ Muc$^\pm$) were obtained (data not shown). As observed with *S. lactis* ML-3/2.202, the elimination of pSRQ2201 and pSRQ2202 from SLA3.2501 correlated with the inability of the derivatives to express the Lac$^+$ and Muc$^+$ phenotypes, respectively.

Table 2 summarizes interspecies transfer frequencies for pSRQ2201 and pSRQ2202 singly and for the cotransfer of pSRQ2202 with the Lac plasmid from *S. lactis* ML-3/2.202 to malty *S. lactis* 4/4.2 and *S. lactis* subsp. *diacetylactis* SLA3.25.

Viscosity measurements on mucoid and nonmucoid cultures are shown in Table 3. In all cases, mucoid cultures had much greater viscosity than nonmucoid strains.

TABLE 3. Viscosity measurements on nonmucoid and mucoid cultures[a]

Strain	Phenotype	Draining time (s)[b]	Viscosity (cP)[c]
S. cremoris MS01	Nonmucoid	8.2	<150[d]
S. cremoris MS	Mucoid	86.0	1,175
S. cremoris MS04	Mucoid	32.0	400
S. cremoris MS0401	Mucoid	77.0	1,050
S. lactis ML-3/2.201	Nonmucoid	7.2	<150
S. lactis ML-3/2.202	Mucoid	59.2	800
S. lactis 4/4.2 (malty)	Nonmucoid	7.0	<150
S. lactis 4/4.201 (malty)	Mucoid	51.2	650
S. lactis subsp. *diacetylactis* SLA3.25	Nonmucoid	6.5	<150
S. lactis subsp. *diacetylactis* SLA3.2501	Mucoid	53.0	650

[a] Refer to Materials and Methods for the procedure used.
[b] Draining time from Zahn cup no. 4 was determined in seconds.
[c] Viscosity determined from graph (draining time in seconds versus centipoises) provided by the manufacturer.
[d] 150 cP is the lower limit of viscosity measurement in the linear portion of the conversion curve.

DISCUSSION

The results presented clearly demonstrate that the mucoid phenotype in the lactic streptococci examined in this study is encoded on plasmid DNA. In this study, the association of the mucoid phenotype with plasmid DNA in wild-type *S. cremoris* MS was initially demonstrated by curing experiments. In these experiments, the presence or absence of an 18.5-MDa plasmid correlated with the mucoid and nonmucoid phenotypes, respectively. The actual confirmation that the mucoid phenotype is encoded on plasmid DNA was obtained in mating experiments, in which the conjugative transfer of the 18.5-MDa plasmid from mucoid *S. cremoris* MS04 to nonmucoid *S. lactis* ML-3/2.201 enabled the transconjugant containing the 18.5-MDa pSRQ2202 plasmid to express the mucoid phenotype. Additionally, the elimination of pSRQ2202 from the mucoid transconjugant *S. lactis* ML-3/2.202 resulted in a nonmucoid phenotype. Subsequently, pSRQ2202 was conjugatively transferred from *S. lactis* ML-3/2.202 to plasmid-free malty *S. lactis* 4/4.2 and *S. lactis* subsp. *diacetylactis* SLA3.25. The phenotypic expression of pSRQ2202 in the respective transconjugants (*S. lactis* 4/4.201 and SLA3.2501) indicates that in general, the mucoid phenotype in lactic streptococci is linked to plasmid DNA. These results also substantiate earlier reports on the instability of the mucoid characteristic among lactic streptococci

TABLE 2. Frequency of interspecies transfer of plasmids pSRQ2201 and pSRQ2202 among lactic streptococci

Plasmid	Mating[a]		Transfer frequency[b]	Transconjugant[a]
	D	R		
pSRQ2201	Sc MS	Sl ML-3/2.2	7.0×10^{-8}	Sl ML-3/2.201
	Sl ML-3/2.201	Sc MS04	0.3×10^{-8}	Sc MS0401
pSRQ2202	Sc MS04	Sl ML-3/2.201	3.0×10^{-4}[c]	Sl ML-3/2.202
Cotransfer of pSRQ2201 and pSRQ2202	Sl ML-3/2.202	MSl 4/4.2	1.7×10^{-8}	MSl 4/4.201
	Sl ML-3/2.202	Sld SLA3.25	2.0×10^{-8}	Sld SLA3.2501

[a] D, Donor; R, recipient. Sc, *S. cremoris*; Sl, *S. lactis*; MSl, malty *S. lactis*; Sld, *S. lactis* subsp. *diacetylactis*. Phenotypic characteristics of donors, recipients, and transconjugants: Sc MS, Muc$^+$ Lac$^+$; Sl ML-3/2.2, nonmucoid, Lac$^-$; Sl ML-3/2.201, nonmucoid, Lac$^+$; Sc MS04, Muc$^+$ Lac$^-$; Sc MS0401, Muc$^+$ Lac$^+$; Sl ML-3/2.202, Muc$^+$ Lac$^+$; MSl 4/4.2, nonmucoid, Lac$^-$; MSl 4/4.201, Muc$^+$ Lac$^+$; Sld SLA3.25, nonmucoid, Lac$^-$; Sld SLA3.2501, Muc$^+$ Lac$^+$.
[b] Frequency is expressed as the number of Lac$^+$ or Muc$^+$ or Lac$^+$ Muc$^+$ (cotransfer) transconjugants per donor CFU. Donor CFU were determined before mating. Frequency values reported are averages of at least two independent experiments.
[c] Results from matings with phage lysate with a titer of $>1 \times 10^9$ PFU/ml.

by Hammer (8), Foster et al. (5), Brooker (3), and Macura and Townsley (11).

The ease with which the Muc plasmid was eliminated by incubation between 38 and 42°C was in keeping with the earlier observation that to retain the desired mucoid characteristic in Scandinavian ropy milks, low-temperature incubation between 13 and 18°C is favored; incubation at temperatures higher than 27 to 30°C results in considerable reduction or loss of desirable high viscosity and mucoidness (11).

In addition to the Muc plasmid, transfer of the Lac plasmid was achieved in this study. The Lac plasmid from the wild-type mucoid *S. cremoris* MS was first transferred to *S. lactis* ML-3/2.2, and subsequently the same plasmid was retransferred from *S. lactis* ML-3/2.201 to the Lac^- Muc^+ derivative of the wild-type mucoid *S. cremoris* (*S. cremoris* MS04). Further, the plasmid was tranferred from the Lac^+ Muc^+ transconjugant *S. lactis* ML-3/2.202 to the malty *S. lactis* 4/4.2 and *S. lactis* subsp. *diacetylactis* SLA3.25. In the latter two matings, the Lac plasmid also mobilized the Muc plasmid and other cryptic plasmids. In all these transfers, the Lac^+ phenotype was expressed in the respective transconjugants, and the elimination of the 75.8-MDa plasmid from the respective transconjugants rendered them Lac^-. Available data suggest that the Muc plasmid either cotransferred with the lactose plasmid or was mobilized by pSRQ2201. Currently, experiments are in progress to determine independent conjugal transfer of pSRQ2202.

Although the transfer of the Muc plasmid was detected in these mating experiments by indirect selection procedures, namely, scoring for the Lac^+ phenotype or phage resistance or both, a direct selection procedure through the use of a suitable differential medium to distinguish between mucoid and nonmucoid colony types would be very useful. Research is currently in progress for the development of a suitable medium.

A significant observation in this study was the association of phage resistance and mucoidness. With the transfer of the Muc plasmid to a nonmucoid, phage-sensitive recipient, the resultant mucoid transconjugant became resistant to the phage. This held true with *S. lactis* and *S. lactis* subsp. *diacetylactis*. Maxted (13) reported that the possession of extracellular material by group A streptococci rendered the cell nonsusceptible to infection by bacteriophage. Marth (12) reported observations in which "rope-producing properties" were induced by phage. The association of phage resistance with mucoid phenotype in transconjugants offers another possible mechanism by which phage-resistant derivatives for starter cultures could be made. Additionally, the selection procedure for the distinction of transconjugants through the use of high-titer lytic phage lysates provides a means for avoiding drug markers for selection. This is especially significant in deriving desired strains for food and feed fermentations. Currently, we are examining the mechanism of phage resistance associated with the Muc plasmid.

At present, the availability of mucoid strains is limited. With the genetic transfer procedures developed in this investigation, it may be possible to derive additional mucoid strains. Such additional strains would be useful in other applications in the dairy industry as described by Vedamuthu and Shah (U.S. patent 4,382,097, May 1983).

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