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Partial Characterization of the Genetic Basis for Sucrose Metabolism and Nisin Production in *Streptococcus lactis*†

JAMES L. STEELE AND LARRY L. McKAY*

Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

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We attempted to identify the genetic loci for sucrose-fermenting ability (Suc^+), nisin-producing ability (Nip^+), and nisin resistance (Nis^r) in certain strains of *Streptococcus lactis*. To obtain genetic evidence linking the Suc^+ Nip^+ Nis^r phenotype to a distinct plasmid, both conjugal transfer and transformation were attempted. A conjugation procedure modified to protect the recipients against the inhibitory action of nisin allowed the conjugal transfer of the Suc^+ Nip^+ Nis^r marker from three Suc^+ Nip^+ Nis^r donors to various recipients. The frequency of transfer ranged from 1.7×10^{-4} to 5.6×10^{-8} per input donor, depending on the mating pair. However, no additional plasmid DNA was apparent in these transconjugants. Transformation of *S. lactis* LM0230 to the Suc^+ Nip^+ Nis^r phenotype by using the plasmid pool of *S. lactis* ATCC 11454 was not achieved, even though other plasmids present in the pool were successfully transferred. However, two results imply the involvement of plasmid DNA in coding for the Suc^+ Nip^+ Nis^r phenotype. The Suc^+ Nip^+ Nis^r marker was capable of conjugal transfer to a recipient deficient in host-mediated homologous recombination (Rec^-), and the Suc^+ Nip^+ Nis^r marker exhibited bilateral plasmid incompatibility with a number of lactose plasmids found in *S. lactis*. Although our results indicate that the Suc^+ Nip^+ Nis^r phenotype is plasmid encoded, no physical evidence linking this phenotype to a distinct plasmid was obtained.

The group N streptococci produce a variety of antibiotic-like substances including nisin, diplococcin, and a number of bacteriocins (6). Nisin is an antibiotic produced by certain strains of *Streptococcus lactis*. It is a polypeptide containing three nonprotein amino acids: lanthionine, β-methyl lanthionine, and dehydroalanine (14). It has a molecular weight of 3,500 and is commonly found as stable, biologically active dimers or tetramers (13). Its spectrum of activity includes almost all gram-positive organisms and their spores (21). Among antibiotics, nisin is unique in that it is used as a food preservative. It is used as such because it has a relatively wide spectrum of activity, it is not used medicinally or in animal feed, and it is not toxic when it is eaten (21).

The involvement of plasmid DNA in the production of nisin was suggested previously. In 1974 Kozak et al. (26) reported that treatment with proflavin or ethidium bromide or growth at an elevated temperature (37°C) resulted in an increased conversion of the nisin-producing (Nip^+) phenotype to the nonproducing (Nip^-) phenotype. More recently, LeBlanc et al. (28) reported that the loss of a 28-megadalton (MDa) plasmid from *S. lactis* ATCC 11454 correlated with the loss of both nisin production and sucrose-fermenting ability (Suc^+). The linkage of nisin production with sucrose metabolism was observed as early as 1951, when it was reported that 12 nisin-producing strains were also able to ferment sucrose (17). In 1984 Gasson (9) reported the en bloc conjugal transfer of the Suc^+ Nip^+ nisin resistance (Nis^r) phenotype. A faint plasmid band of approximately 30 MDa was occasionally visualized on agarose gels after lysis of these Suc^+ Nip^+ Nis^r transconjugants. However, this DNA degraded during storage and was not cut by restriction endonucleases. Recently, Gonzalez and Kunka (11) reported plasmid curing data which correlated the loss of a 29.1-MDa plasmid from *S. lactis* ATCC 11454 with the loss of the ability to ferment sucrose and produce nisin and the loss of resistance to nisin. These results are consistent with the previously reported results of LeBlanc et al. (28). Gonzalez and Kunka (11) also reported the conjugal transfer of the Suc^+ Nip^+ Nis^r phenotype in the absence of detectable plasmid DNA.

The results presented in this paper also suggest that plasmid DNA is associated with the ability of some strains of *S. lactis* to ferment sucrose, produce nisin, and grow in the presence of nisin. However, physical evidence linking the Suc^+ Nip^+ Nis^r marker to a distinct plasmid was not obtained, even though the Suc^+ Nip^+ Nis^r phenotype was conjugally transferred into a Rec^- recipient and was incompatible with a number of lactose plasmids found in *S. lactis*.

MATERIALS AND METHODS

Bacterial strains. All streptococcal strains used in this study were maintained by biweekly transfers at 32°C in M17 broth (42) containing 0.5% glucose, lactose, or sucrose; the strains are described in Table 1.

Screening for nisin resistance and production. Nisin resistance was determined by adding a 2% inoculum of the test strain to M17-sucrose broth with or without 100 ng of nisin (Alpin and Barrett Ltd.) per ml; the nisin was prepared as a stock solution of 0.4 mg/ml in 0.2 N HCl. The tubes were incubated for 32 h at 32°C and visually examined for growth.

Nisin production was detected by a method previously described for detection of bacteriocin production (39). One loopful of a 16-h M17-sucrose broth culture was spot inoculated onto the surface of an Elliker agar plate and incubated at 32°C for 16 to 18 h. The agar was detached from the edges of the petri dish with a sterile spatula and flipped into the lid. The nisin-sensitive strain *S. lactis* LM2306 was grown in M17-glucose broth at 32°C for 18 h. A suspension (0.7 ml) of the cells was then mixed with 7 ml of 45°C-tempered Elliker broth (Difco Laboratories, Detroit, Mich.) which contained 0.7% agar, and the mixture was poured over the surface of

* Corresponding author.

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TABLE 1. Strains of *S. lactis* and *S. cremoris* used in this study[a]

Strain	Relevant phenotype	Comment (reference)
S. lactis		
ATCC 11454	Lac^+ Suc^+ Nip^+ Nis^r Str^s Ery^s	Parent (28; DR1251)
354/07	Lac^- Suc^+ Nip^+ Nis^r Str^s Ery^s	Parent (19)
DL16	Lac^+ Suc^+ Nip^+ Nis^r Str^s Ery^s	Parent (28; DR1253)
ML3	Lac^+ Suc^- Nip^- Nis^s Str^s Ery^s	Parent (27)
ML3S	Lac^+ Suc^- Nip^- Nis^s Str^r Ery^s	Str^r derivative of ML3
LM3301	Lac^- Suc^- Nip^- Nis^s Str^r Ery^s	Spontaneous Lac^- Str^r mutant of ML3 (45)
MMS362	Lac^- Suc^- Nip^- Nis^s Str^r Ery^s	Lac^- Rec^- Str^r mutant of ML3 (1)
EB101	Lac^+ Suc^- Nip^- Nis^s Str^s Ery^r	Lac^+ transconjugant of EB_7 × LM3302 (41)
LM0230	Lac^- Suc^- Nip^- Nis^s Str^s Ery^s	Plasmid-cured derivative of C2 (7)
LM2306	Lac^- Suc^- Nip^- Nis^s Str^r Ery^r	Mal^- Str^r Ery^r derivative of LM0230
D6528	Lac^- Suc^- Nip^- Nis^r Str^r Ery^s	LM2301 containing pNP40 which codes for nisin resistance (32)
PW2	Lac^+ Suc^- Nip^- Nis^s Str^r Ery^s	Lac^+ transconjugant of ML3 × LM2301 capable of HFC of the Lac^+ phenotype (45)
4621	Lac^+ Suc^- Nip^- Nis^s Str^r Ery^s	LM2301 containing a Lac plasmid from *S. lactis* subsp. *diacetylactis* DRC3
LM2201	Lac^- Suc^- Nip^- Nis^s Str^r Ery^s	Str^r derivative of LM0220
KB21	Lac^+ Suc^- Nip^- Nis^s Str^r Ery^s	Lac^+ Prt^+ stabilized transductant of LM2201 (31)
JK301	Lac^- Suc^- Nip^- Nis^s Str^s Em^r	Transformant of LM0230 with pGB301 Em^r Cm^r (25)
JK18	Lac^+ Suc^- Nip^- Nis^s Str^s Em^r	Transformant of LM0230 with pGB301 containing a *BclI* fragment of pLM2001 which encodes for lactose metabolism (25)
JS1	Lac^- Suc^+ Nip^- Nis^r Str^r Ery^s	Suc^+ Str^r transconjugant of ATCC 11454 × D6528 (this study)
JS21	Lac^- Suc^+ Nip^+ Nis^r Str^r Ery^r	Suc^+ Str^r Ery^r transconjugant of ATCC 11454 × LM2306 (this study)
JS51	Lac^- Suc^+ Nip^+ Nis^r Str^r Ery^s	Suc^+ Str^r transconjugant of 354/07 × LM3301 (this study)
JS61	Lac^- Suc^+ Nip^+ Nis^r Str^r Ery^s	Suc^+ Str^r transconjugant of 354/07 × MMS362 (this study)
S. cremoris		
BC102	Lac^- Suc^- Nip^- Nis^s Str^s Ery^r	Spontaneous Ery^r mutant of an AO-induced plasmid-cured mutant of *S. cremoris* B1 which is Prt^+ (45)
JS71	Lac^- Suc^+ Nip^+ Nis^r Str^s Ery^r	Suc^+ Ery^r transconjugant of JS1 × BC102 (this study)

[a] Lac^+, Lactose-fermenting ability; Lac^-, lactose negative; Suc^+, sucrose-fermenting ability; Mal^+, maltose-fermenting ability; Prt^+, proteinase activity; Nip^+, nisin producing; Nis^r, nisin resistant; Nis^s, nisin sensitive; Str^r, streptomycin resistant; Ery^r, erythromycin resistant; Em^r, plasmid-coded erythromycin resistance; Cm^r, plasmid-coded chloramphenicol resistance; Rec^-, recombination deficient; HFC, high-frequency conjugal transfer.

the inverted agar. Plates were incubated at 32°C for 16 to 20 h and were examined for zones of growth inhibition. The Nip^- strain *Streptococcus cremoris* BC102 was used as a negative control spot.

The nisin produced was tested for sensitivity to various proteolytic enzymes to confirm the protein nature of the inhibitory substance. Enzymes used included alpha-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.), trypsin (Sigma), and pronase (Calbiochem-Behring, La Jolla, Calif.). Each enzyme was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, to a final concentration of 10 mg/ml, and 0.4 ml of the enzyme solution was spread on the inverted agar before the nisin-sensitive strain was overlaid (39). As a control, the bacteriocin produced by *S. lactis* NP2 (39) was tested for sensitivity to the proteolytic enzymes.

Curing trials. Curing of plasmids was accomplished through growth at an elevated temperature (37°C) in the presence of acridine orange (AO). *S. lactis* ATCC 11454 was grown overnight in M17-sucrose broth. A 2% inoculum was then added to M17-glucose and M17-glucose broth containing 54, 131, 250, 359, and 458 μg of AO per ml. All tubes were incubated at 37°C for 14 h. The cultures were serially diluted with 0.85% sodium chloride, were plated on bromocresol purple (BCP)-lactose and BCP-sucrose indicator agar plates (35), and were incubated at 32°C for 40 h. Lac^- and Suc^- isolates were tested for resistance to nisin and for nisin production. The plasmid complements of the Lac^- and Suc^- isolates were determined as described below.

Conjugal matings. Conjugal matings between various Suc^+ Nip^+ Nis^r donors and Suc^- Nip^- Nis^s recipients were initially conducted as previously described (33); however, the inhibitory effect of the donor-produced nisin on the recipient cells necessitated the following modifications. Before they were added to the conjugation mixture, the donor cells were pelleted by centrifugation and resuspended in the original volume of fresh medium. Alpha-chymotrypsin was added to the conjugal mixture to a final concentration of 400 μg/ml to degrade residual nisin. Suc^+ transconjugants were scored on BCP-sucrose agar plates containing 600 μg of streptomycin per ml or 15 μg of erythromycin per ml or both to select against the donor strain. Suspected transconjugants were confirmed by bacteriophage typing (39), by their ability to ferment maltose or lactose, or by comparing their plasmid profiles with those of the donor and recipient.

Transduction and transformation were eliminated as modes of genetic transfer in the conjugation procedure by methods previously described (41).

Plasmid isolation. Plasmid DNA was routinely isolated from 1 liter of culture by the method of Anderson and McKay (2). In some cases, 0.7% (vol/vol) diethylpyrocarbonate (Sigma) was added before sodium dodecyl sulfate lysis to inactivate nucleases (5, 23, 29, 46). The purified plasmid band from a CsCl-ethidium bromide density gradient was dialyzed against 10 mM Tris–1 mM EDTA buffer at pH 8.0, precipitated with ethanol, and resuspended in 100 μl of the buffer.

Agarose gel electrophoresis of plasmid DNA was performed at 4 V/cm for 4.5 h with a horizontal submerged 0.6% agarose gel in 40 mM Tris-acetate–2 mM EDTA buffer (pH 8.0).

UV irradiation sensitivity. The UV sensitivities of *S. lactis*

MMS362 and its Suc$^+$ Nip$^+$ Nis^r transconjugants were determined as described by Anderson and McKay (1). Cells from 1 ml of culture were washed and suspended in 10 ml of 0.85% saline solution. The cell suspension was transferred to a sterile glass petri dish (100 by 15 mm) and swirled constantly during exposure to UV doses of 0, 5, 10, 20, 30, and 40 s emitted from a 15-W General Electric germicidal lamp located 38.5 cm above the suspension. Irradiated cells were diluted in saline solution, plated on Elliker broth (Difco) plus 1.5% agar, incubated for 48 h at 32°C, and then counted.

Transformation. Transformation of *S. lactis* LM0230 with *S. lactis* ATCC 11454 CsCl–ethidium bromide-purified plasmid DNA was accomplished as described by Kondo and McKay (24). Transformants were selected on M17-sucrose agar with 0.5 M lactose as a stabilizer and BCP (40 mg/liter) as a pH indicator; the amount of β-glycerophosphate was reduced to 5 g/liter to lower the buffering capacity to a level where acid-producing colonies could be scored. These colonies, which appeared within 6 days, were transferred with sterile toothpicks to BCP-lactose and BCP-sucrose agar plates to determine their sugar-fermenting phenotypes.

Transduction. Transducing phage was induced from *S. lactis* 8 (31) by UV irradiation by the method of McKay and Baldwin (30). Transduction of lactose-fermenting ability to *S. lactis* LM2306 and its Suc$^+$ Nip$^+$ Nis^r transconjugant was attempted as described by McKay et al. (34).

RESULTS

Confirmation of the nisin-producing phenotype. Nisin is inactivated by alpha-chymotrypsin but not by pronase or trypsin (20). Therefore, the inhibitors produced by the three Suc$^+$ parental strains and by the Suc$^+$ transconjugants derived from matings with the donors *S. lactis* ATCC 11454 and 354/07 were tested to determine if they were inactivated by these three proteolytic enzymes. The bacteriocin produced by *S. lactis* NP2 is inactivated by these enzymes (39) and was therefore used to confirm their activities. When plates containing the inhibitor-producing parental strains, the transconjugants, or NP2 were treated with pronase or trypsin and overlaid with agar containing the indicator strain LM2306, zones of inhibition were observed above the Suc$^+$ parental strains and their transconjugants but not above *S. lactis* NP2. When plates treated with alpha-chymotrypsin were overlaid with LM2306, no zones of inhibition were observed, regardless of the culture used.

Curing trials. Since growth of *S. lactis* ATCC 11454 in the presence of 250 μg of AO per ml resulted in a 99% reduction in viability as compared with growth in the control tube containing no AO, this concentration of AO was used to isolate Suc$^-$ or Lac$^-$ derivatives. Two Suc$^-$ and five Lac$^-$ derivatives were isolated by screening 2,000 and 500 colonies, respectively. Both Suc$^-$ isolates were Lac$^+$, sensitive to nisin (Nis^s), and did not produce nisin (Nip$^-$). The Lac$^-$ isolates were Suc$^+$, resistant to nisin (Nis^r), and produced nisin (Nip$^+$). Plasmid profiles revealed that of the plasmids present in the Lac$^+$ parental strain Lac$^-$ isolates were missing only a 35-MDa plasmid. Both of the Suc$^-$ Nip$^-$ Nis^s isolates lacked a 30-MDa plasmid which was present in the Suc$^+$ Nip$^+$ Nis^r parental strain.

Conjugal matings. Attempts to use the standard conjugation procedure to transfer sucrose-fermenting ability from Suc$^+$ Nip$^+$ Nis^r donors to *S. lactis* LM2306 (Suc$^-$ Nip$^-$ Nis^s) were not successful. Since the Suc$^-$ recipient is not selected against by the indicator agar, a confluent lawn of Suc$^-$ growth would be expected, but was not observed. This growth inhibition of recipient cells was most likely a result of the inhibitory effect of the nisin produced by the donor. When *S. lactis* D6528 (Suc$^-$ Nip$^-$ Nis^r) (32), which contains pNP40 coding for Nis^r, served as the recipient, the ability to metabolize sucrose was transferred from *S. lactis* ATCC 11454 or *S. lactis* 354/07 at low frequency (Table 2, standard protocol). The standard conjugation procedure was then modified to decrease the inhibitory effect of the donor-produced nisin on the recipient cells. Washing the donor cells to remove free nisin did not result in the transfer of the Suc$^+$ phenotype to LM2306. Furthermore, by itself the addition of alpha-chymotrypsin to the conjugal mixture (final concentration of 400 μg/ml) had no effect on the transfer of sucrose-fermenting ability to LM2306. However, when the donor cells were washed and alpha-chymotrypsin was added to the conjugal mixture, sucrose-fermenting ability was transferred from *S. lactis* ATCC 11454 and *S. lactis* 354/07 to *S. lactis* LM2306 (Table 2, nisin-modified protocol). Additional matings done by using the nisin-modified procedure and using *S. lactis* DL16 as the donor yielded no Suc$^+$ transconjugants, and the Suc$^-$ confluent lawn of growth was still absent from the conjugation plates. The nisin-modified conjugation procedure was then used in matings of the three Suc$^+$ Nip$^+$ Nis^r parental donors with the Nis^r recipient *S. lactis* D6528. Transfers of the Suc$^+$ phenotype from ATCC 11454, 354/07, and DL16 to D6528 were achieved with frequencies of 1.0×10^{-7}, 1.7×10^{-6}, and 4.4×10^{-7}, respectively. Therefore, the nisin-modified conjugation procedure was used for all remaining conjugation attempts.

TABLE 2. Conjugation of sucrose-fermenting ability to *S. lactis* LM2306 and D6528

Donor[a] × recipient[b]	Selected phenotype	Transfer frequency with conjugation protocol[c]:	
		Standard	Nisin modified[d]
DL16 × LM2306	Suc$^+$ Str^r Ery^r	0	0
11454 × LM2306	Suc$^+$ Str^r Ery^r	0	2.2×10^{-6}
354/07 × LM2306	Suc$^+$ Str^r Ery^r	0	2.6×10^{-6}
DL16 × D6528	Suc$^+$ Str^r	0	4.4×10^{-7}
11454 × D6528	Suc$^+$ Str^r	2.2×10^{-8}	1.0×10^{-7}
354/07 × D6528	Suc$^+$ Str^r	4.4×10^{-8}	1.7×10^{-6}

[a] All donors were Suc$^+$ Nip$^+$ Nis^r Str^s Ery^s.
[b] *S. lactis* LM2306 was Suc$^-$ Nip$^-$ Nis^s Str^r Ery^r; *S. lactis* D6528 was Suc$^-$ Nip$^-$ Nis^r Str^r Ery^s.
[c] Frequencies calculated as transconjugants per input donor; zero indicates that no transconjugants were observed, and the frequency was less than 10^{-9} per input donor.
[d] The nisin-modified conjugation procedure was the same as the standard procedure except that the donor cells were washed and alpha-chymotrypsin was added.

The Suc$^+$ transconjugants of recipients LM2306 and D6528 were then tested for the ability to produce nisin. Regardless of the donor used, all the Suc$^+$ transconjugants of LM2306 produced nisin; however, none of the Suc$^+$ transconjugants of D6528 produced any detectable nisin. To determine if the genes responsible for nisin production were present, but were not phenotypically expressed, in D6528 transconjugants, a Suc$^+$ Nip$^-$ transconjugant of D6528, designated *S. lactis* JS1, was used as the donor in conjugal matings with three recipients (Table 3). These matings were designed to determine if the Suc$^+$ phenotype could be transferred independently of the Nis^r determinant of pNP40, and result in Suc$^+$ Nip$^+$ transconjugants. The Nip$^-$ phenotype of JS1 was most likely due to the nisin resistance determinant of pNP40, since Suc$^+$ Nip$^+$ transconjugants

TABLE 3. Frequency of conjugal transfer of sucrose-fermenting ability by a Suc$^+$ Nip$^-$ transconjugant

Donor[a] × recipient[b]	Frequency of Suc$^+$ transfer[c]	Nisin production
S. lactis JS1 × *S. lactis* LM2306	2.1×10^{-6}	+
S. lactis JS1 × *S. lactis* EB101	1.1×10^{-5}	+
S. lactis JS1 × *S. cremoris* BC102	1.8×10^{-7}	+

[a] The phenotype of the JS1 donor strain was Suc$^+$ Nip$^-$ Nis^r Ery^s.
[b] The phenotype of all recipients was Suc$^-$ Nip$^-$ Nis^s Ery^r. The selected phenotype was Suc$^+$ Ery^r.
[c] Calculated as transconjugants per input donor.

were obtained for all three recipients in the absence of this plasmid.

To determine if the Suc$^+$ Nip$^+$ Nis^r marker codes for its own transfer or if its transfer is dependent on the presence of a fertility plasmid, a mating of *S. cremoris* JS71 with *S. lactis* LM2201 was attempted. *S. cremoris* JS71 is a Suc$^+$ Nip$^+$ Nis^r transconjugant which contains no detectable plasmid DNA and is derived from the JS1 × BC102 mating. *S. cremoris* JS71 conjugally transferred the Suc$^+$ Nip$^+$ Nis^r traits to LM2201 at a frequency of 2.3×10^{-7} per donor. Since Nip$^+$ cells must be Nis^r to survive, the Nip$^+$ Nis^r phenotype will be designated simply as Nip$^+$.

Plasmid isolation. The Suc$^+$ Nip$^+$ transconjugants of LM2306, the Suc$^+$ Nip$^-$ Nis^r transconjugants of D6528, and the Suc$^+$ Nip$^+$ transconjugants from matings in which JS1 was the donor were lysed and their plasmid profiles were determined. No additional plasmid DNA was detected in the transconjugants when plasmid profiles were compared to those of the recipients (data not shown), although exhaustive attempts were made to isolate a Suc$^+$ Nip$^+$ plasmid. The addition of diethylpyrocarbonate to the lysis protocol to inactivate nucleases did not result in the detection of any additional plasmid DNA.

Conjugal transfer of the Suc$^+$ Nip$^+$ marker to a Rec$^-$ recipient. The lack of an additional, detectable plasmid in the Suc$^+$ Nip$^+$ transconjugants suggested that the Suc$^+$ Nip$^+$ phenotype may be chromosomally encoded. Chromosomally encoded genes are dependent on a host-mediated homologous recombination enzyme system (Rec) for successful transfer and can thus be differentiated from plasmid-encoded genes (1). To determine if the Suc$^+$ Nip$^+$ phenotype was chromosomally encoded, *S. lactis* LM3301 and its Rec$^-$ derivative *S. lactis* MMS362 were used as recipients in conjugal matings with *S. lactis* ATCC 11454 and *S. lactis* 354/07. The Suc$^+$ Nip$^+$ trait was conjugally transferred into both the Rec$^+$ and Rec$^-$ recipients (Table 4). The resulting Suc$^+$ Str^r transconjugants were Nip$^+$. The frequency of conjugal transfer of the Suc$^+$ Nip$^+$ marker into the Rec$^-$ recipients was 100- to 1,000-fold lower than the frequency of transfer into the Rec$^+$ recipients. The lower frequency of transfer into the Rec$^-$ strain is consistent with the observations of Goering (10), who compared transduction of plasmid DNA into Rec$^+$ and Rec$^-$ recipients of *Staphylococcus aureus*. To confirm that the Suc$^+$ Nip$^+$ transconjugants of MMS362 were Rec$^-$, the UV sensitivities of MMS362, JS51, and JS61 were determined. Tests with MMS362 and JS61 yielded UV dose-survival curves that were consistent with those of Rec$^-$ strains, whereas tests with JS51 yielded a UV dose-survival curve that was consistent with those of Rec$^+$ strains (data not shown; 1).

Transformation of *S. lactis* LM0230 with *S. lactis* ATCC 11454 plasmid DNA. To determine if the transfer of the Suc$^+$ Nip$^+$ or Lac$^+$ phenotypes was possible by transformation and if this mode of transfer would result in a detectable Suc$^+$ Nip$^+$ plasmid, transformation of *S. lactis* LM0230 with *S. lactis* ATCC 11454 plasmid DNA was attempted. Transformants were selected by using M17-sucrose agar with lactose as a stabilizer and BCP as a pH indicator; therefore, Suc$^+$ and Lac$^+$ transformants would both exhibit the acid-producing phenotype. Of the 1,100 acid-producing transformants obtained, 750 were screened for the ability to utilize lactose or sucrose or both. All transformants fermented lactose but none metabolized sucrose. Plasmid profiles revealed that all eight Lac$^+$ transformants examined contained a 35-MDa plasmid. One transformant also contained a 5.0-MDa plasmid, and two transformants also contained both 5.0- and 1.0-MDa plasmids.

Incompatibility of the Suc$^+$ Nip$^+$ marker with the lactose plasmids of *S. lactis* ML3, *S. lactis* C2, and *S. lactis* subsp. *diacetylactis* DRC3. We attempted to construct a strain with both Nip$^+$ and fast-acid-producing (Lac$^+$; proteinase positive, Prt$^+$) capabilities when the strain was grown in milk. Although we were able to conjugally transfer certain lactose plasmids into Suc$^-$ Nip$^-$ recipients, we were unable to transfer lactose-fermenting ability into the corresponding Suc$^+$ Nip$^+$ transconjugants (Table 5). *S. lactis* ML3, a strain which harbors a conjugative Lac plasmid (33), transferred lactose-fermenting ability to *S. lactis* LM3301 at a frequency of 8.2×10^{-6} per donor cell, but did not transfer lactose-fermenting ability to *S. lactis* JS51, a Suc$^+$ Nip$^+$ transconjugant of LM3301 (Table 5, experiment 1). Similarly, *S. lactis* 4621 contains a conjugative Lac plasmid from *S. lactis* subsp. *diacetylactis* DRC3 (32) which was transferred to *S. cremoris* BC102 at a frequency of 3.0×10^{-6} per donor cell; however, 4621 was unable to transfer lactose-fermenting ability to *S. cremoris* JS71, a Suc$^+$ Nip$^+$ transconjugant of *S. cremoris* BC102 (Table 5, experiment 2).

To determine whether the lactose plasmid in *S. lactis* ML3 would inhibit the conjugal transfer of the Suc$^+$ Nip$^+$ marker, additional matings were attempted. *S. lactis* 354/07 transferred the Suc$^+$ Nip$^+$ marker to *S. lactis* LM3301 at a frequency of 1.7×10^{-4} per donor cell, but did not transfer the marker to *S. lactis* ML3S, the Lac$^+$ parent of LM3301 (Table 5, experiment 3).

Matings were then performed to ascertain whether a chromosomally integrated lactose plasmid could inhibit transfer of the Suc$^+$ Nip$^+$ marker. *S. lactis* ATCC 11454 was the donor of the Suc$^+$ Nip$^+$ marker, and the recipients were *S. lactis* LM2201 (Str^r derivative of LM0220) and *S. lactis* KB21, a Lac$^+$ Prt$^+$ transductant of LM0220 (31). *S. lactis* KB21 has part or all of pLM2103, the transductionally shortened lactose plasmid of *S. lactis* C2, integrated into its chromosome. *S. lactis* ATCC 11454 transferred the Suc$^+$

TABLE 4. Frequency of conjugal transfer of sucrose-fermenting ability into Rec$^-$ and Rec$^+$ recipients

Donor[a] × recipient[b]	Frequency of Suc$^+$ transfer[c]
354/07 × LM3301	1.7×10^{-4}
354/07 × MMS362	1.9×10^{-6}
11454 × LM3301	1.3×10^{-5}
11454 × MMS362	5.6×10^{-8}

[a] The phenotype of both donors was Suc$^+$ Str^s.
[b] The phenotype of LM3301 was Suc$^-$ Str^r Rec$^+$; the phenotype of MMS362 was Suc$^-$ Str^r Rec$^-$.
[c] Calculated as transconjugants per input donor.

TABLE 5. Conjugal matings demonstrating plasmid incompatibility between the Suc$^+$ Nip$^+$ phenotype and certain lactose plasmids

Expt no.	Donor		Recipient		Selected phenotype	Frequency of Lac$^+$ or Suc$^+$ transfer[a]
	Strain	Phenotype	Strain	Phenotype		
1	ML3	Lac$^+$ Str^s	LM3301	Lac$^-$ Suc$^-$ Str^r	Lac$^+$ Str^r	8.2×10^{-6}
			JS51	Lac$^-$ Suc$^+$ Str^r	Lac$^+$ Str^r	0
2	4621	Lac$^+$ Ery^s	BC102	Lac$^-$ Suc$^-$ Ery^r	Lac$^+$ Ery^r	3.0×10^{-6}
			JS71	Lac$^-$ Suc$^+$ Ery^r	Lac$^+$ Ery^r	0
3	354/07	Suc$^+$ Str^s	LM3301	Lac$^-$ Suc$^-$ Str^r	Suc$^+$ Str^r	1.7×10^{-4}
			ML3S	Lac$^+$ Suc$^-$ Str^r	Suc$^+$ Str^r	0
4	11454	Suc$^+$ Str^s	LM2201	Lac$^-$ Suc$^-$ Str^r	Suc$^+$ Str^r	6.2×10^{-6}
			KB21	Lac$^+$ Suc$^-$ Str^r	Suc$^+$ Str^r	0
5	11454	Suc$^+$ Ery^s	JK301	Lac$^-$ Suc$^-$ Em^r	Suc$^+$ Em^r	1.7×10^{-6}
			JK18	Lac$^+$ Suc$^-$ Em^r	Suc$^+$ Em^r	2.0×10^{-6}
6	PW2	Lac$^+$ Ery^s	BC102	Lac$^-$ Suc$^-$ Ery^r	Lac$^+$ Ery^r	5.1×10^{-2}
			JS71	Lac$^-$ Suc$^+$ Ery^r	Lac$^+$ Ery^r	3.0×10^{-6}

[a] Calculated on a per donor basis. Zero indicates that no transconjugants were observed and therefore the frequency was less than 10^{-9} per donor.

Nip$^+$ phenotype to *S. lactis* LM2201 at a frequency of 6.2×10^{-6}, but was unable to transfer the Suc$^+$ Nip$^+$ phenotype to *S. lactis* KB21 (Table 5, experiment 4).

Additional matings were attempted to determine if the *lac* genes directly inhibited conjugal transfer of the Suc$^+$ Nip$^+$ marker or if an additional locus on the lactose plasmid was required. *S. lactis* ATCC 11454 was the donor of the Suc$^+$ Nip$^+$ marker, and the recipients were *S. lactis* JK301 and *S. lactis* JK18. *S. lactis* JK301 (25) is a transformant of *S. lactis* LM0230 containing pGB301 (4), a streptococcal cloning vehicle coding for erythromycin resistance. *S. lactis* JK18 is a transformant of *S. lactis* LM0230 containing pJK18 (25), a derivative of pGB301 with an inserted 19.4-kilobase-pair *Bcl*I fragment containing the *lac* genes from pLM2001. *S. lactis* ATCC 11454 transferred the Suc$^+$ Nip$^+$ marker into both *S. lactis* JK301 and *S. lactis* JK18 (Table 5, experiment 5).

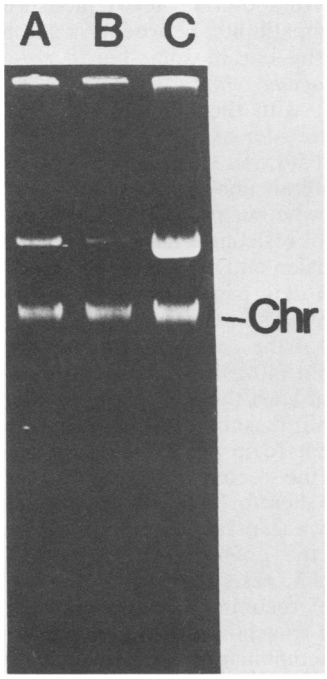

FIG. 1. Agarose gel electrophoretic patterns of the 60-MDa Lac plasmids from *S. lactis* PW2 (lane A), an unstable Lac$^+$ Suc$^+$ transconjugant (lane B), and a stable Lac$^+$ Suc$^+$ transconjugant (lane C). The band above the chromosome (Chr) represents the Lac plasmid. Plasmid DNA was isolated from 5.0 ml of 4-h cultures and analyzed without CsCl-ethidium bromide density gradient purification (2).

The last set of matings was done to ascertain whether the Suc$^+$ Nip$^+$ marker would inhibit the transfer of a lactose plasmid which exhibited high-frequency conjugal transfer. *S. lactis* PW2, a strain which harbors such a plasmid (45), served as the donor. *S. lactis* PW2 transferred the ability to ferment lactose to *S. cremoris* BC102 at a frequency of 5.1×10^{-2}. *S. lactis* PW2 also transferred its lactose plasmid to *S. cremoris* JS71, a Suc$^+$ Nip$^+$ transconjugant of *S. cremoris* BC102, but at a reduced frequency of 3.0×10^{-6}. To stably maintain the Lac$^+$ Suc$^+$ Nip$^+$ phenotype in the JS71 recipients, the transconjugants were propagated in M17-lactose broth containing 2.0 ng of nisin per ml. Four Lac$^+$ Suc$^+$ Nip$^+$ transconjugants were tested for the stability of the Lac$^+$ Suc$^+$ phenotype after nonselective growth. After plating on Elliker agar, three of the transconjugants exhibited partitioning of the two phenotypes; about 50% of the cells became Lac$^-$ Suc$^+$, and the remaining cells became Lac$^+$ Suc$^-$. The other Lac$^+$ Suc$^+$ Nip$^+$ transconjugant stably maintained the Lac$^+$ Suc$^+$ phenotype without selective pressure. It also appeared to maintain the lactose plasmid at a significantly higher copy number than the other three transconjugants, based on the intensities of the plasmid bands in agarose gels (Fig. 1).

The results obtained to this point indicated that there was replicon incompatibility between the Suc$^+$ Nip$^+$ marker and certain Lac plasmids found in *S. lactis* and suggested that the type of replicon incompatibility occurring was plasmid incompatibility. Transduction of lactose metabolism was used to determine whether plasmid incompatibility was occurring rather than entry exclusion. A lactose-transducing lysate was obtained from *S. lactis* 8, a Lac$^+$ transductant of *S. lactis* LM0220, and mixed with *S. lactis* LM2306 or its Suc$^+$ Nip$^+$ transconjugant, *S. lactis* JS21. *S. lactis* LM2306 gave rise to 7.2×10^3 Lac$^+$ transductants per ml of phage lysate, whereas no Lac$^+$ transductants of *S. lactis* JS21 were observed.

DISCUSSION

The inhibitors produced by *S. lactis* ATCC 11454, *S. lactis* 354/07, *S. lactis* DL16, and by transconjugants from matings using *S. lactis* ATCC 11454 or *S. lactis* 354/07 as the donor

had identical proteolytic enzyme resistance patterns. These inhibitors were inactivated by alpha-chymotrypsin but not by pronase or trypsin. These results agree with those reported for the antibiotic nisin (21). Since *S. lactis* ATCC 11454 was previously reported to be a nisin producer (11, 28), the results suggest that all three parental strains are nisin producers.

Since the involvement of plasmid DNA with the Suc$^+$ Nip$^+$ Nis^r phenotype in *S. lactis* ATCC 11454 was previously reported (9, 11, 26, 28), we examined the stability of the Suc$^+$ Nip$^+$ Nis^r trait when *S. lactis* ATCC 11454 was grown under conditions which selectively inhibited plasmid replication (16). In the presence of AO, 0.1% of the culture became Suc$^-$ Nip$^-$ Nis^s and 1.0% became Lac$^-$. The greater stability of the Suc$^+$ Nip$^+$ Nis^r trait may be due in part to selection against Nis^s cells by the nisin produced by Suc$^+$ Nip$^+$ Nis^r cells. Loss of the Suc$^+$ Nip$^+$ Nis^r phenotype appeared to correlate with the loss of a 30-MDa plasmid, and loss of the Lac$^+$ phenotype correlated with the loss of a 35-MDa plasmid. Plasmid curing results for *S. lactis* ATCC 11454 were previously reported by LeBlanc et al. (28) who correlated the loss of a 28-MDa plasmid and a 32-MDa plasmid with the loss of the Suc$^+$ Nip$^+$ Nis^r and Lac$^+$ phenotypes, respectively. Similarly, Gonzalez and Kunka (11) correlated the loss of a 29.1-MDa plasmid and a 31.8-MDa plasmid with the loss of the Suc$^+$ Nip$^+$ Nis^r and Lac$^+$ phenotypes, respectively. The differences in molecular weights reported are likely due to the different conditions used for the sizing of plasmid DNA.

Conjugal transfer of the Suc$^+$ Nip$^+$ trait by *S. lactis* ATCC 11454, *S. lactis* 354/07, and *S. lactis* DL16 was demonstrated. However, to allow successful transfer, the conjugation procedure was modified to reduce the inhibitory effect of nisin on the recipient cells. This was achieved by washing the donor cells to remove the free nisin and by the addition of alpha-chymotrypsin to inactivate the residual and cell-bound nisin. Gasson washed filters containing the conjugal mixture with fresh medium, performed short-term matings, and obtained similar transfer frequencies (9). Gonzalez and Kunka harvested the donor cells early in the log phase, performed short-term matings, and also obtained similar transfer frequencies (11).

When *S. lactis* D6528(pNP40, Nis^r) was the conjugal recipient, all Suc$^+$ transconjugants were Nip$^-$ Nis^r, regardless of the donor that was used. However, when a Suc$^+$ Nip$^-$ Nis^r transconjugant of D6528 was used as the donor, the resulting transconjugants were all Suc$^+$ Nip$^+$ Nis^r. This suggested that the Nis^r determinant of pNP40 was masking the Nip$^+$ genotype of the Suc$^+$ transconjugants of D6528.

Since the Suc$^+$ Nip$^+$ marker originally came from a strain containing many plasmids, it was necessary to determine whether it was self-transmissible or whether it was being mobilized by a different plasmid. *S. cremoris* JS71, a Suc$^+$ Nip$^+$ strain which contained no detectable plasmid DNA, was constructed and was mated with *S. lactis* LM2201. The results indicated that the Suc$^+$ Nip$^+$ marker coded for its own transfer; this was also suggested by Gonzalez and Kunka (11).

No additional plasmid DNA was detected when the Suc$^+$ Nip$^+$ phenotype was conjugally transferred. Exhaustive attempts were made to isolate a Suc$^+$ Nip$^+$ plasmid by using a lysis technique which was effective in isolating plasmids up to a size of 88 MDa (39) and at a copy number as low as 1.0 copy per cell (3). The addition of a nuclease inhibitor to the lysis procedure did not lead to the recovery of a Suc$^+$ Nip$^+$ plasmid; therefore, the inability to isolate a Suc$^+$ Nip$^+$ plasmid is probably not because of nuclease activity. An inability to isolate any additional plasmid DNA from Suc$^+$ Nip$^+$ transconjugants, even though three different plasmid isolation techniques were used, was reported previously by Gonzalez and Kunka (11). Similar results were reported by Gasson (9) who also used three plasmid isolation procedures. However, sometimes a faint plasmid band of approximately 30 MDa was visualized; this DNA was apparently lost upon storage or restriction (9). One of the following factors may account for our inability to isolate a Suc$^+$ Nip$^+$ plasmid: the integration of a Suc$^+$ Nip$^+$ plasmid upon conjugal transfer; the existence of a determinant other than plasmid DNA for the Suc$^+$ Nip$^+$ traits; or the presence of a Suc$^+$ Nip$^+$ plasmid which is undetectable by the isolation procedures employed (even though six different plasmid isolation techniques have been used).

The conjugal transfer of genetic information in the absence of plasmid DNA is well documented for the streptococci (8, 15, 18, 33). McKay et al. (33) reported the conjugal transfer of chromosomally encoded streptomycin resistance and the transfer of chromosomally linked *lac* genes from an integrated Lac plasmid in *S. lactis*. Guild et al. (15) demonstrated conjugal transfer of the chromosomally linked R determinants *cat* and *tet* in the absence of plasmid DNA. Cotransformation of the *cat* and *tet* genes and a chromosomal gene implied that the three genes were linked and were present at a chromosomal location. Further support for the chromosomal location of the *cat-tet* determinant was obtained by showing that *cat* transforming ability cosediments with chromosomal DNA markers in dye-buoyant gradients. Horodniceanu et al. (18) reported low-frequency conjugal transfers of multiple-antibiotic resistance markers in the absence of detectable plasmid DNA for nine streptococcal strains. In addition, they demonstrated entry exclusion and incompatibility between an autonomous plasmid (pIP501) and the chromosome-borne *cat-tet-erm* markers from *Streptococcus agalactiae* B109. The entry exclusion was unilateral, with the *cat-tet-erm* determinant of B109 inhibiting the transfer of pIP501 but not vice versa. Incompatibility of pIP501 and the resistance determinants of B109 was also unilateral, and the frequency of pIP501 loss was dependent on whether pIP501 was incoming or whether it was the resident resistance marker. The unilateral nature of the entry exclusion and incompatibility between pIP501 and the *cat-tet-erm* determinant of B109 is the result expected between an integrated and an autonomous plasmid (22). Further study of the *cat-tet-erm* element was reported by Smith and Guild (40); they showed that it was capable of insertion into at least three different locations on the conjugative hemolysin plasmid pAD1. The ability of the *cat-tet-erm* determinant to insert at different locations on pAD1 suggests that the recombination event is not related to homology and therefore implies that the *cat-tet-erm* genes are encoded by a transposable element. Franke and Clewell (8) described the conjugal transfer of a chromosomally linked tetracycline resistance determinant. The determinant was capable of recombining with plasmid DNA and was capable of self-transmission independent of host-mediated homologous recombination enzymes. The determinant was located on the 10-MDa transposon Tn*916*. Therefore, the conjugal transfer of genetic information in the absence of plasmid DNA in the streptococci has involved a chromosomally integrated plasmid, transfer of chromosomal DNA, transposable elements, and an element with characteristics of both plasmid and transposon origins.

Bacterial genetic information is coded for by three basic

types of determinants: chromosomal DNA, plasmid DNA, and transposable elements. Chromosomal genes can be differentiated from plasmid and transposon genes by their dependence on the host *rec* gene products for integration into chromosomal DNA. Transferred chromosomal markers must be integrated into host replicons for replication and expression to occur, whereas replication and expression of transferred plasmid or transposon genes are independent of the host *rec* gene products. Our results indicate that the conjugal transfer of the Suc^+ Nip^+ marker was a *rec*-independent event; thus, we conclude that the Suc^+ Nip^+ marker is not chromosomally encoded.

We next attempted to construct a Lac^+ Prt^+ Suc^+ Nip^+ strain. The Suc^+ Nip^+ replicon and certain Lac plasmids interacted such that there was a reduced frequency of conjugal transfer when we tried to combine the Suc^+ Nip^+ and Lac^+ phenotypes (Table 5). The transfer frequency of lactose metabolism coded for by pPW2, a plasmid capable of high-frequency conjugal transfer (45), was reduced approximately 10,000-fold in the presence of the Suc^+ Nip^+ marker. Thus, it was not surprising that we were unable to isolate Lac^+ Suc^+ Nip^+ transconjugants when the trait being transferred was incapable of high-frequency conjugation. This reduced frequency of conjugal transfer is most likely a result of replicon incompatibility. Two types of replicon incompatibility are known: entry exclusion and plasmid incompatibility (36). Entry exclusion occurs because of a specific cell surface barrier which prevents conjugal transfer between two strains containing closely related or isogenic conjugative plasmids; this barrier can be bypassed if the entering plasmid is introduced by transduction (36). Plasmid incompatibility occurs when two plasmid-linked traits cannot be stably maintained simultaneously within the same cell line (38). Two results support the hypothesis that plasmid incompatibility was operative in our work rather than entry exclusion. First, lactose metabolism was transduced into *S. lactis* LM2306 but not into its Suc^+ Nip^+ derivative *S. lactis* JS21. Second, the Lac^+ Suc^+ transconjugants from the PW2 × JS71 mating were unable to stably maintain the Lac^+ Suc^+ phenotype. Instability of the Lac^+ Suc^+ phenotype in these transconjugants was bilateral, suggesting that both the *suc nip* determinant and the Lac plasmid exist as independent replicons. The ability to replicate autonomously, which is implied by the bilateral incompatibility, suggests that the *suc nip* determinant is not coded for by a classical transposable element. However, in light of the discovery of a multiple-antibiotic resistance determinant in *S. agalactiae* B109 which seems to exhibit features of both a transposable element and a plasmid (18, 40), the possible involvement of such an element with the Suc^+ Nip^+ phenotype cannot be ruled out.

Plasmid incompatibility and copy number control are related (43, 44); a shared regulatory mechanism may be responsible for both phenomena (43). Therefore, it is not surprising that a Lac^+ Suc^+ transconjugant which stably maintained the Lac^+ Suc^+ phenotype appeared to maintain its lactose plasmid at a higher copy number. A cell cannot differentiate between two incompatible plasmids with regard to replication and assortment (37). Therefore, the partitioning of two incompatible plasmids is dependent on their relative copy numbers. The equal segregation of three of the four Lac^+ Suc^+ transconjugants to Lac^+ Suc^- and Lac^- Suc^+ phenotypes implies that the *lac* replicon and *suc nip* replicon are maintained at similar copy numbers, if random assortment of replicons is assumed (37). Because pPW2 is reportedly held at approximately 1.9 copies per cell (3), it is likely that the *suc nip* replicon is maintained at a similar copy number. Further evidence for a low copy number for the Suc^+ Nip^+ marker is its incompatibility with the integrated lactose plasmid of *S. lactis* KB21 (Table 5, experiment 4). Incompatibility with an integrated plasmid implies a copy number of less than two, because any plasmid with a copy number greater than two should be stable in the presence of an integrated plasmid belonging to the same incompatibility group (12).

Transformation of *S. lactis* LM0230 with the plasmid pool of *S. lactis* ATCC 11454 resulted in 750 Lac^+ transformants and no Suc^+ transformants. The transformation results confirm our lactose fermentation curing data. In addition, our results agree with those reported previously (11, 28) that the 35-MDa plasmid of *S. lactis* ATCC 11454 codes for the ability to metabolize lactose. Because three of the eight Lac^+ transformants examined contained small plasmids in addition to the 35-MDa Lac plasmid, it may be possible to use transformation, followed by curing of the Lac^+ phenotype, to isolate cryptic plasmids in the lactic streptococci. Suc^+ transformants were not obtained, even though the 30-MDa plasmid was visualized in the plasmid pool that was used. This contradicts our curing results and the previously reported curing studies which linked sucrose metabolism to a plasmid of approximately 30 MDa in *S. lactis* ATCC 11454 (11, 28).

In *S. lactis* ATCC 11454, the role of the 30-MDa plasmid in sucrose metabolism and nisin production remains unclear. If the 30-MDa plasmid does code for the Suc^+ Nip^+ phenotype, it must be nontransformable and must undergo a structural change to an undetectable form upon conjugal transfer. Such a change could involve the formation of an undetectable cointegrate plasmid (i.e., a plasmid-protein complex) or the integration of the transferred plasmid into the chromosomal DNA of the recipient by a *rec*-independent event. Another possibility is that the 30-MDa plasmid may be required for the stable maintenance of a currently undetectable plasmid in *S. lactis* ATCC 11454 which codes for the Suc^+ Nip^+ phenotype.

In conclusion, our results suggest that the Suc^+ Nip^+ phenotype is plasmid coded, although the involvement of a genetic element exhibiting characteristics of both plasmid and transposon origins is possible. The involvement of plasmid DNA is suggested by the curing data, *rec*-independent transfer, and bilateral incompatibility with known plasmids. The segregation data and the incompatibility with an integrated Lac plasmid imply that the suggested Suc^+ Nip^+ plasmid is maintained at a copy number of less than two. In addition, we confirmed the suggestive evidence of LeBlanc et al. (28) and Gonzalez and Kunka (11) that the 35-MDa plasmid of *S. lactis* ATCC 11454 codes for lactose metabolism.

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