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Conjugal Transfer of Nisin Plasmid Genes from *Streptococcus lactis* 7962 to *Leuconostoc dextranicum* 181†

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Acriflavine-generated mutants of *Streptococcus lactis* 7962 with various combinations of plasmid molecular masses were screened for nisin production. Nisin was produced by both the wild type and mutants that contained a 17.5-megadalton plasmid, which was obscured by chromosomal fragments. No nisin was produced by plasmid-free mutants. Sucrose fermentation and nisin production were simultaneously expressed. A transconjugant obtained from nisin-producing donor *S. lactis* 7962 and recipient *Leuconostoc dextranicum* 181 was a "supernisin" producer. The *L. dextranicum* Nis$^+$ transconjugant was resistant to *S. lactis* 7962 phage and vancomycin (>1,000 μg/ml), and it contained an extra 17.5-megadalton plasmid.

Nisin is a polypeptide antibiotic with a molecular weight of about 7,500, and it is produced by group N lactic streptococci (11). It is a potential food preservative since it is able to inhibit outgrowth of spores of *Bacillus* sp. and *Clostridium* sp. (31, 33), which are the primary causes of canned food spoilage. The level of nitrite used as a preservative can be reduced when nisin is used as an adjunct in fermented or acidified meats (30, 31). In addition, heat treatment of many processed foods can be reduced by the use of nisin so that organoleptic and nutritional qualities are improved (3). The late gas blowing defect of Emmental, Swiss, and Gruyere cheese types can be prevented by using nisin as well (10, 17). Nisin is considered nontoxic because it can be digested by α-chymotrypsin, is unstable at pH 7, is ineffective against most gram-negative bacteria, and is produced by bacteria used in manufacturing numerous fermented foods (3, 12). It is widely used in European countries as a food preservative in dairy products and canned foods. In view of this, it would be worthwhile to improve nisin yields in cultures by genetic means.

Nisin production and resistance are believed to be mediated by plasmid DNA in *Streptococcus lactis* (18, 34). For example, a 40-megadalton (MDa) plasmid in *S. lactis* subsp. *diacetylactis* DRC3 (23) and ME2 (16) is responsible for nisin resistance. A 30-MDa plasmid in eight strains of *S. lactis* (7) and a 28-MDa plasmid in *S. lactis* 11454 (19) appear to be involved in nisin production and resistance. Also, a relationship between plasmids and nisin production has been shown by conjugal transfer of this plasmid gene from donor to negative-phenotype recipients (7, 8, 23). However, some investigators have obtained conflicting results which suggest that nisin production could be mediated by either chromosomal or plasmid DNA (2, 5).

The reasons for these inconsistent results may be strain variability, different indicator strain responses, phenotype-testing methods, procedures for cell lysis, and use of different plasmid DNA isolation protocols. In this study, *S. lactis* 7962 was chosen because it is resistant to at least a 40-fold higher concentration of nisin than are other strains tested (28).

The relationship between plasmid content and nisin phenotype of *S. lactis* 7962 was studied. Mutants with various sizes of plasmids were screened for nisin production and plasmid profiles. A 17.5-MDa plasmid (pNS17.5) mediated nisin production. When pNS17.5 was conjugally transferred to *Leuconostoc dextranicum* 181, a selected transconjugant was a nisin super-producer. This is the first report of genetically engineered nisin production by *Leuconostoc*, normally a nisin nonproducer. Also, physical evidence linking nisin production and resistance to a distinct plasmid was obtained from conjugation studies.

MATERIALS AND METHODS

Mutant isolation. Acriflavine treatment of lactic streptococci generates lactose-negative mutants (22, 24) and was used to obtain such mutants with various sizes of plasmids. Acriflavine was added to a final concentration of 6 μg/ml in M17-glucose broth. Cultures were incubated at 30°C for 24 h, and then 1% of treated cultures were transferred to fresh M17-glucose broth containing the same concentration of acriflavine. After second and third treatments, prospective plasmid-cured cultures were spread over the surface of lactose indicator plates such as Miller's (25) lactose indicator agar with 0.005% (2,3,5-triphenyltetrazolium chloride; Sigma Chemical Co., St. Louis, Mo.), McKay modified lactic agar (22), M17 agar containing 0.004% bromocresol purple (Sigma) with 0.5% sodium glycerophosphate, fast-slow differential agar (32), or SALT agar (29). On these media, the red, white, less yellow, or small colonies (<1.0 mm) with minute red centers were collected, respectively, and screened for plasmid content. The Anderson and McKay method (1) was used for rapid screening of plasmids.

Test for nisin production. For rapid screening and qualitative assay, the flip-over agar method described by Kékessy and Piguet (14) was followed. For quantitative assay, the agar plate diffusion method described by Tramer and Fowler (36) was used. The medium used in the Kékessey and Piguet method was M17-glucose agar. Each plate was incubated anaerobically at 30°C for 24 h (wild type and mutants growing normally) or for 48 h (slow-growing mutants) before

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inoculation of the reverse side of the agar with a 1/10 dilution of indicator strain. Then the plates were incubated aerobically at 30°C for another 24 h.

The agar plate diffusion method required transferring 1% overnight cultures into 25 ml of fresh broth and then incubating them at 30°C until cells reached the stationary phase. The supernatant was obtained by centrifugation at 10,000 × *g* for 12 min at 4°C. The supernatant was filtered through a 0.45-μm (pore size) filter. Uniform quantities (90 μl) of a known standard nisin concentration and unknown supernatants were delivered into the agar wells. Nisin standard or stock solution was prepared from purified nisin (40×10^6 IU/g; Aplin and Barrett, Trowbridge, England) dissolved in 0.02 N HCl at 10 mg/ml and filter sterilized through a 0.45-μm (pore size) filter (Millipore Corp., Bedford, Mass.). The assay procedure, medium, and organisms were as described by Tramer and Fowler (36) except that 20 ml of assay agar medium was added to the petri dishes.

Test for nisin resistance. M17-glucose agar (for lactic streptococci) or MRS agar (for *Leuconostoc* colonies) (27) fortified with 1% of a 1:1 mixture of Tween 20 and sterile distilled water (0.15 ml/15 ml of agar) was used to make gradient concentrations of nisin. From an overnight culture, 0.1 ml was taken and spread on agar. Plates were incubated anaerobically at 30°C for 24 to 48 h. Eight *Leuconostoc* strains and one transconjugant (*L. dextranicum* HT200) were tested with various concentrations from 10 to 300 μg/ml.

Test for vancomycin resistance. All preparations were as described above except that the vancomycin stock solution was prepared by dissolving vancomycin (Sigma) in distilled water at 10 mg/ml. To determine the vancomycin concentration required to eliminate donor cells when transconjugants were selected, vancomycin resistance from 10 to 70 μg/ml was tested.

Sucrose-fermenting ability. Sucrose indicator agar (25) with 0.005% 2,3,5-triphenyltetrazolium chloride was used to examine the acriflavine-generated Lac^- mutants. Strains capable of fermenting sucrose produced white colonies, while nonfermenters produced red colonies. Eight variants of *S. lactis* 7962 were examined.

Solid-surface conjugation. Cultures of nisin donor strain *S. lactis* 7962, grown overnight in M17 broth, and recipient *L. dextranicum* 181, grown overnight in MRS broth, each were inoculated (2%) into fresh broth and incubated at 30°C for 2.5 h. Mating was performed at a donor-recipient ratio of 1:4 (20 μl of donor, 80 μl of recipient). This mixture was spread on the surface of Elliker agar (4), and the plates were placed in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 30°C for 16 h. After incubation, the mating mixtures were harvested with 3 ml of MRS broth. Cells were collected by centrifugation at 9,900 × *g* at 4°C for 12 min. To eliminate donor cells, the pellet was suspended in 2 ml of high-titer *S. lactis* 7962 phage lysate (3.8×10^8 PFU/ml) containing 0.2 ml of 0.2 M $CaCl_2$ and gently mixed thoroughly; on control plates without recipient cells, no phage- or vancomycin-resistant colonies appeared. Samples (100 μl) of this mixture were spread on the surface of 22 plates of MRS agar containing 30 μg of nisin per ml, 100 μg of vancomycin per ml, and 0.1% (vol/vol) Tween 20. Plates were incubated anaerobically at 30°C for 48 h. Survival colonies were enumerated, and each was transferred to MRS broth containing 100 μg of nisin per ml. Viable cultures of the second selection were grown in MRS broth, diluted, and spread on MRS agar containing 200 μg of nisin per ml. In this manner, a single colony exhibiting high resistance to nisin was obtained and designated as *L. dextranicum* HT200.

Donor strain cells were diluted and plated on M17 agar to determine CFU/ml. Conjugation frequency was calculated from the number of nisin-resistant surviving colonies appearing on the MRS agar plates containing nisin (30 μg/ml) and vancomycin (100 μg/ml) relative to the number of input donor cells.

Control mating experiments were done (i) in the presence of DNase (100 μg/ml), (ii) by using cell-free filtrates of donor cells, or (iii) by using heat-killed donor cells mixed with recipient cells so that transformation and transduction mechanisms of gene transfer would be excluded. Cell-free filtrates were obtained by removing the centrifuged cell pellet and passing the supernatant through a sterile 0.22-μm [pore size] membrane filter. Heat-treated cells were obtained by boiling for 10 min.

Nisin confirmation. The agar plate diffusion method of Tramer and Fowler (36) and Fowler et al. (6) was used to assay for nisin production. In addition, the supernatant obtained from the transconjugant *L. dextranicum* HT200 was adjusted in separate samples to pH 2.0 or 11. After treatment by immersion in boiling water for 10 min, the solutions were sterilized by being passed through a 0.22-μm filter. Solution (90 μl) was delivered into a well of the assay agar.

***S. lactis* 7962 phage assay.** The titer determination and stock maintenance of *S. lactis* 7962 phage were performed as described by Terzaghi and Sandine (35).

Cell lysis and plasmid DNA isolation. The cultures prepared for cell lysis were grown in lysis broth (16) and incubated at 23°C for 10 h or until they reached the mid-log phase. The methods used for rapid small-scale plasmid DNA screening were described by Anderson and McKay (1), Kado and Liu (13), and Orberg and Sandine (26). The methods used for large-scale isolation were the alkali-sodium dodecyl sulfate procedure as described by Maniatis et al. (21) and the Anderson and McKay procedure (1); in the latter method, the following modifications were made. The concentration of lysozyme was 20 mg/ml, but for obtaining lysates of *L. dextranicum* 181 and HT200, and *S. lactis* SN10, SN1, and SA703, 1,000 μl of mutanolysin (5,000 U/ml; Sigma) was added with lysozyme to facilitate cell lysis. Vortexing of the lysate was omitted. After the lysate was transferred into a glass graduated cylinder, sodium hydroxide was added, and the cylinder was shaked gently from side to side for 10 min. The DNA pellet was washed once in 70% alcohol and centrifuged again at room temperature, and the pellet was suspended in TE buffer (0.04 M Tris acetate–0.001 M EDTA).

Density gradient centrifugation and purification. For preparing a CsCl-EtBr density gradient, the final density of the solution was 1.55 to 1.57 g/ml. This solution was transferred into Beckman Quick-seal centrifuge tubes (13 by 51 mm) and centrifuged for 16 h at 65,000 rpm in a Beckman VTi 80 rotor at 20°C. The lower band, the CsCl-purified covalently closed circular form of plasmid DNA, was removed with a no. 18 hypodermic needle. EtBr was extracted with an equal volume of 1-butanol saturated with water, whereas CsCl was removed by dialysis against TE buffer (pH 8.0). Purified plasmids were concentrated by keeping them for 1 to 2 h at −20°C in the presence of 2 volumes of cold 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 6.6). DNA pellets were obtained by centrifugation at 9,880 × *g* (model J2-21; Beckman) for 12 min at room temperature. The ethanol was aspirated, and the pellets were dried in a vacuum for 30 min and suspended in TE buffer (pH 8.0).

Agarose gel electrophoresis. Agarose gel (0.7%) electro-

phoresis was performed in Tris-acetate buffer containing 40 mM Tris-acetate and 1 mM sodium EDTA (pH 8.0).

RESULTS

Plasmid profile of *S. lactis* 7962. Methods of small-scale rapid screening of plasmid DNA of *S. lactis* 7962 were studied, and the results were compared with those obtained with large-scale isolation and CsCl-EtBr purification procedures. Plasmid profiles obtained by the latter technique (Fig. 1, lane D) revealed that two plasmids, 17.5 and 18.5 MDa, were obscured by chromosomal fragments appearing in gels of samples purified by screening methods (lanes A, B, and C).

Phenotypic characteristics of 7962 variants. After acroflavine treatment, eight Lac$^-$ mutants of *S. lactis* 7962 were obtained. Five of them contained various sizes of plasmids. Two, SN1 and SA703, were plasmid-free derivatives. One, SN10, had two plasmids not present in the wild type. Plasmid profiles of the mutants used in this study are listed in Table 1.

Nisin production was 1,300 IU/ml for all variants harboring pNS17.5. In variants in which pNS17.5 could not be detected, nisin was not produced. These data generally are in agreement with the results of Grushina et al. (9) and Lee and Kim (20). Also, there was no nisin production by mutant SN10, which contained only the 2.7- and 6-MDa plasmids.

S. lactis 7962 and mutants containing pNS17.5, such as strains HT20, HT25, HT33, HT35, and HT43, were sucrose fermenters, whereas plasmid-free mutants and *S. lactis* SN10 were not (Table 1).

Nisin resistance survey among *Leuconostoc* spp. To select a suitable recipient candidate for the nisin gene, eight strains of *Leuconostoc* species (Table 2) were chosen to test for nisin resistance. Six strains could not survive levels of >10 μg of nisin per ml. On the other hand, some cells of *L. dextranicum* 226 were resistant to nisin at >50 μg/ml. Since nisin producers would not survive unless they were also nisin resistant, nisin-sensitive *L. dextranicum* 181 was chosen as a recipient organism.

Vancomycin resistance. Donor *S. lactis* 7962 cells were tested for resistance to vancomycin. The results showed that there were no surviving colonies on plates containing from 10 to 70 μg of vancomycin per ml when 10^8 cells per ml (0.1 ml per plate) were tested. Therefore, the selective concentrations of vancomycin and nisin were set at 100 and 30 μg/ml, respectively.

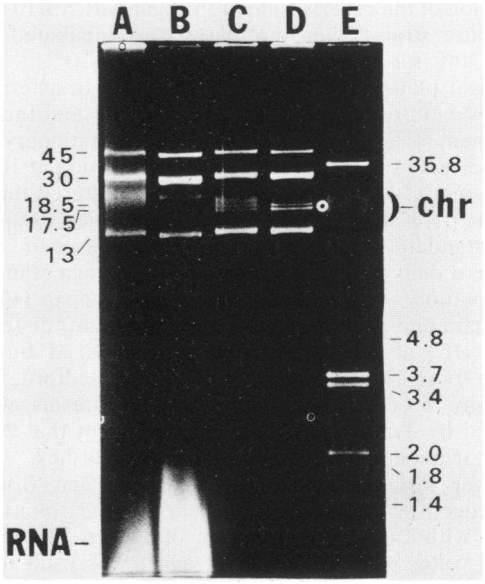

FIG. 1. Plasmid profiles of *S. lactis* 7962 as determined by the following four methods (lanes): A, Kado and Liu (13); B, Anderson and McKay (1); C, Orberg and Sandine (26); D, Maniatis et al. (21). Lane E, *E. coli* V517 standard. The white circle between lanes D and E locates the two plasmids masked in preparations shown in lanes A, B, and C. The numbers to the left indicate plasmid molecular masses in megadaltons, and those on the right indicate molecular mass standards in megadaltons. chr, chromosomal fragment area.

Conjugation. The average number of colonies to appear on the 22 plates of selective medium containing 30 μg of nisin per ml and 100 μg of vancomycin per ml are listed in Table 3. Since nisin resistance genes transferred during matings, the number of recipient cells having high nisin resistance increased 30 to 40 times over that of the control group with cell-free filtrates of donor cells and heat-killed donor cells. The number of input donor cells on M17-glucose agar was 1.9×10^7. Therefore, the conjugation frequency was 4.1×10^{-7} for the experimental group with donor cells. Frequencies for the control groups with cell filtrates of the donor and the heat-killed donor, respectively, were 9.5×10^{-9} and 1.2×10^{-8}. Colonies, presumably transconjugants, appearing on the selective agar (Table 3, donor and donor cells + DNase) all grew when cultured in MRS broth containing 100 μg of

TABLE 1. Plasmid profiles of *S. lactis* 7962 and mutant derivatives thereof

Strain	Description[a]	Plasmid contents (MDa)	Source
S. lactis			
7962	Wild type, Nis$^+$ Suc$^+$, donor	45, 30, 18.5, 17.5, 14.5, 13	This laboratory
HT20	Mutant, Nis$^+$ Suc$^+$	30, 17.5	This study
HT25	Mutant, Nis$^+$ Suc$^+$	18.5, 17.5, 14.5, 13	This study
HT33	Mutant, Nis$^+$ Suc$^+$	30, 17.5, 13	This study
HT35	Mutant, Nis$^+$ Suc$^+$	45, 18.5, 17.5, 14.5, 13	This study
HT43	Mutant, Nis$^+$ Suc$^+$	45, 30, 18.5, 17.5	This study
SN1	Mutant, Nis$^-$ Suc$^-$	0	This study
SN10	Mutant, Nis$^-$ Suc$^-$	6.0, 2.7	This study
SA703	Mutant, Nis$^-$ Suc$^-$	0	This study
E. coli V517	Source of reference plasmids	35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4	E. Lederberg[b]

[a] Nis$^+$, Nisin production; Nis$^-$, nisin negative; Suc$^+$, sucrose fermenting; Suc$^-$, sucrose negative.
[b] Stanford University School of Medicine.

TABLE 2. Bacterial strains used

Strain	Description[a]	Vancomycin resistance (μg/ml)	Nisin (50 μg/ml) resistance (no. of colonies of ≥10^7 organisms)	Source
L. cremoris				
CAF7	Wild type	>1,000[b]	4	This laboratory
104	Wild type	Unknown	0	This laboratory
225	Wild type	500[b]	0	This laboratory
L. dextranicum				
180	Wild type	>1,000[b]	0	This laboratory
181	Wild type, Nis$^-$, recipient	>1,000[b]	0	This laboratory
226	Wild type	Unknown	75	This laboratory
ATCC 8358	Wild type	Unknown	0	This laboratory
ATCC 8359	Wild type	Unknown	0	This laboratory
HT200	Transconjugant, Nis$^+$	>1,000	10^7	This study
M. flavus				
NCIB 8166	Sensitive indicator for nisin assay	Unknown	0	Tramer and Fowler

[a] Nis$^+$, Nisin production; Nis$^-$, nisin negative.
[b] Data from reference 27.

nisin per ml. No colonies appearing on control plates (Table 3, donor filtrate and heat-treated donor) would grow in such medium. When subcultured in MRS broth and spread on MRS agar containing 200 μg of nisin per ml, one of the Nis^r transconjugant cultures produced a single colony. This colony, designated *L. dextranicum* HT200, was further tested for resistance to nisin, and it grew on MRS agar containing nisin at 300 μg/ml. Also, this transconjugant formed a 167% larger inhibition zone on an indicator lawn than did *S. lactis* 7962 when tested by the plate diffusion assay (Fig. 2). By comparison with the standard curve, the potency of nisin produced by *L. dextranicum* HT200 was 1.5×10^6 IU/ml, about 1,000 times higher than that of *S. lactis* 7962.

Nisin confirmation. A significant zone of inhibition occurred surrounding the well of the untreated supernatant, as well as around the well containing the heat-treated (100°C) and acidified (pH 2.0) supernatant of the transconjugant. However, the alkaline (pH 11) heat-treated supernatant of the transconjugant was inactivated, thereby confirming the presence of nisin in the supernatant of *L. dextranicum* HT200.

***S. lactis* 7962 phage assay and vancomycin resistance test for the donor, recipient, and transconjugant.** Unlike donor cells, transconjugant and recipient cells were resistant both to 7962 phage and to >1,000 μg of vancomycin per ml.

Plasmid profiles. Plasmid profiles after large-scale isolation and CsCl-EtBr purification revealed that transconjugant *L. dextranicum* HT200 harbored an extra 17.5-MDa plasmid, a novel species (Fig. 3).

DISCUSSION

S. lactis 7962 has been reported to contain three species of plasmids, with molecular masses of 13, 30, and 45 MDa (P. K. Orberg, Ph.D. thesis, Oregon State University, Corvallis, 1985). However, when the plasmid DNA was purified by a CsCl-EtBr density gradient, three extra species of plasmids, 14.5, 17.5, and 18.5 MDa, were evident. The 17.5- and 18.5-MDa plasmids had been obscured because they electrophoresed within the chromosomal fragments. Also, they were of weak intensity. In rapid screening by the Kado and Liu method (13), these two plasmid species were totally obscured by the broad chromosomal fragments, whereas in screening by the Anderson and McKay method (1), they could hardly be seen; with the Orberg and Sandine method (26), they were barely visible. Therefore, for an accurate determination of the plasmid profile of *S. lactis* 7962, large-scale isolation and CsCl-EtBr purification were necessary. This could be done with either the Anderson and McKay procedure (1) or the alkali sodium dodecyl sulfate method of Maniatis et al. (21).

The frequency of colonies appearing per donor cell on selective agar containing nisin and vancomycin was as follows: donor plus recipient, 4×10^{-7}; donor plus recipient plus DNase, 3.6×10^{-7}; cell-free donor filtrates plus recipient, 9.5×10^{-9}; and heat-treated donor plus recipient, 1.2×10^{-8}. Several thoughts are brought to mind by these data. First, conjugal transfer of the nisin gene does occur during mating, resulting in a 40-fold greater survival than that of the control group when either donor cells treated with heat or donor cell filtrates were used. The frequency obtained from the group treated with DNase was approximate to that of the experimental group. This indicated that transformation and transduction did not occur under the experimental conditions used. Second, whereas spontaneous mutants of *L. dextranicum* 181 resistant to 30 μg of nisin per ml occurred rarely (Table 3), such mutants with higher resistance were not found. When surviving colonies (from the control group

TABLE 3. Conjugal transfer of nisin resistance gene from *S. lactis* 7962 to *L. dextranicum* 181

Donor in mating[a]	Avg no. of colonies[b]	Frequency/donor[c]
Donor	7.7	4×10^{-7}
Cell-free filtrate of donor	0.18	9.5×10^{-9}
Donor in presence of DNase[d]	6.8	3.6×10^{-7}
Heat-treated donor[e]	0.23	1.2×10^{-8}

[a] At a donor-recipient ratio of 1:4.
[b] Colonies appearing on selective agar containing 30 μg of nisin per ml and 100 μg of vancomycin per ml when 0.1 ml of the 2.2 ml of postconjugation cells was spread over 22 plates.
[c] Calculated from 1.9×10^7 CFU of donor cells per ml.
[d] At 100 μg/ml.
[e] At 100°C for 10 min.

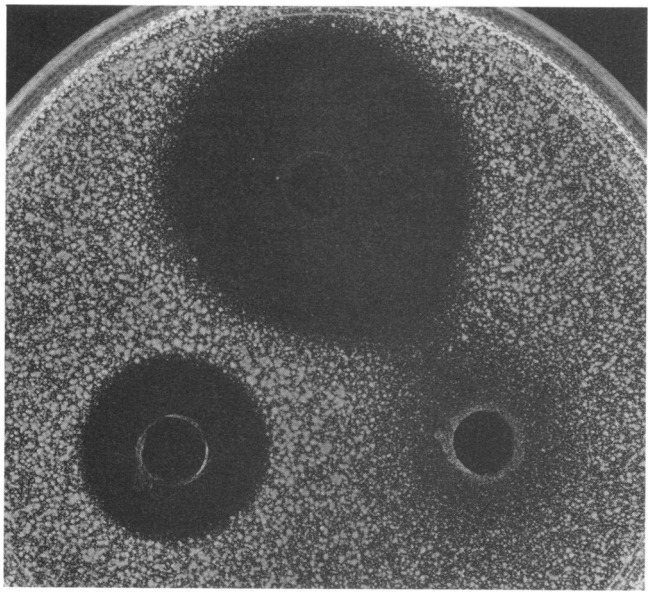

FIG. 2. Tramer and Fowler plate diffusion assay used to quantitate the nisin produced by the nisin gene donor, *S. lactis* 7962 (lower left), the recipient, *L. dextranicum* 181 (lower right), and the transconjugant, *L. dextranicum* HT200 (top). The indicator organism (lawn) was *Micrococcus flavus* NCIB 8166.

with both donor cells treated with heat and donor cell filtrates) were tested for survival at 100 μg of nisin per ml, no colonies survived from the control group. However, the transconjugant obtained, *L. dextranicum* HT200, was resistant to 300 μg of nisin per ml, and it also overproduced the antibiotic. Plasmid profiles of this transconjugant revealed an extra plasmid with a molecular mass of 17.5 MDa. This indicated that donor pNS17.5 was transferred to the

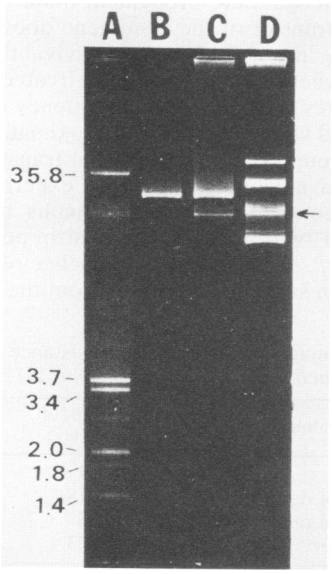

FIG. 3. Plasmid profiles. Lanes: A, *E. coli* V517 standard; B, the recipient, *L. dextranicum* 181; C, the transconjugant, *L. dextranicum* HT200; D, the nisin gene donor, *S. lactis* 7962. pNS17.5 is shown (arrow) both in lane C, the transconjugant, and in lane D, the donor organism. The numbers on the left indicate molecular mass standards in megadaltons.

Leuconostoc recipient intact and that the nisin gene was well expressed in this new host.

Davey and Pearce (2) studied plasmid-free derivatives of nisin-producing *S. lactis* H1 which retained antibiotic-producing ability; therefore, they proposed that the nisin gene was located on the chromosome. On the other hand, there are reports that indicate that the nisin phenotype is plasmid mediated (7, 16, 19, 23). Gonzalez and Kunka (8) transferred the nisin gene from *S. lactis* to *S. lactis* and subsequently retransferred it to *S. lactis* and *S. lactis* subsp. *diacetylactis*. Steele and McKay (34) suggested that the plasmid is associated with sucrose-fermenting ability, nisin resistance, and nisin production in some strains of *S. lactis*. Their results indicated that nisin production and resistance were plasmid encoded. However, the conjugal-transfer plasmid could not be isolated or detected in lysates of transconjugants (7, 8, 35). The present study indicated that the nisin phenotype of *S. lactis* 7962 is mediated by a plasmid, pNS17.5, and physical evidence linking nisin production and resistance to this plasmid was obtained. The linkage of sucrose-fermenting ability and nisin production has been previously reported (7, 8, 12, 19); the present study supports this possibility.

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