APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1991, p. 517–524
0099-2240/91/020517-08$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 57, No. 2

Genetic Construction of Nisin-Producing *Lactococcus lactis* subsp. *cremoris* and Analysis of a Rapid Method for Conjugation†

JEFFERY R. BROADBENT AND JEFFERY K. KONDO*

Western Dairy Foods Research Center, Department of Nutrition and Food Sciences, Utah State University, Logan, Utah 84322-8700

Received 29 May 1990/Accepted 25 November 1990

Conjugation was used to construct nisin-producing *Lactococcus lactis* subsp. *cremoris* strains. Recipients were obtained by electroporation of *L. lactis* subsp. *cremoris* strains with the drug resistance plasmid pGK13 or pGB301. A method, direct-plate conjugation, was developed in which donor and recipient cells were concentrated and then combined directly on selective media. This method facilitated transfer of the nisin-sucrose (Nip^+ Suc^+) phenotype from the donor strain, *L. lactis* subsp. *lactis* 11454, to three *L. lactis* subsp. *cremoris* recipient strains. Nip^+ Suc^+ *L. lactis* subsp. *cremoris* transconjugants were obtained at frequencies which ranged from 10^{-7} to 10^{-8} per donor CFU. DNA-DNA hybridization to transconjugant DNAs, performed with an oligonucleotide probe synthesized to detect the nisin precursor gene, showed that this gene was transferred during conjugation but was not associated with detectable plasmid DNA. Further investigation indicated that *L. lactis* subsp. *cremoris* Nip^+ Suc^+ transconjugants retained the recipient strain phenotype with respect to bacteriophage resistance and acid production in milk. Results suggested that it would be feasible to construct nisin-producing *L. lactis* subsp. *cremoris* strains for application as mixed and multiple starter systems. Additionally, the direct-plate conjugation method required less time than filter or milk agar matings and may also be useful for investigations of conjugal mechanisms in these organisms.

Nisin is a peptide antibiotic produced by some strains of *Lactococcus lactis* subsp. *lactis*. The antibiotic inhibits a variety of gram-positive bacteria (21) and has been used frequently in Europe as an additive to prevent clostridial blowing in processed cheese (24). The Food and Drug Administration recently awarded a nisin preparation generally regarded as safe status within the United States for use in certain pasteurized cheese spreads to prevent contamination by *Clostridium botulinum* (13).

Investigators have attempted to use nisin-producing starter cultures, alone or with the usual industrial starter strains of lactococci, lactobacilli, and propionibacteria, to control clostridial spoilage in rennet-set Dutch and Swiss cheeses (Edam, Emmenthal). The nisin producers controlled clostridial growth but adversely affected cheese quality even when the industrial starters were included, because they were also inhibited by nisin (21, 24). Later experiments which combined nisin producers with nisin-resistant mutants of the industrial starters also proved ineffective because the latter organisms suffered from prolonged lag phase, increased bacteriophage sensitivity, lower heat resistance, and lower acid production in milk (24). The nisin sensitivity of cultures used in mixed and multiple starter systems has continued to prevent the inclusion of a single nisin-producing strain and has limited the use of nisin to direct addition in processed products. Nisin-producing cheese starters have remained appealing, however, because the antibiotic has been shown to inhibit pathogenic and spoilage organisms such as *Listeria*, *Staphylococcus*, and *Clostridium* spp., which occasionally contaminate cheeses (5, 7, 21, 24). Additionally, U.S. cheese processors that utilized cheeses made with nisin-producing starters could reduce the amount of added nisin needed in cheese spread blends. Genetic studies have recently suggested that it is possible to construct nisin-producing starter cultures which would avoid the problems observed by early investigators.

Although the nisin precursor gene, *nisA*, has been cloned (6, 9, 22), nisin production by clones has not been reported. Expression will likely require cloning of the gene(s) needed for nisin activation (9, 21) and nisin immunity (if the cloning host is sensitive). Beginning in 1984, however, Gasson (15) reported conjugal transfer of nisin production and immunity (Nip^+) among strains of *L. lactis* subsp. *lactis* and the simultaneous transfer of sucrose-fermenting ability (Suc^+). Gonzales and Kunka (17) later described transfer of the Nip^+ Suc^+ phenotype into a strain of *L. lactis* subsp. *lactis* biovar *diacetylactis* and discovered that the transconjugant had acquired resistance to a lytic bacteriophage. Murphy et al. (29) also reported reduced bacteriophage sensitivity (Rbs^+) in Suc^+ transconjugants of *L. lactis* subsp. *lactis*. Steele and McKay, in 1986 (35), reported on the genetic basis of Nip^+ Suc^+ and mentioned conjugal transfer of these genes into a strain of *L. lactis* subsp. *cremoris*. These reports indicated that natural gene transfer might be utilized to obtain Nip^+ starters.

This report describes the method of direct-plate conjugation (DPC), a technique which enhanced transfer of Nip^+ Suc^+ among *L. lactis* subsp. *lactis* strains. When DPC was applied to three *L. lactis* subsp. *cremoris* recipients obtained by transformation of parental strains with drug resistance plasmids, Nip^+ Suc^+ transconjugants from all three recipients were obtained. These transconjugants retained the recipient strain phenotype with respect to bacteriophage resistance and acid production in milk. DNA-DNA hybridization data also indicated that the *nisA* gene was transferred during conjugation but was not associated with detectable plasmid DNA in either donor or transconjugant cells. Finally, the DPC method was used to investigate features of Nip^+ Suc^+ transfer.

* Corresponding author.

† Approved as journal paper no. 3985 of the Utah Agricultural Experiment Station.

TABLE 1. Lactococcal strains used in this study

Strain	Relevant phenotype	Description (reference)
L. lactis subsp. *lactis*		
ATCC 11454	Nip^+ Suc^+ Em^s	Nip^+ Suc^+ donor (35)
ML3	Lac^+ Em^s	Lac^+ donor (2)
LM2306	Nip^- Suc^- Lac^- Em^r	Plasmid-cured recipient derived from strain C2 (35)
NS5406	Nip^+ Suc^+ Em^r Rbs^+	Nip^+ Suc^+ transconjugant from 11454 × LM2306 (this study)
JBML06	Lac^+ Em^r	Lac^+ transconjugant from ML3 × LM2306 (this study)
7962	Nip^+ Suc^+ Em^s	Nip^+ Suc^+ donor (40)
DL16	Nip^+ Suc^+ Em^s	Nip^+ Suc^+ donor (35)
L. lactis subsp. *cremoris*		
CS224	Lac^+ Nip^- Suc^- Em^s	Parental strain (43)
SW224	Lac^+ Nip^- Suc^- Em^r	CS224 transformed with pGB301 by protoplast transformation (43)
NS224	Lac^+ Nip^+ Suc^+ Em^r	Nip^+ Suc^+ transconjugant from 11454 × SW224 (this study)
EB7	Lac^+ Nip^- Suc^- Em^s	Parental strain (33)
JKEB7	Lac^+ Nip^- Suc^- Em^r	EB7 electrotransformed with pGB301 (this study)
NSEB7	Lac^+ Nip^+ Suc^+ Em^r	Nip^+ Suc^+ transconjugant from 11454 × JKEB7 (this study)
C3	Lac^+ Nip^- Suc^- Em^s	Parental strain (33)
JBC3	Lac^+ Nip^- Suc^- Em^r	C3 electrotransformed with pGK13 (this study)
NSC3	Lac^+ Nip^+ Suc^+ Em^r	Nip^+ Suc^+ transconjugant from 11454 × JBC3 (this study)

MATERIALS AND METHODS

Bacterial strains. Lactococcal strains used in this study are described in Table 1. Cultures were stored at 4°C and maintained by biweekly transfers in M17 broth (38), which contained 0.5% glucose or lactose (M17-G or M17-L) as the sole carbohydrate source. Lactococcal cultures were grown at 30°C. *Escherichia coli* V517 (25) was grown in brain-heart infusion (BBL Microbiology Systems, Cockeysville, Md.) at 37°C with aeration.

Electroporation. To develop suitable *L. lactis* subsp. *cremoris* recipients for conjugation, we transformed strains by electroporation with either the 4.9-kb plasmid pGK13 (37) or the 9.8-kb plasmid pGB301 (4). These plasmids each contain resistance determinants to erythromycin and chloramphenicol which are expressed in lactococcal hosts. Cells for electroporation were grown to an optical density at 600 nm of 0.3 in M17-L, centrifuged at 4,300 × *g* for 10 min, washed with 5 ml of ice-cold electroporation buffer (1 mM potassium phosphate buffer [pH 7.4], 0.5 M sucrose, 1 mM $MgCl_2$) (31), and suspended in 0.01 culture volume of cold electroporation buffer. Cells (200 μl) were then mixed with 1 μg of CsCl-purified plasmid DNA (in 10 mM Tris hydrochloride [pH 8.0]) and loaded into a 2.5-mm electroporation cuvette of a Prototype Design Services model ZA1000 electroporation unit (Madison, Wis.). A single pulse of 1.75 kV (7.0 kV/cm, 15 μF capacitance) was delivered to the mixture, and then the cells were cooled on ice for 10 min, 200 μl of cold 2× M17-G was added, and the mixture was chilled on ice for an additional 15 min. The cells were incubated for 1 h to allow expression of the erythromycin resistance (Em^r) determinant and then plated on M17-G agar which contained 0.5 M sucrose and 5 μg of erythromycin per ml. Plates were examined for Em^r colonies after 48 h of incubation. Agarose gel electrophoresis was used to detect pGK13 or pGB301 in lysates prepared from the putative transformants.

DPC matings. Observations in this laboratory indicated that conjugation would occur if donor and recipient cells were mixed directly on selective media. To prepare cells for conjugation, we made 1.5% inoculations into fresh broth from 18-h-old cultures. Donors were grown in M17-L broth, and recipients were grown in M17-G or M17-L containing 5 μg of erythromycin per ml. Donor and recipient cultures were incubated for 4 h (>10^7 CFU/ml), collected by centrifugation at 4,300 × *g*, washed in 5 ml of 0.85% saline (Nip^+ Suc^+ donor cells were washed three times to remove residual nisin), suspended in 0.1 volume of saline, and placed on ice. Donors and recipients were then mixed 1:2, to a final volume of 100 μl, directly on selective media. Conjugal transfer of lactose-fermenting ability (Lac^+) was performed on bromocresol purple-lactose indicator agar (28) which contained 5 μg of erythromycin per ml, and Nip^+ Suc^+ was transferred on bromocresol purple-sucrose agar (35) that contained the antibiotic. Donor and recipient controls received the respective cell ratio blended with saline. After 48 h of incubation, plates were examined for large yellow transconjugant colonies (e.g., see Fig. 1), and transconjugants were verified by phenotypic characterization and plasmid analysis. Transfer frequencies were expressed as the number of transconjugants per donor CFU, and the values reported were the average of at least four separate experiments.

Controls against other forms of gene transfer were performed to verify that conjugation was the mechanism of transfer on selective media. Transduction controls were performed by blending filtered (0.45-μm pore size) donor growth supernatant with recipient cells (1:1), adding 1 M $CaCl_2$ to a final concentration of 10 mM, and plating the mixture (100 μl) onto selective media. For transformation controls, 0.5 ml each of donor and recipient cell preparation was placed into sterile 1.5-ml microcentrifuge tubes and centrifuged for 10 s at 16,000 × *g* to pellet cells. The donor and recipient cell pellets were each suspended in 0.5 ml of filter-sterilized 0.85% saline which contained 100 mg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, and matings were performed as described above. Conjugation controls were performed with nonviable donor cells prepared by two methods: heating for 1 h at 55°C, and the Clorox treatment method described by Hershfield (18). Treated cells were plated onto Elliker agar (Difco Laboratories, Detroit, Mich.) to confirm donor cell death.

The cross-streak DPC assay was developed as a modification of the Franke and Clewell (14) procedure for detection of high-frequency conjugal events. Donor and recipient cells were streaked perpendicular to one another on selective

TABLE 2. Comparison of conjugal transfer frequencies obtained by DPC and milk agar plate methods

Mating (donor × recipient)	Selected phenotype	Method	Transfer frequency (transconjugants/donor CFU)
ML3 × LM2306	Lac^+ Em^r	Milk agar; RP[a]	2.2×10^{-9}
ML3 × LM2306	Lac^+ Em^r	Milk agar; harvest[b]	1.1×10^{-7}
ML3 × LM2306	Lac^+ Em^r	DPC	1.1×10^{-7}
ML3 filtrate × LM2306	Lac^+ Em^r	DPC	$<7.2 \times 10^{-10}$
ML3 + DNase × LM2306 + DNase	Lac^+ Em^r	DPC	6.2×10^{-8}
Nonviable ML3[c] × LM2306	Lac^+ Em^r	DPC	$<7.2 \times 10^{-10}$
ATCC 11454 × LM2306	Suc^+ Em^r	Milk agar; RP[a]	2.4×10^{-8}
ATCC 11454 × LM2306	Suc^+ Em^r	Milk agar; harvest[b]	1.6×10^{-6}
ATCC 11454 × LM2306	Suc^+ Em^r	DPC	7.0×10^{-6}
ATCC 11454 filtrate × LM2306	Suc^+ Em^r	DPC	$<1.9 \times 10^{-9}$
ATCC 11454 + DNase × LM2306 + DNase	Suc^+ Em^r	DPC	8.7×10^{-7}
Nonviable ATCC 11454[c] × LM2306	Suc^+ Em^r	DPC	$<1.9 \times 10^{-9}$

[a] Transconjugants were detected by replica plating onto selective media (2).
[b] Cell mixture was harvested from the milk agar plate with 1 ml of 0.85% saline, and then cells were plated on selective media to detect transconjugants (27).
[c] Donors were heat killed (55°C for 1 h) or Clorox treated (18).

media with sterile cotton applicators. Three potential high-frequency donor strains with a common recipient were assayed on each plate. Plates were examined for transconjugant colonies after 48 h of incubation, and transconjugants were verified by phenotypic characterization and plasmid analysis.

Solid surface milk agar conjugation. Solid surface milk agar matings were performed by the method of McKay et al. (27), except that for Nip^+ Suc^+ matings, the α-chymotrypsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment of Steele and McKay (35) was used. Transconjugants from solid surface milk agar matings were detected by replica plating onto selective media (2) or cells were harvested from the milk agar plate (27) in 1 ml of 0.85% saline and then 0.1-ml volumes were plated onto selective media. Transfer frequencies were calculated as described above for DPC matings.

DNA isolation and purification. Plasmids were isolated by the method of Anderson and McKay (1) and, if needed, purified by CsCl density gradient centrifugation (26). The presence of plasmids in the cells lysates was established by electrophoresis in 0.6% agarose gels at 3 V/cm for 7 h with CsCl-purified plasmids from *E. coli* V517 (25) included as plasmid size standards.

Genomic DNA was isolated by a modification of the Anderson and McKay procedure (1). After sodium dodecyl sulfate was added, the cell mixture was vortexed at high speed for 15 s. NaCl was added to a final concentration of 3%, and then the solution was extracted with 1 volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was collected after centrifugation at 4,000 × *g* for 10 min at 4°C, and the extraction was repeated. DNA was precipitated with 2 volumes of absolute ethanol and stored overnight at −20°C. The DNA was collected by centrifugation at 4°C for 20 min at 7,600 × *g*, dried, and suspended in 10 mM Tris hydrochloride, pH 8.0. Residual protein was removed by the addition of an equal volume of 5 M ammonium acetate followed by centrifugation at 16,000 × *g* for 15 min. The supernatant was collected, and the DNA was precipitated with ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and suspended in 5 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

Restriction analysis of genomic DNA was performed according to the enzyme manufacturer's directions (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or International Biotechnologies Inc., New Haven, Conn.). Prior to electrophoresis, 3 μl of RNase A (Sigma Chemical Co.) (1 mg/ml in 10 mM Tris hydrochloride, pH 8.0) was added to each sample. Restricted genomic DNA was separated in 0.8% agarose gels at 1.4 V/cm for 18 h. Adenovirus type 2 DNA digested with *Eco*RI and *Bam*HI (International Biotechnologies Inc.) was used for fragment size standards.

Oligonucleotide probe synthesis and DNA-DNA hybridizations. To detect the *nisA* gene in cells, a 14-mer oligonucleotide probe was synthesized (Utah State University Biotechnology Center, Logan) with an Applied Biosystems model 380B DNA synthesizer (Foster City, Calif.) and then purified by high-pressure liquid chromatography. The synthetic oligonucleotide, 5′-ATGTTACAACCCAT-3′, was complementary to a portion of the published *nisA* gene sequence of *L. lactis* subsp. *lactis* 11454 (6, 9, 22). 5′-end labeling of the *nisA* probe with [γ-^{32}P]ATP, hybridization to plasmid and genomic DNA in NaCl-sodium citrate solution, and autoradiography were performed as described by Ausubel et al. (3). Transfer of DNA to GeneScreen Plus nylon membranes (E. I. du Pont de Nemours & Co., Inc., NEN Research Products, Boston, Mass.) was performed by the method of Southern (34).

Assays. Nisin production by transconjugants was verified by the agar overlay method described by Steele and McKay (35). To determine whether Nip^+ Suc^+ transconjugants expressed Rbs^+, we performed spot assays for bacteriophage sensitivity as described by Terzaghi and Sandine (38). Fast acid production in milk was measured by the ability to clot 11% nonfat dry milk at 21°C within 16 to 18 h.

RESULTS

Development of DPC and methods comparison. Transfer frequencies of the Lac^+ and Nip^+ Suc^+ phenotypes that were obtained from DPC and solid surface milk agar matings (27) were compared. The recipient for both matings was *L. lactis* subsp. *lactis* LM2306 (35). The Lac^+ donor was *L. lactis* subsp. *lactis* ML3 (2), and *L. lactis* subsp. *lactis* 11454 (17, 35) served as the Nip^+ Suc^+ donor. Results of the comparison are shown in Table 2. DPC provided a similar rate of Lac^+ transfer but produced a 4- to 100-fold-higher frequency for Nip^+ Suc^+ transfer. Plasmid analysis of transconjugants showed that all Lac^+ isolates screened contained either the 33-MDa Lac plasmid and/or the 60-MDa cointegrate (2), while Suc^+ isolates contained no detectable

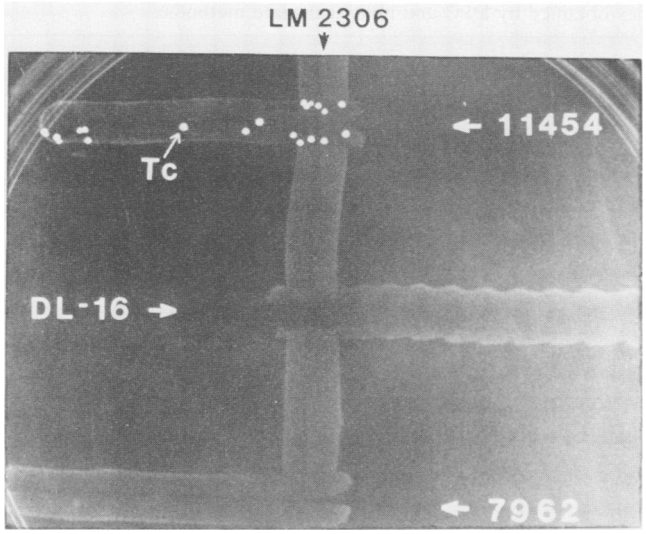

FIG. 1. Cross-streak DPC assay for high-frequency Nip$^+$ Suc$^+$ transfer. Arrows next to strain designations indicate the direction cells were streaked with sterile cotton applicators. The recipient, LM2306, was streaked first, and then donors were streaked perpendicular to the recipient. The bromocresol purple-sucrose plate, which contained 5 μg of erythromycin per ml, was photographed after 48 h of incubation at 30°C. Tc, Suc$^+$ Em^r transconjugant colony.

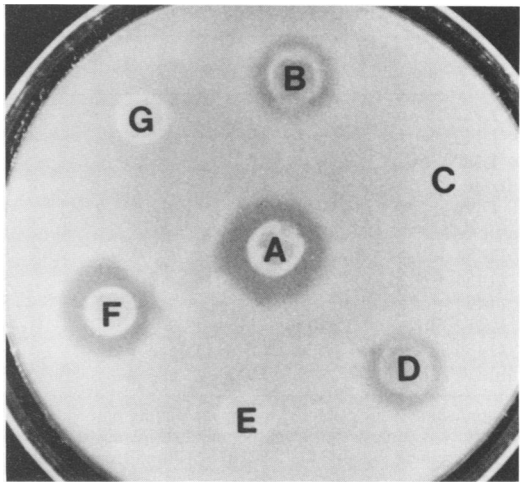

FIG. 2. Agar overlay assay for nisin production. Included in the assay were Nip$^+$ Suc$^+$ donor *L. lactis* subsp. *lactis* 11454 (A), *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NS224 (B), *L. lactis* subsp. *cremoris* parental CS224 (C), *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NSC3 (D), *L. lactis* subsp. *cremoris* parental C3 (E), *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NSEB7 (F), and *L. lactis* subsp. *cremoris* parental EB7 (G).

plasmid DNA (35). When assayed, over 30 Suc$^+$ transconjugants were all found to be Nip$^+$ (data not shown).

Gene transfer controls indicated that transfer had not occurred with nonviable donors or filtered growth supernatant and was not eliminated by DNase I (Table 2). The mechanism of gene transfer detected on antibiotic-containing media was therefore conjugation.

An example of the cross-streak DPC assay for high-frequency Nip$^+$ Suc$^+$ transfer is shown in Fig. 1. The results demonstrated that the transfer of Nip$^+$ Suc$^+$ from 11454 to LM2306, which occurred at a frequency of 7×10^{-6} transconjugants per donor CFU with DPC (Table 2), could be detected by the assay. Nip$^+$ Suc$^+$ transfer from *L. lactis* subsp. *lactis* donor DL16 (35) or 7962 (40) to LM2306 was not demonstrated by any of the conjugal techniques used in this study.

Although transfer of Lac$^+$ from ML3 to LM2306 was not detected by cross-streak DPC, experiments in this laboratory have shown that the assay readily identified Lac$^+$ transconjugants that subsequently became high-frequency donors of this trait (16, 41; data not shown).

Conjugation of Nip$^+$ Suc$^+$ into *L. lactis* subsp. *cremoris*. To obtain recipients for conjugation, *L. lactis* subsp. *cremoris* EB7 (33) was transformed by electroporation with the plasmid pGB301 and strain C3 (33) was electrotransformed with plasmid pGK13. Protoplast transformation of strain CS224 with pGB301 was described previously (43). The *L. lactis* subsp. *cremoris* transformants, SW224, JKEB7, and JBC3, all expressed resistance to 5 μg of erythromycin per ml in broth, and plasmid profiles revealed a new plasmid band which comigrated with the respective CsCl-purified drug resistance plasmid during agarose gel electrophoresis (data not shown).

DPC was used for matings between the Nip$^+$ Suc$^+$ donor *L. lactis* subsp. *lactis* 11454 and the *L. lactis* subsp. *cremoris* transformants SW224, JKEB7, and JBC3. Suc$^+$ transconjugants were obtained which were also Nip$^+$ (Fig. 2). The frequency of Nip$^+$ Suc$^+$ transfer ranged from slightly under 4.0×10^{-7} transconjugants per donor CFU for *L. lactis* subsp. *cremoris* recipient strains SW224 and JBC3 to 4.5×10^{-8} per donor CFU for strain JKEB7. When solid surface milk agar matings were performed, only recipient SW224 yielded Nip$^+$ Suc$^+$ transconjugants. The frequency of transfer, 1.4×10^{-8}, was 25 times lower than that obtained by DPC.

Characteristics of Nip$^+$ Suc$^+$ transconjugants. Reduced bacteriophage sensitivity in Nip$^+$ Suc$^+$ transconjugants of *L. lactis* subsp. *lactis* has been reported previously (17, 29). Bacteriophage spot assays detected a difference in phage sensitivity between the *L. lactis* subsp. *lactis* recipient LM2306 and the Nip$^+$ Suc$^+$ transconjugant of that strain, NS5406. Phage *c*2 (4.5×10^7 PFU/ml) formed a zone of inhibition on a lawn of *L. lactis* subsp. *lactis* LM2306 cells, while no zone of inhibition was observed when the phage was spotted onto lawns of NS5406 or 11454. This result indicated that the *L. lactis* subsp. *lactis* transconjugant acquired Rbs$^+$ with Nip$^+$ Suc$^+$. Alterations in bacteriophage sensitivity patterns were not detected among the *L. lactis* subsp. *cremoris* recipients and Nip$^+$ Suc$^+$ transconjugants.

To further characterize *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugants, we examined acid production in milk. All *L. lactis* subsp. *cremoris* parentals and Nip$^+$ Suc$^+$ transconjugants were initially fast acid producers. Strains CS224 and C3, however, slowly lost this capability after repeated transfer in nonfat dry milk (28), and Nip$^+$ Suc$^+$ transconjugants of these strains behaved similarly. *L. lactis* subsp. *cremoris* EB7 and Nip$^+$ Suc$^+$ transconjugants of this strain remained fast.

Finally, DNA-DNA hybridizations were performed to determine whether the *nisA* gene was transferred during conjugation and whether it could be detected on plasmid DNA. Restriction digests with the enzymes *Cla*I, *Eco*RI, and *Hin*dIII were performed on genomic DNA isolated from *L. lactis* subsp. *lactis* LM2306 and the Nip$^+$ Suc$^+$ transcon-

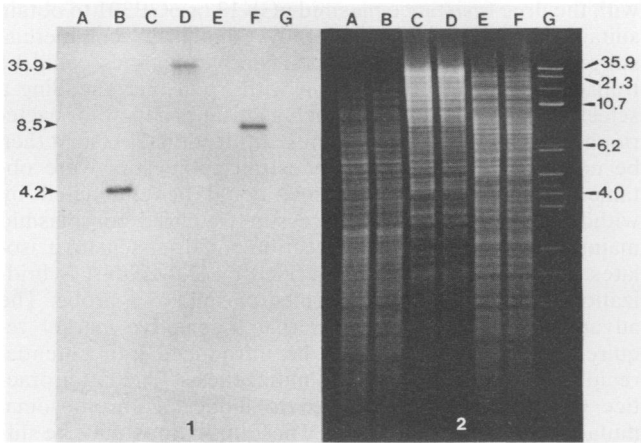

FIG. 3. Hybridization of the oligonucleotide *nisA* probe to restricted genomic DNA from *L. lactis* subsp. *lactis* recipient LM2306 and its Nip$^+$ Suc$^+$ transconjugant, NS5406. Panel 1 shows the autoradiogram obtained after a 72-h exposure of the blot, made from the agarose gel shown in panel 2, which was probed with the labeled oligonucleotide. Hybridization was performed at room temperature for 24 h as described by Ausubel et al. (3). DNA samples which corresponded to the lanes in each part were: A, *Hin*dIII-digested LM2306; B, *Hin*dIII-digested NS5406; C, *Cla*I-digested LM2306; D, *Cla*I-digested NS5406; E, *Eco*RI-digested LM2306; F, *Eco*RI-digested NS5406; and G, *Bam*HI-*Eco*RI-digested adenovirus type 2 DNA fragment size standards. Numbers on the left and right of the gel indicate sizes (in kilobase pairs).

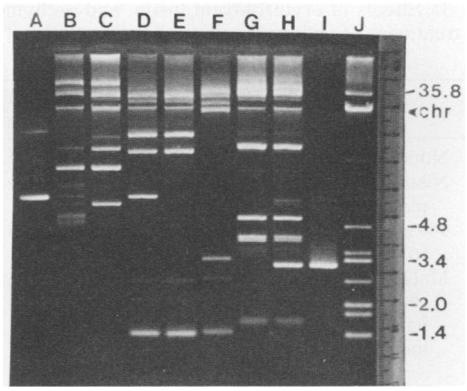

FIG. 4. Agarose gel electrophoresis of plasmids isolated from *L. lactis* subsp. *cremoris* parental strains, the Nip$^+$ Suc$^+$ transformant-transconjugants, and the Nip$^+$ Suc$^+$ donor. The transconjugants were obtained after mating the Nip$^+$ Suc$^+$ donor with parental strains which had been transformed with either pGB301 or pGK13, as described in the text. Lanes: A, CsCl-purified pGB301 (4); B, *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NSEB7, which contained pGB301; C, *L. lactis* subsp. *cremoris* parental EB7; D, *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NS224, with pGB301; E, *L. lactis* subsp. *cremoris* parental CS224; F, Nip$^+$ Suc$^+$ donor *L. lactis* subsp. *lactis* 11454; G, *L. lactis* subsp. *cremoris* parental C3; H, *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugant NSC3, which contained pGK13; I, CsCl-purified pGK13 (37); and J, *E. coli* V517 size standard plasmids (25). chr, Chromosome. Numbers on the right of the gel indicate sizes (in megadaltons).

jugant of that strain, NS5406. The DNA fragments were separated in an agarose gel and then transferred to a hybridization membrane and probed with the 5′-end-labeled oligonucleotide constructed to detect the *nisA* gene. Autoradiography showed that the probe hybridized to DNA fragments in the Nip$^+$ Suc$^+$ transconjugant, NS5406, but did not hybridize to Nip$^-$ Suc$^-$ LM2306 recipient DNA. The bands observed on the autoradiogram (Fig. 3) correlated with NS5406 DNA fragments of approximately 35.9. 8.5, and 4.2 kb in *Cla*I, *Eco*RI, and *Hin*dIII digests, respectively. To investigate whether the *nisA* gene might be plasmid associated, we isolated plasmid DNA from the *L. lactis* subsp. *cremoris* recipients, their Nip$^+$ Suc$^+$ transconjugants, and the Nip$^+$ Suc$^+$ donor *L. lactis* subsp. *lactis* 11454 (Fig. 4). The plasmid DNAs were transferred to a hybridization membrane and then probed with the oligonucleotide. Autoradiography demonstrated that the probe hybridized to chromosomal DNA bands of the Nip$^+$ Suc$^+$ donor and *L. lactis* subsp. *cremoris* transconjugants, but hybridization to plasmid DNA was not detected (data not shown).

Investigation of Nip$^+$ Suc$^+$ conjugation. Modifications to DPC and solid surface milk agar matings were performed to investigate events which affected Nip$^+$ Suc$^+$ transfer (Table 3). To determine whether transfer could occur if the selective media contained an agent that was bactericidal rather than bacteriostatic, matings were performed on bromocresol purple-sucrose agar which contained 10 μg of nisin (Aplin and Barett, Ltd., Wiltshire, United Kingdom; 3.7×10^7 IU/g) per ml and 5 μg of erythromycin per ml. Transfer of Nip$^+$ Suc$^+$ was detected on these plates but at a greatly reduced frequency (3.4×10^{-8}).

Results from the comparison of conjugation methods (Table 2) indicated that the transfer frequencies obtained by DPC could account for all transconjugants that resulted from milk agar matings. This suggested that conjugation occurred predominantly on selective media rather than on the milk agar plates. To test this possibility, we determined the efficiency with which donor and recipient cells could conjugate on selective media after 18 h of incubation on a milk agar plate. The frequency of Nip$^+$ Suc$^+$ transfer obtained with a standard milk agar mating was compared with that from a DPC mating made between donor and recipient cells which had been harvested from separate milk agar plates after 18 h of incubation (Table 3). Conjugation between the harvested cells was detected, but the frequency, presented in Table 3, was an order of magnitude lower than that obtained from the standard milk agar matings. Other modified conjugal matings were performed to investigate the use of α-chymotrypsin in Nip$^+$ Suc$^+$ matings. Results from these matings confirmed that the protease improved transfer of Nip$^+$ Suc$^+$ on milk agar (35) but that enhanced transfer of Nip$^+$ Suc$^+$ could be obtained without α-chymotrypsin if donor cells were rigorously washed in saline prior to mating.

DISCUSSION

The objective of this study was to develop an efficient method to conjugally construct nisin-producing strains of *L. lactis* subsp. *cremoris*. Nisin production in these strains would be desirable because many commercially important mesophilic starters are mixed or multiple strains of *L. lactis* subsp. *cremoris* (20). Prior use of nisin-producing *L. lactis* subsp. *lactis* starters has shown that these organisms effectively controlled some of the bacterial contaminants associated with cheese production (24). Unfortunately, the success of these experiments was limited by frequent defects in the cheese quality. This problem was attributed to nisin activity against the other bacterial cultures in the starter blend and because, alone, the nisin-producing *L. lactis* subsp. *lactis* produced an inferior quality cheese (21, 24). *L. lactis* subsp. *lactis* has been associated with the production of bitter

TABLE 3. Effects of erythromycin, nisin, and α-chymotrypsin treatment of cells and cell washing on conjugal transfer of Nip$^+$ Suc$^+$[a]

Method	Modification	Transconjugants/donor
DPC	None[b]	7.0×10^{-6}
DPC	Nisin (10 μg/ml) included in selective plates	3.4×10^{-8}
DPC	400 μg of α-chymotrypsin per ml added to mating mixture	5.2×10^{-6}
DPC	Mating performed between donor and recipient cells which had been harvested from milk agar after 18 h of incubation[c]	1.0×10^{-7}
Milk[d]	α-Chymotrypsin treatment of Steele and McKay (35)	1.6×10^{-6}
Milk[d]	α-Chymotrypsin not included	6.5×10^{-7}
Milk[d]	Donor washed three times in 5 ml of 0.85% saline before mating, then suspended in 1 ml of saline. α-Chymotrypsin included.	9.1×10^{-6}
Milk[d]	Same as above except no α-chymotrypsin was added	7.2×10^{-6}

[a] *L. lactis* subsp. *lactis* 11454 × *L. lactis* subsp. *lactis* LM2306.
[b] Standard DPC mating; for details, see text.
[c] Donor and recipient cells were prepared by the addition of either 66 μl of recipient and 33 μl of saline or 33 μl of donor and 66 μl of saline to milk agar. The plates were incubated for 18 h, and then cells were harvested as described for milk agar matings. DPC was then performed.
[d] Cells were recovered from milk plates in 1 ml of saline.

peptides and other off flavors in cheese (20, 39). For these reasons, inclusion of a single nisin producer among *L. lactis* subsp. *cremoris* starters has been impossible in the past, and the exclusive use of nisin-producing *L. lactis* subsp. *lactis* starters has remained unappealing. These shortcomings should be avoided if Nip$^+$ Suc$^+$ is conjugally introduced into commercially proven *L. lactis* subsp. *cremoris* starters. Investigators have demonstrated nisin inhibition of *Listeria*, *Clostridium*, *Staphylococcus*, *Bacillus*, and other undesirable gram-positive bacteria (5, 7, 21, 24). Thus, construction of nisin-producing strains from commercial *L. lactis* subsp. *cremoris* starters may lead to enhanced product safety and shelf life without any compromise in product quality.

The data presented in Fig. 2 and 4 demonstrated that Nip$^+$ Suc$^+$ was conjugally transferred from *L. lactis* subsp. *lactis* 11454 to *L. lactis* subsp. *cremoris* recipient strains SW224, JBC3, and JKEB7. Although transfer of Nip$^+$ Suc$^+$ into SW224 was achieved by the solid surface milk agar method of conjugation, transfer of this trait into JKEB7 and JBC3 was detected only by the DPC method. Characteristics of DPC indicate that this technique offers advantages over previous methods. Aside from the improvement in Nip$^+$ Suc$^+$ transfer frequency (Table 2), media and time requirements are reduced compared with either milk agar (27) or filter (16) matings. Additionally, because cells may be concentrated prior to mating, the DPC technique may allow for improved detection of very low frequency conjugal events. High-frequency events were readily detected by the cross-streak DPC assay (Fig. 1).

Electroporation can be used to construct recipient strains for conjugation from a variety of lactic or other bacteria lacking useful genetic markers (8, 10, 19, 31). This technique was used to transform two *L. lactis* subsp. *cremoris* strains with the drug resistance plasmid pGK13 or pGB301 to obtain suitable recipients for the study. Similarly, commercial starters which already possess desirable qualities could be transformed by electroporation with a plasmid encoding a selective marker, and traits such as nisin production, bacteriophage resistance (32), or lactose utilization (27) might then be conjugally introduced. Once transconjugants were obtained, the undesirable plasmid could be eliminated by withdrawing the antibiotic pressure required for plasmid maintenance, followed by selection for drug-sensitive isolates. Plasmid loss could be verified by DNA-DNA hybridizations which utilized the purified plasmid as a probe. The advantage to this approach is that a selective marker required for conjugation could be introduced into potential recipients without resorting to mutagenesis. The latter practice has been commonly used to induce a chromosomal mutation for drug resistance. These mutations may be difficult to reverse, and the mutagenic treatment may adversely affect other desirable functions in the cell. Electroporation of cells with a drug resistance plasmid should be less likely to damage desirable cell functions and would rapidly yield suitable recipient cells, and subsequent removal of the selective marker could be easily confirmed.

Transfer of Nip$^+$ Suc$^+$ into all *L. lactis* subsp. *cremoris* recipients was somewhat surprising because of the extreme sensitivity toward nisin exhibited by *L. lactis* subsp. *cremoris*. Hurst (21) has suggested that because *L. lactis* subsp. *lactis* and subsp. *cremoris* are struggling for dominance in the relatively new substrate of "milk in a container," they produce antibiotics aimed at one another. Thus, *L. lactis* subsp. *cremoris* has been reported to be the organism most sensitive to nisin, and *L. lactis* subsp. *lactis* is the most vulnerable to diplococcin, a bacteriocin produced by some *L. lactis* subsp. *cremoris* strains (21). Despite this, *L. lactis* subsp. *cremoris* strains were readily obtained which expressed nisin production and immunity.

The bacteriophage spot assays indicated that *L. lactis* subsp. *lactis* NS5406 Nip$^+$ Suc$^+$ transconjugants expressed Rbs$^+$. This observation was consistent with other reports (17, 29). Although Rbs$^+$ was not detected among the Nip$^+$ Suc$^+$ *L. lactis* subsp. *cremoris* transconjugants, many commercial strains of *L. lactis* subsp. *cremoris* are relatively bacteriophage resistance (20, 32). Because the Nip$^+$ Suc$^+$ *L. lactis* subsp. *cremoris* transconjugants generated in the study appeared to retain the recipient phenotype for phage resistance, it seems probable that nisin-producing transconjugants of commercial strains would also retain this parental property. Another characteristic of the *L. lactis* subsp. *cremoris* Nip$^+$ Suc$^+$ transconjugants was that acquisition of Nip$^+$ Suc$^+$ did not appear to affect the capability for acid production in milk. These features indicated that Nip$^+$ Suc$^+$ *L. lactis* subsp. *cremoris* transconjugants retained parental characteristics which are important in starter cultures. The characterization studies suggested that blends of mixed and multiple nisin-producing *L. lactis* subsp. *cremoris* starters, obtained by conjugal transfer of Nip$^+$ Suc$^+$ into proven commercial strains, would be suited to commercial applications.

DNA-DNA hybridizations demonstrated that the nisin precursor gene was transferred during conjugation. This result agreed with a recent report by Dodd et al. (9). The 8.5-kb *Eco*RI and 4.2-kb *Hin*dIII fragments of the Nip$^+$ Suc$^+$ transconjugant DNA which hybridized to the oligonucleotide probe were also in close agreement with reported data (6, 9, 22). The hybridization data indicated that at least 4.2 kb and perhaps over 8.5 kb of DNA was transferred with

Nip^+ Suc^+. Although several investigators have suggested plasmid involvement in nisin production (17, 35, 40), evidence linking plasmid DNA to nisin production has not been confirmed. The hybridization performed between the oligonucleotide *nisA* probe and plasmid DNAs showed that the *nisA* gene was not associated with detectable plasmid DNA in the Nip^+ Suc^+ *L. lactis* subsp. *lactis* donor or any of the Nip^+ Suc^+ transconjugants. Although this result does not preclude the existence of a plasmid encoding nisin production that may have been lost during the plasmid isolation technique used in the study, the plasmid hybridization data demonstrated that the 29.1-MDa plasmid of strain 11454 does not encode the *nisA* gene. Investigators have previously linked this plasmid to Nip^+ Suc^+ through curing studies (17, 35).

The investigations of Nip^+ Suc^+ conjugal transfer (Table 3) provided a few interesting pieces of information. Erythromycin, a bacteriostatic agent and inhibitor of protein synthesis, was used to select against donor cells with DPC. Data for DPC, shown in Tables 2 and 3, strongly indicated that transfer frequency was not impaired by the presence of erythromycin. This result suggested that de novo protein synthesis is not required within donor cells for conjugation to proceed. This condition has been demonstrated in *E. coli*, in which genes on the conjugative F plasmid which encode transfer capability are expressed constitutively (42). Table 3 shows that the observed transfer frequencies from matings performed in the presence of nisin and erythromycin were approximately 200 times lower than those observed on media which contained only erythromycin. This observation suggested that the action of nisin on recipient cells inhibited conjugative transfer of Nip^+ Suc^+ to a greater extent than did erythromycin inhibition of donor cells. It is possible that donor cells in the presence of erythromycin were capable of DNA transfer beyond a time which corresponded to that when recipient participation was prevented by nisin in the modified mating plates. These results also implied that de novo protein synthesis in donor cells was not essential for conjugation.

Successful transfer on media which contained nisin indicated that transfer and expression of Nip^+ Suc^+ occurred relatively quickly on selective media. This inference was based on spectrophotometric data which indicated that recipient cell lysis occurred approximately 4 h after the addition of 10 μg of nisin per ml (data not shown). Transfer on the nisin-erythromycin plates also suggested that the expression of nisin immunity in transconjugants allowed at least some of these cells to survive despite exposure to the antibiotic before gene transfer and expression were completed.

Results from the DPC mating performed between donor and recipient cells which had been harvested from milk agar suggested that solid surface milk agar conjugation involved gene transfer both on milk agar plates and on the selective media. The demonstrated ability of harvested cells to conjugate on selective media (Table 3) also suggested that reports of lactococcal conjugation in broth (41), in calcium alginate beads (36), and on filters (16) require further investigation to confirm conjugation under the study conditions as opposed to possible subsequent transfer on selective media only.

Data presented in Table 3 also confirmed a report that Nip^+ Suc^+ transfer by the milk agar technique was improved if cells were suspended in fresh media and α-chymotrypsin was added to the conjugal mixture (35). α-Chymotrypsin is known to inactivate nisin, and it has been used to protect recipient cells from the antibiotic during conjugation (35). Inclusion of the protease in DPC matings did not improve the transfer frequency of Nip^+ Suc^+. Additionally, results showed that washing cells with saline eliminated the need for α-chymotrypsin in all matings (Table 3).

In conclusion, conjugation has been widely described for a variety of phenotypic traits in lactic acid bacteria (12, 23, 30). This method of gene transfer has been useful in studies which have investigated the genetics and plasmid biology of these organisms. Conjugation has been applied practically to obtain improved phage resistance in lactococcal strains for the dairy industry (32). Organisms which are genetically improved by conjugation bypass many obstacles associated with the commercial application of strains which contain recombinant DNA molecules (12). For this reason, conjugation will probably continue to be an important means to improve strains for these applications.

Despite this background of study and application, mechanisms of conjugation among gram-positive bacteria, except for the pheromone-induced cell-to-cell adhesion between strains of *Enterococcus faecalis* (11), remain poorly understood. An improved understanding of conjugal mechanisms would facilitate the scope of genetic manipulations that may be performed by conjugation. Results of this study suggest that certain aspects of conjugation can be examined through manipulation of the DPC technique.

ACKNOWLEDGMENTS

This work was supported by a National Science Foundation Presidential Young Investigator award to J.K.K., a U.S. Department of Agriculture-Agricultural Research Service National Needs graduate fellowship to J.R.B., the Utah Agricultural Experiment Station, and the Western Dairy Foods Research Center.

Thanks are extended to Larry L. McKay for providing strains ML3, LM2306, DL16, C3, JK301 (which contained pGB301), and EB7; William E. Sandine for strains 7962 and CS224 and for phage 7962; and Jan Kok for vector pGK13. Gratitude is also expressed to Peggy Doelling and Kirk Housley for technical assistance and the Utah State University Biotechnology Center for synthesis and purification of the oligonucleotide probe.

REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Anderson, D. G., and L. L. McKay.** 1984. Genetic and physical characterization of recombinant plasmids associated with cell aggregation and high-frequency conjugal transfer in *Streptococcus lactis* ML3. J. Bacteriol. **158**:954–962.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
4. **Behnke, D., M. S. Gilmore, and J. J. Ferretti.** 1981. Plasmid pGB301, a new multiple resistance streptococcal cloning vehicle and its use in molecular cloning of a gentamicin/kanamycin resistance determinant. Mol. Gen. Genet. **182**:414–421.
5. **Benkerroum, N., and W. E. Sandine.** 1988. Inhibitory action of nisin against *Listeria monocytogenes*. J. Dairy Sci. **71**:3237–3245.
6. **Buchman, G. W., S. Banerjee, and J. N. Hansen.** 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. **263**:16260–16266.
7. **Carminati, D., G. Giraffa, and M. G. Bossi.** 1989. Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes*. J. Food Prot. **52**:614–617.
8. **Chassy, B. M., and J. L. Flickinger.** 1987. Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett.

44:173–177.

9. **Dodd, H. M., N. Horn, and M. J. Gasson.** 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. **136**:555–566.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
11. **Dunny, G. M., P. J. Christie, M. L. Tortorello, J. C. Adsit, R. Z. Korman, and S. A. Zahler.** 1987. *Streptococcus faecalis* plasmid pCF10 as a model for analysis of conjugation in streptococci and other gram-positive bacteria, p. 11–16. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
12. **Fitzgerald, G. F., and M. J. Gasson.** 1988. *In vivo* gene transfer systems and transposons. Biochimie **70**:489–502.
13. **Food and Drug Administration.** 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Regist. **53**:11247–11251.
14. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.
15. **Gasson, M. J.** 1984. Transfer of sucrose fermenting ability, nisin resistance, and nisin production into *Streptococcus lactis* 712. FEMS Microbiol. Lett. **21**:7–10.
16. **Gasson, M. J., and F. L. Davies.** 1980. High-frequency conjugation associated with *Streptococcus lactis* donor cell aggregation. J. Bacteriol. **143**:1260–1264.
17. **Gonzales, C. F., and B. S. Kunka.** 1985. Transfer of sucrose-fermenting ability and nisin production phenotype among lactic streptococci. Appl. Environ. Microbiol. **49**:627–633.
18. **Hershfield, V.** 1979. Plasmids mediating multiple drug resistance in group B *Streptococcus*: transferability and molecular properties. Plasmid **2**:137–149.
19. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55**:3119–3123.
20. **Huggins, A. R.** 1984. Progress in dairy starter culture technology. Food Technol. **38**:41–50.
21. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27**:85–123.
22. **Kaletta, C., and K.-D. Entian.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product. J. Bacteriol. **171**:1597–1601.
23. **Kondo, J. K., and L. L. McKay.** 1985. Gene transfer systems and molecular cloning in group N streptococci: a review. J. Dairy Sci. **68**:2143–2159.
24. **Lipinska, E.** 1977. Nisin and its applications, p. 103–130. *In* M. Woodbine (ed.), Antibiotics and antibiosis in agriculture. Butterworths, London.
25. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **McKay, L. L., K. A. Baldwin, and P. M. Walsh.** 1980. Conjugal transfer of genetic information in group N streptococci. Appl. Environ. Microbiol. **40**:84–91.
28. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23**:1090–1096.
29. **Murphy, M. C., J. L. Steele, C. Daly, and L. L. McKay.** 1988. Concomitant transfer of reduced-bacteriophage sensitivity with lactose- and sucrose-fermenting ability in lactic streptococci. Appl. Environ. Microbiol. **54**:1951–1956.
30. **Neve, H., A. Geis, and M. Teuber.** 1987. Conjugation, a common plasmid transfer mechanism in lactic acid streptococci of dairy starter cultures. Syst. Appl. Microbiol. **9**:151–157.
31. **Powell, I. B., M. C. Achen, A. J. Hillier, and B. E. Davidson.** 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. **54**:655–660.
32. **Sanders, M. E., P. J. Leonhard, W. D. Sing, and T. R. Klaenhammer.** 1986. Conjugal strategy for construction of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. Appl. Environ. Microbiol. **52**:1001–1007.
33. **Snook, R. J., and L. L. McKay.** 1981. Conjugal transfer of lactose-fermenting ability among *Streptococcus cremoris* and *Streptococcus lactis* strains. Appl. Environ. Microbiol. **42**:904–911.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
35. **Steele, J. M., and L. L. McKay.** 1986. Partial characterization of the genetic basis for sucrose metabolism and nisin production in *Streptococcus lactis*. Appl. Environ. Microbiol. **51**:57–64.
36. **Steenson, L. R., and T. R. Klaenhammer.** 1987. Conjugal transfer of plasmid DNA between streptococci immobilized in calcium alginate gel beads. Appl. Environ. Microbiol. **53**:898–900.
37. **Suvorov, A., J. Kok, and G. Venema.** 1988. Transformation of group A streptococci by electroporation. FEMS Microbiol. Lett. **56**:95–100.
38. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
39. **Thomas, T. D., and O. E. Mills.** 1981. Proteolytic enzymes of starter bacteria. Neth. Milk Dairy J. **35**:255–273.
40. **Tsai, H.-J., and W. E. Sandine.** 1987. Conjugal transfer of nisin plasmid genes from *Streptococcus lactis* 7962 to *Leuconostoc dextranicum* 181. Appl. Environ. Microbiol. **53**:352–357.
41. **Walsh, P. M., and L. L. McKay.** 1981. Recombinant plasmid associated with cell aggregation and high frequency conjugation of *Streptococcus lactis* ML3. J. Bacteriol. **146**:937–944.
42. **Willetts, N., and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48**:24–41.
43. **Woskow, S. A., and J. K. Kondo.** 1987. Effect of proteolytic enzymes on transfection and transformation of *Streptococcus lactis* protoplasts. Appl. Environ. Microbiol. **53**:2583–2587.