JOURNAL OF BACTERIOLOGY, Feb. 1992, p. 1280–1287
0021-9193/92/041280-08$02.00/0

Vol. 174, No. 4

Characterization of the Novel Nisin-Sucrose Conjugative Transposon Tn*5276* and Its Insertion in *Lactococcus lactis*

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Received 13 September 1991/Accepted 5 December 1991

A novel, chromosomally located conjugative transposon in *Lactococcus lactis*, Tn*5276*, was identified and characterized. It encodes the production of and immunity to nisin, a lanthionine-containing peptide with antimicrobial activity, and the capacity to utilize sucrose via a phosphotransferase system. Conjugal transfer of Tn*5276* was demonstrated from *L. lactis* NIZO R5 to different *L. lactis* strains and a recombination-deficient mutant. The integration of Tn*5276* into the plasmid-free strain MG1614 was analyzed by using probes based on the gene for the nisin precursor (*nisA*) and the gene for sucrose-6-phosphate hydrolase (*sacA*). The transposon inserted at various locations in the MG1614 chromosome and showed a preference for orientation-specific insertion into a single target site (designated site 1). By using restriction mapping in combination with field inversion gel electrophoresis and DNA cloning of various parts of the element including its left and right ends, a physical map of the 70-kb Tn*5276* was constructed, and the *nisA* and *sacA* genes were located. The nucleotide sequences of Tn*5276* junctions in donor strain NIZO R5 and in site 1 of an MG1614-derived transconjugant were determined and compared with that of site 1 in recipient strain MG1614. The results show that the A+T-rich ends of Tn*5276* are flanked by a direct hexanucleotide repeat in both the donor and the transconjugant but that the element does not contain a clear inverted repeat.

Gene transfer in gram-positive bacteria by bacterial mating or conjugation is a natural process that has received increasing attention in recent years (8, 12, 29, 34). The mechanism of this process is unknown, but, like that in gram-negative bacteria, it requires intimate cell-to-cell contact, is insensitive to nucleases, and does not involve a transducing bacteriophage. Two kinds of conjugative elements in gram-positive bacteria have been described: conjugative transposons and conjugative plasmids. Conjugative transposons, which have only been found in streptococci and enterococci, confer resistance to antibiotics (29, 34). Some streptococcal transposons, such as Tn*916* (8), can be conjugally transferred to other genera and have become important genetic tools. Conjugative plasmids have been identified in many genera, and most encode antibiotic resistances (29).

Lactococcus lactis strains that are used in industrial dairy fermentations do not carry transmissable antibiotic resistance genes but can be used as hosts for conjugative transposons and plasmids (12). In addition, naturally occurring *L. lactis* strains harbor metabolic plasmids that are often conjugative and are known to contain genes that code for the ability to ferment carbohydrates, production of proteinases, reduced sensitivity to bacteriophages, and production of and resistance to bacteriocins (12). Some of these plasmids can integrate into the chromosome of recombination-proficient *L. lactis*, as is the case with a large plasmid that encodes lactose metabolism and bacteriophage insensitivity and shows properties of an episome (47).

For a long time, it has been assumed that a conjugative *L. lactis* plasmid encodes the production of nisin, the immunity to nisin, the ability to ferment sucrose via a phosphotransferase system, and an unidentified mechanism conferring reduced sensitivity to isometric bacteriophages (16, 20, 30, 36). However, physical evidence for the presence of an actual plasmid carrying these functions has never been provided. Recent interest in the biosynthesis of nisin, a 34-residue peptide containing lanthionine and dehydrated amino acids with antimicrobial activity (25), has resulted in the identification and sequence analysis of identical copies of the *nisA* gene (27) for the nisin precursor in *L. lactis* ATCC 11454 (2) and in *L. lactis* 6F3 (27). Subsequent hybridization experiments with specific DNA probes indicated the presence of the *nisA* gene on a large plasmid in *L. lactis* 6F3 (27) and on the chromosomes of *L. lactis* K1 (10) and *L. lactis* ATCC 11454 (48). Evidence in favor of a chromosomal location of the *nisA* gene was provided in the analysis of the nisin-producing and sucrose-fermenting transconjugant *L. lactis* FI5876, obtained from a mating between NCFB 894 and MG1614 (9). In that study a junction fragment of chromosomal DNA and the *nisA* gene was identified. Further analysis of this junction fragment showed that it contained an additional copy, relative to the number in the recipient strain, of the insertion sequence IS*904*, which is located upstream of the *nisA* gene (9). Part of the conflicting results on the genetic location of the *nisA* gene may be attributed to insensitive techniques or strain differences. As a consequence, no conclusive evidence exists with respect to the nature of the mobile genetic element that encodes nisin production and sucrose fermentation. It could be a conjugative plasmid, a conjugative episome, or a conjugative transposon. We characterized this mobile element in the starter strain *L. lactis* NIZO R5 and in nisin-producing and sucrose-utilizing *L. lactis* transconjugants. In this report we provide genetic and physical evidence for the existence of a novel, 70-kb conjugative transposon, designated Tn*5276*, that codes for nisin biosynthesis and sucrose fermentation and is capable of orientation-specific insertion at a preferential site in the *L. lactis* chromosome and also insertion into secondary sites.

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TABLE 1. *L. lactis* strains used in this study

Strain	Relevant phenotype[a] and description	Reference or source
NIZO R5	Lac$^+$ Suc$^+$ Nip$^+$ Nim$^+$, multiplasmid strain	14, 33
MG1614	Lac$^-$ Suc$^-$ Nip$^-$ Nim$^-$ Rif^r Str^r; antibiotic-resistant, plasmid-free derivative of NCDO 712	15
MG1390	Lac$^-$ Suc$^-$ Nip$^-$ Nim$^-$ Spc^r; antibiotic resistant, plasmid-free derivative of NCDO 712	17
T165.1-8	Lac$^-$ Suc$^+$ Nip$^+$ Nim$^+$ Rif^r Str^r; plasmid-free, antibiotic-resistant transconjugants derived from matings between NIZO R5 (donor) and MG1614 (recipient)	This study
MMS36	Lac$^+$ Suc$^-$ Nip$^-$ Nim$^-$; multiplasmid, recombination-deficient derivative of ML3	1
MMS36S	Lac$^+$ Suc$^-$ Nip$^-$ Nim$^-$ Str^r; multiplasmid strain, antibiotic-resistant derivative of MMS36	This study

[a] Abbreviations for bacterial phenotypes: Lac$^+$, lactose fermenting; Lac$^-$, lactose negative; Suc$^+$, sucrose fermenting; Suc$^-$, sucrose negative; Nip$^+$, nisin producer; Nip$^-$, nisin nonproducer; Nim$^+$, immune to nisin; Nim$^-$, sensitive to nisin; Rif^r, Str^r, and Spc^r, resistant to rifampin, streptomycin, and spectinomycin, respectively.

(A preliminary account of part of this work was presented previously [40].)

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The lactococcal strains used in this study are listed in Table 1. The following *L. lactis* phages were used: φR5 (Netherlands Institute for Dairy Research collection), which is specific for strain NIZO R5, and the prolate phage φ763 (obtained from the National Collection of Dairy Organisms), which is specific for strains MG1614 and MMS36S. *Escherichia coli* MC1061 (6), TG1 (19), and MB406 (obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden) were used as hosts for the pACYC184 derivative pNZ84 (52), M13mp18 and M13mp19 (57), and bacteriophage λEMBL3 (13), respectively.

Growth and culture conditions. *E. coli* strains were grown in L broth-based medium as described previously (44). *L. lactis* strains were routinely grown at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5% glucose, lactose, or sucrose. For nisin production and immunity assays, cells were grown in 10% reconstituted skimmed milk containing 1% glucose and 0.05% Casamino Acids. The ability to ferment sugars was tested on indicator agar based on Elliker broth (11) containing 0.004% bromocresol purple and 0.5% of the suitable sugar. When appropriate, media were supplemented with antibiotics in the following amounts: ampicillin, 50 μg/ml; rifampin, 100 μg/ml in liquid medium or 50 μg/ml in plates; streptomycin, 200 μg/ml; spectinomycin, 100 μg/ml.

Conjugal matings. Conjugal matings were carried out on milk agar plates as described previously (46) with a donor/recipient ratio of 1:2 and conjugation times of 4 and 20 h. When appropriate, DNase I (20 μg/ml) was included in the media. Transconjugants were initially selected for their capacity to ferment sucrose and for antibiotic resistance. The identity of putative transconjugants was confirmed by comparing their sensitivities to strain-specific bacteriophages, their capacities to ferment lactose, their plasmid complements, and their sensitivities to mitomycin C (for MMS36S-derived transconjugants) with those of donor and recipient strains. Conjugation frequencies are expressed as number of transconjugant CFU per donor CFU.

DNA manipulations. Plasmid and bacteriophage DNAs were isolated from *E. coli* cells or lysates essentially by using established protocols (44). Lactococcal plasmid DNA was isolated as described previously (55). Total DNA was isolated from *L. lactis* by the addition of 4 volumes of 50 mM Tris-HCl (pH 8.0)–5 mM EDTA–50 mM NaCl–0.5% sodium dodecyl sulfate to a protoplast suspension prepared as described previously (55) and then phenol-chloroform extraction and ethanol precipitation. DNA was digested with restriction enzymes (Gibco/BRL Life Technologies, Gaithersburg, Md., and New England BioLabs Inc., Beverly, Mass.) as recommended by the manufacturers. DNA fragments were recovered from agarose gels with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). Standard cloning procedures were used throughout (44).

Agarose gel electrophoresis, DNA transfer, and hybridization. Agarose gel electrophoresis was performed as described previously (44). For field inversion gel electrophoresis (FIGE), DNA was isolated and digested with restriction enzymes in agarose plugs (44), which were inserted into a 20- by 20-cm 1% agarose gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA). FIGE was carried out at 4°C with a Chromopulse control unit (ICN Biomedicals, Inc., Amsterdam, The Netherlands) connected to a power supply. Unless indicated otherwise, FIGE run conditions were as follows: (i) for high-range separation (50 to 500 kb), 7 V/cm, a voltage ratio of 0.38, and pulses from 1 to 40 s, increasing at 1.5 s/h; (ii) for medium-range separation (10 to 50 kb), 7 V/cm, a voltage ratio of 0.66, constant 0.8-s pulse for 14 h and then an increase to 6 s at 0.8 s/h. A *Hin*dIII digest or concatemers of bacteriophage lambda DNA (New England BioLabs) or a 5-kb ladder purchased from Bio-Rad Laboratories, Richmond, Calif., was used as size markers.

GeneScreen Plus nylon membranes (Du Pont, NEN Research Products, Wilmington, Del.) were used in all DNA transfers; unless indicated otherwise, transfer, hybridization, washing, and deprobing conditions were as recommended by the manufacturer. DNA was transferred from FIGE gels by alkaline capillary blotting (43) after a 10-min UV light (302-nm wavelength) treatment.

Design, construction, and use of DNA probes. The following DNA probes (Fig. 1) were used in the characterization of the Tn*5276* transposition process.

(i) Nisin production (*nisA* probe). The *nisA* gene and flanking sequences were isolated from strain NIZO R5 before the publication of the *nisA* gene sequence from strain ATCC 11454 (2) in the following way. A library of NIZO R5 DNA, partially digested with *Sau*3A, was prepared in λEMBL3 by using a Packagene Lambda DNA packaging system (Pharmacia LKB) and then screened by using an oligonucleotide with the sequence 5′-ATGGGTTGTAATA TGAAAAC (*nisA* probe). Bacteriophage λNZ700 was found to carry a 20-kb insert that hybridized to the *nisA* probe. A 1.7-kb *Sau*3A fragment from this insert was subcloned into M13mp18-M13mp19 and found to contain an *iso*-IS*904* element (38) and a *nisA* gene with a sequence identical to the published sequences of *nisA* genes of other strains (2, 9, 27). It appeared from this analysis that the sequence of the *nisA* probe differed in one nucleotide from the corresponding *nisA*

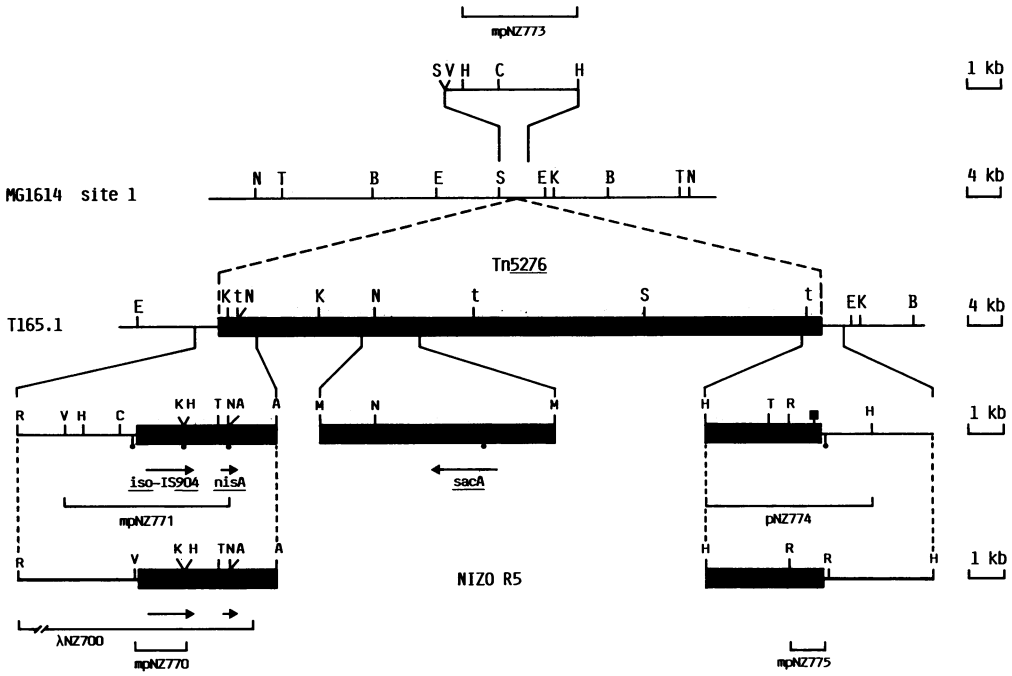

FIG. 1. Physical and genetic map of Tn*5276* in MG1614 transconjugant T165.1 and donor NIZO R5. Tn*5276* is represented by a black bar, MG1614 DNA is represented by a thin line, and NIZO R5 DNA is indicated by a thick line. Some regions are enlarged to show more detail. Arrows indicate the positions and directions of *iso*-IS*904* (the direction of the arrow indicates the direction of the putative transposase open reading frame [38]), *nisA*, and *sacA*. The positions of the oligonucleotide probes used in the restriction mapping (●) and the identification of the right end of Tn*5276* in NIZO R5 (■) are indicated. Cloned fragments used for the isolation and sequencing of both Tn*5276* borders and of site 1 are indicated (——). Complete restriction maps are shown for each contiguous DNA fragment, except for the *Tth*111I sites (t) in Tn*5276*. Other restriction enzyme abbreviations: A, *Sac*I; B, *Bgl*I; C, *Sca*I; E, *Eag*I; H, *Hin*dIII; K, *Kpn*I; M, *Bam*HI; N, *Nci*I; R, *Eco*RI; S, *Sac*II; T, *Tth*111I; V, *Eco*RV.

sequence (5′-ATGGGTTGTAACATGAAAAC). However, at a temperature of 45°C the *nisA* probe appeared to hybridize specifically to *nisA*-containing sequences.

(ii) Sucrose fermentation (*sacA* probe). The sucrose-6-phosphate hydrolase (*sacA*) gene of strain NIZO R5, encoding a key enzyme in the sucrose phosphotransferase pathway, was cloned and sequenced (41). An oligonucleotide with the sequence 5′-GATCTCGTCCACTTTG (*sacA* probe) was deduced on the basis of the *sacA* gene sequence and used in hybridizations at a temperature of 46°C.

(iii) IS*904* element (IS*904* probe). An insertion sequence was found upstream from the NIZO R5 *nisA* gene (38) that was almost identical in sequence and location to IS*904* in strain FI5876 (9). An oligonucleotide with the sequence 5′-AGCCGTGAATATCGAC (IS*904* probe) was based on the nucleotide sequence of this *iso*-IS*904* insertion sequence (positions 784 through 799 [38]) and used at a hybridization temperature of 46°C.

(iv) Insertion site 1 (site 1 probe). The 3.2-kb *Hin*dIII insert of mpNZ773/1 (see below) was used as a probe for the preferred site of insertion (designated site 1) of Tn*5276* in the chromosome of strain MG1614.

(v) Left and right junctions (L and R probes, respectively). As probes for the left and right junctions of Tn*5276* in transconjugant T165.1 (Fig. 1), oligonucleotides with the sequences 5′-GTATGAACTAGGGCTG (L probe) and 5′-AAACTGGCAAATCATGG (R probe) were used at hybridization temperatures of 46 and 52°C, respectively. These oligonucleotides were based on the nucleotide sequence of MG1614 site 1 (147 nucleotides left and 85 nucleotides right of the site of integration, respectively).

All oligonucleotide probes were end labeled, and the DNA fragment probes were labeled by nick translation with γ-^{32}P- and α-^{32}P-labeled ATP as described previously (44). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (MilliGen Biosearch Division, San Rafael, Calif.).

Identification and cloning of insertion site 1 and Tn*5276* junctions. (i) Left junction in NIZO R5. A 1.4-kb *Eco*RV-*Hin*dIII fragment of λNZ700 that hybridized with the IS*904* probe was isolated and cloned into M13mp19 digested with *Hin*cII and *Hin*dIII, resulting in mpNZ770/1. The insert containing the left junction of Tn*5276* in NIZO R5 was then subcloned into M13mp18, resulting in mpNZ770/2.

(ii) Left junction in T165.1. A 4.5-kb *Eco*RV-*Sac*I fragment of T165.1 DNA that hybridized to the *nisA* probe was cloned into M13mp18 digested with *Hin*cII and *Sac*I, resulting in mpNZ771 (Fig. 1). Then a 3.0-kb *Sca*I-*Sac*I subfragment containing the Tn*5276* left border (Fig. 1) was cloned into M13mp18 and M13mp19, resulting in mpNZ772/1 and mpNZ772/2, respectively.

(iii) Insertion site 1 from MG1614. A 3.2-kb *Hin*dIII fragment of MG1614 DNA containing integration site 1 was cloned in *Hin*dIII-linearized M13mp18, resulting in the constructs mpNZ773/1 and mpNZ773/2, with different insert orientations (Fig. 1). This *Hin*dIII fragment was identified by hybridization to the 1.5-kb *Eco*RV-*Sca*I fragment of the mpNZ771/1 insert, containing only MG1614 DNA.

(iv) Right junction in T165.1. A 4.6-kb *Hin*dIII fragment of T165.1 DNA was identified by hybridization to the 3.2-kb *Hin*dIII insert of mpNZ773/1 and cloned into *Hin*dIII-linearized pNZ84, resulting in pNZ774/1.

(v) Right junction in NIZO R5. A 1.0-kb *Eco*RI fragment

from NIZO R5 DNA was identified by hybridization to the oligonucleotide (5'-CTAACCAAGAGACTAACC; hybridization temperature, 48°C; Fig. 1) that matched the sequence of the right end of Tn*5276*. This 1.0-kb *Eco*RI fragment was cloned into *Eco*RI-linearized M13mp18 and M13mp19, resulting in mpNZ775/1 and mpNZ775/2, respectively.

DNA sequencing. The nucleotide sequences of DNA fragments cloned in M13mp18 and M13mp19 were determined from both strands by the dideoxy-chain termination method (45) adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with either the M13 universal primer or synthesized primers. In pNZ773, the nucleotide sequences of both strands were determined by using a double-stranded DNA sequencing method (21) adapted for Sequenase version 2.0 and synthesized primers. The sequence data were assembled and analyzed using the PC/Gene program version 5.01 (Genofit, Geneva, Switzerland).

Determination of nisin production and immunity. Nisin production by *L. lactis* strains was determined by using an agar-diffusion bioassay with *Micrococcus flavus* DSM1719 (50). Nisin immunity was determined by following the growth of *L. lactis* strains in milk containing 0.5% glucose, 0.1% yeast extract, and various amounts of commercial nisin (Koch-Light Ltd., Haverhill, Suffolk, England).

Nucleotide sequence accession numbers. The nucleotide sequences of the left and right junctions of Tn*5276* in *L. lactis* T165.1 will appear in the EMBL and GenBank nucleotide sequence data bases under accession numbers M84769 and M84770, respectively.

RESULTS

Transfer of the conjugative nisin-sucrose element of NIZO R5. The capacity to ferment sucrose could be transferred in DNase-insensitive matings of *L. lactis* NIZO R5 and the plasmid-free and prophage-free strain *L. lactis* MG1614 (15) with frequencies that varied from 10^{-8} (20-h matings) to 10^{-6} (4-h matings) CFU per CFU of donor. Transconjugants obtained from different matings produced nisin, showed immunity to nisin, and were sensitive to recipient-specific phage φ763 and resistant to donor-specific phage φR5. In addition, transconjugants were able to transfer the ability to ferment sucrose and produce nisin with similar frequencies to the differently marked recipient strain MG1390 (17) (data not shown).

Transfer of the capacity to ferment sucrose was also studied in matings of NIZO R5 and a streptomycin-resistant derivative (MMS36S) of the recombination-deficient strain *L. lactis* MMS36, which is unable to mediate homologous recombination (more than 10^4 reduction of chromosomal transduction [1]). Sucrose-proficient and nisin-producing MMS36S transconjugants, which all showed high sensitivity to mitomycin C (42), were obtained with a frequency of transfer (3×10^{-9} CFU per donor CFU) that was only 100-fold lower than the transfer frequency to MG1614 in a simultaneous experiment. Since *rec*-independent gene transfer is known to be reduced similarly in strain MMS36 (1), these data indicate that transfer of the conjugative sucrose-nisin element is independent of homologous recombination.

The nisin-sucrose element is a conjugative transposon, Tn*5276*. The fates of the *nis* and *sac* genes for nisin biosynthesis and the sucrose phosphotransferase system, respectively, were followed in matings of NIZO R5 and MG1614. Since no plasmid DNA could be detected (data not shown), we isolated total DNA from overnight cultures of colony-purified MG1614 transconjugants derived from a single mating. Hybridization analysis (Fig. 2) indicated that both *nisA*- and *sacA*-specific sequences were present in the DNA of the transconjugants but not in the recipient MG1614. These results also showed that several transconjugants (5 out of 12 tested) had acquired two or three copies of both *nisA* and *sacA*. This was evident from the additional bands obtained with the *nisA* probe (Fig. 2A) and the intensity of the hybridizing bands obtained with the *sacA* probe (Fig. 2B). The analysis was repeated with transconjugants obtained from independent matings of strains NIZO R5 and MG1614, and similar hybridization patterns were obtained (data not shown). The presence of an identical number of copies of *nisA* and *sacA* in all transconjugants demonstrates that nisin production and sucrose fermentation are linked at the gene level.

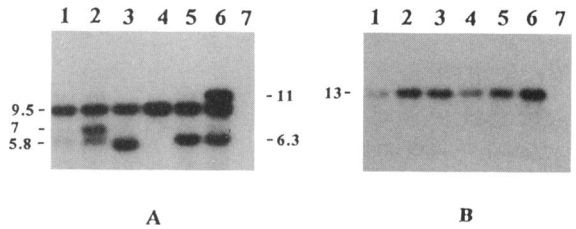

FIG. 2. Presence of *nisA*- and *sacA*-specific sequences in *L. lactis* transconjugants. Equal amounts of total DNA from six transconjugants from the mating between NIZO R5 and MG1614 and from strain MG1614 were digested with *Eco*RI and separated by agarose gel electrophoresis. A Southern blot was hybridized first to the *nisA* probe (A) and, after deprobing, to the *sacA* probe (B). The lanes in panels A and B contain DNA from the following strains: 1, T165.1; 2, T165.2; 3, T165.4; 4, T165.5; 5, T165.6; 6, T165.8; 7, MG1614. The estimated sizes of hybridizing fragments are indicated in kilobases. The intensity of the bands in panel B reflects the number of *sacA* copies per chromosome.

Since the transconjugants with multiple copies of the nisin-sucrose element contain *Eco*RI fragments of a different size that hybridize to the *nisA* probe, the *nisA* gene must be close to one border of the element. This border has been designated the left border (Fig. 1). Since five different *Eco*RI fragments hybridized to the *nisA* probe in the transconjugants (Fig. 2A), the nisin-sucrose element is able to insert into at least five sites in the MG1614 genome. These results and the observation that nisin-sucrose transfer is nuclease insensitive and independent on homologous recombination (see above) led us to conclude that the nisin-sucrose element of NIZO R5 is a conjugative transposon (8) that has been designated Tn*5276* (Fig. 1; registered with the Plasmid Reference Centre Registry [32]).

Orientation-specific insertion of Tn*5276* into a preferred site (site 1) in the MG1614 chromosome. All MG1614-derived transconjugants containing a single or multiple copies of the nisin-sucrose element share the 9.5-kb *Eco*RI fragment that hybridizes to the *nisA* probe (Fig. 2A). Similarly, digestion of DNA of these transconjugants with several other restriction enzymes always showed a single, unique fragment that hybridized to the *nisA* probe (data not shown). These results indicate a preferred site of insertion (designated site 1) for Tn*5276* in the genome of *L. lactis* MG1614 and that insertion into this site is orientation specific.

Tn*5276* is a 70-kb element located in the chromosomes of the donor and transconjugants. To further analyze the location and the size of Tn*5276*, hybridizations to large DNA fragments were performed. DNA from the donor strain NIZO R5, the recipient strain MG1614, and transconjugants con-

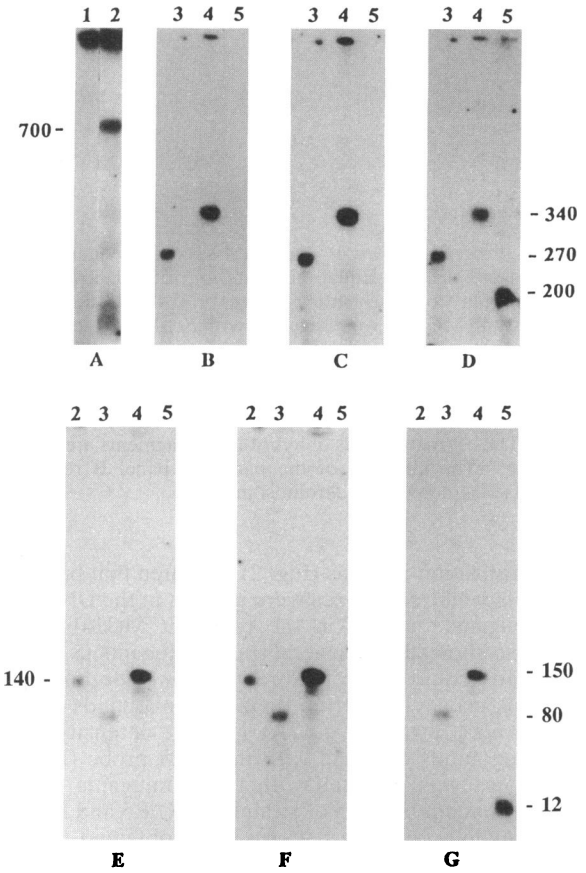

FIG. 3. Determination of size and location of the nisin-sucrose element. Undigested DNA from NIZO R5 (A, lane 1) and *Sma*I digests (A, lane 2; B through D) and *Eag*I digests (E through G) of DNA from strains NIZO R5 (lanes 2), T165.5 (lanes 3), T165.6 (lanes 4), and MG1614 (lanes 5) were separated by FIGE (the high-range regime was used except for panel A DNA, which was separated by using pulses from 1 to 60 s, increasing at 2.5 s/h). Southern blots were hybridized to the *nisA* probe (A, B, E), the *sacA* probe (C, F), and the site 1 probe (D, G). The estimated sizes of the hybridizing fragments are indicated in kilobases.

taining a single copy (T165.5) or two copies (T165.6) of Tn*5276* was digested with the infrequently cutting restriction enzymes *Sma*I and *Eag*I. The resulting DNA fragments were separated by FIGE and hybridized to the Tn*5276*-specific probes and a site 1 probe, which was specific for the preferred site of integration. The results (Fig. 3) show that donor and transconjugant strains contain large *Sma*I fragments that hybridize to both the *nisA* and *sacA* probes. The site 1 probe appeared to hybridize to the same *Eag*I and *Sma*I fragments as the *nisA* and *sacA* probes in both transconjugants. The sizes of these fragments were invariably larger than those of the fragments of MG1614 DNA hybridizing to the site 1 probe. These results demonstrate that the nisin-sucrose element has been inserted into the chromosome of the transconjugants. Furthermore, the DNA of NIZO R5 that hybridizes to the *nisA* probe (and the *sacA* probe; data not shown) does not enter the gel without digestion and generates a 700-kb fragment after digestion with *Sma*I (Fig. 3A, lanes 1 and 2), indicating that Tn*5276* is present as an integral part of the chromosomal DNA in this donor strain.

The 12-kb *Eag*I fragment and the 200-kb *Sma*I fragment of MG1614 are increased to 80 and 270 kb, respectively, after a single integration of Tn*5276* as in T165.5 (Fig. 3). Since T165.5 can conjugally transfer Tn*5276* in a second mating to MG1390 (17; data not shown), it is very likely that it contains a complete copy of the transposon. Therefore, Tn*5276* has a size of approximately 70 kb.

In the digests of the DNA from transconjugant T165.6, containing two Tn*5276* copies, the hybridizing *Eag*I and *Sma*I fragments are another 70 kb larger, confirming the estimated element size. The hybridizing *Eag*I fragment in the MG1614 DNA is only 12 kb in size, so both Tn*5276* copies have inserted in close proximity to each other in the chromosome of transconjugant T165.6.

Physical and genetic map of Tn*5276*. A physical map of Tn*5276* and its flanking regions after insertion into site 1 of MG1614 was constructed (Fig. 1) based on hybridizations of site 1- and element-specific probes to restriction fragments separated by normal gel electrophoresis or FIGE (medium- or high-range separations) and subcloning and mapping of the left and right ends of the element. By using the restriction sites deduced from their nucleotide sequences, the orientation and location of the *nisA* gene, the *iso*-IS*904* element (38), and the *sacA* gene (41) could be determined (Fig. 1). By using the IS*904* probe, it was found that Tn*5276* in TS165.5 contains only a single copy of *iso*-IS*904*. Also, in other MG1614 transconjugants the number of additional IS*904* copies was equal to that of Tn*5276* (data not shown). The MG1614 chromosome contains at least seven copies of elements hybridizing to the IS*904* probe (not shown), but there is no copy of IS*904* in the 12-kb *Eag*I fragment that contains the hot spot site 1 and at least one secondary site (Fig. 3).

Cloning and sequence analysis of the junction regions of Tn*5276* in the donor and the transconjugant. The left and right junction regions of Tn*5276* inserted into site 1 of MG1614 (in transconjugant T165.1) were cloned, and the nucleotide sequences of relevant parts of the resulting constructs, mpNZ771 and pNZ774 (Fig. 1), were determined. The results (Fig. 4A) show that Tn*5276* contains A+T-rich termini (76% A+T in the first 50 bp) without obvious inverted repeats. There is a perfect 11-bp repeat in the right end of Tn*5276*. There is a similar but less perfect repeat in the left end. The results also show that the left terminus is separated from the left end of the *iso*-IS*904* copy by a region of 249 bp, the sequence of which differs considerably from that preceding the IS*904* copy in FI5876 (9) (Fig. 4). This excludes the possible involvement of the *iso*-IS*904* copy in the transposition of Tn*5276*.

The nucleotide sequences of the Tn*5276* junctions in site 1 of transconjugant T165.1 were compared with those in strain NIZO R5 and with the sequence of site 1 from strain MG1614 (Fig. 4B). In both the donor and the transconjugant, Tn*5276* is flanked by a direct repeat of the hexanucleotide with the sequence TTTTTG, which is present once in the integration site 1. In NIZO R5 this hexanucleotide is part of a larger, 25-bp perfect direct repeat. Apart from this hexanucleotide sequence, there is no apparent homology between the ends of Tn*5276* and target site 1.

DISCUSSION

In this report we describe a physical and genetic characterization of the *L. lactis* conjugative nisin-sucrose element from strain NIZO R5, a known nisin producer (14, 33), and its insertion into the chromosome of strain MG1614. The

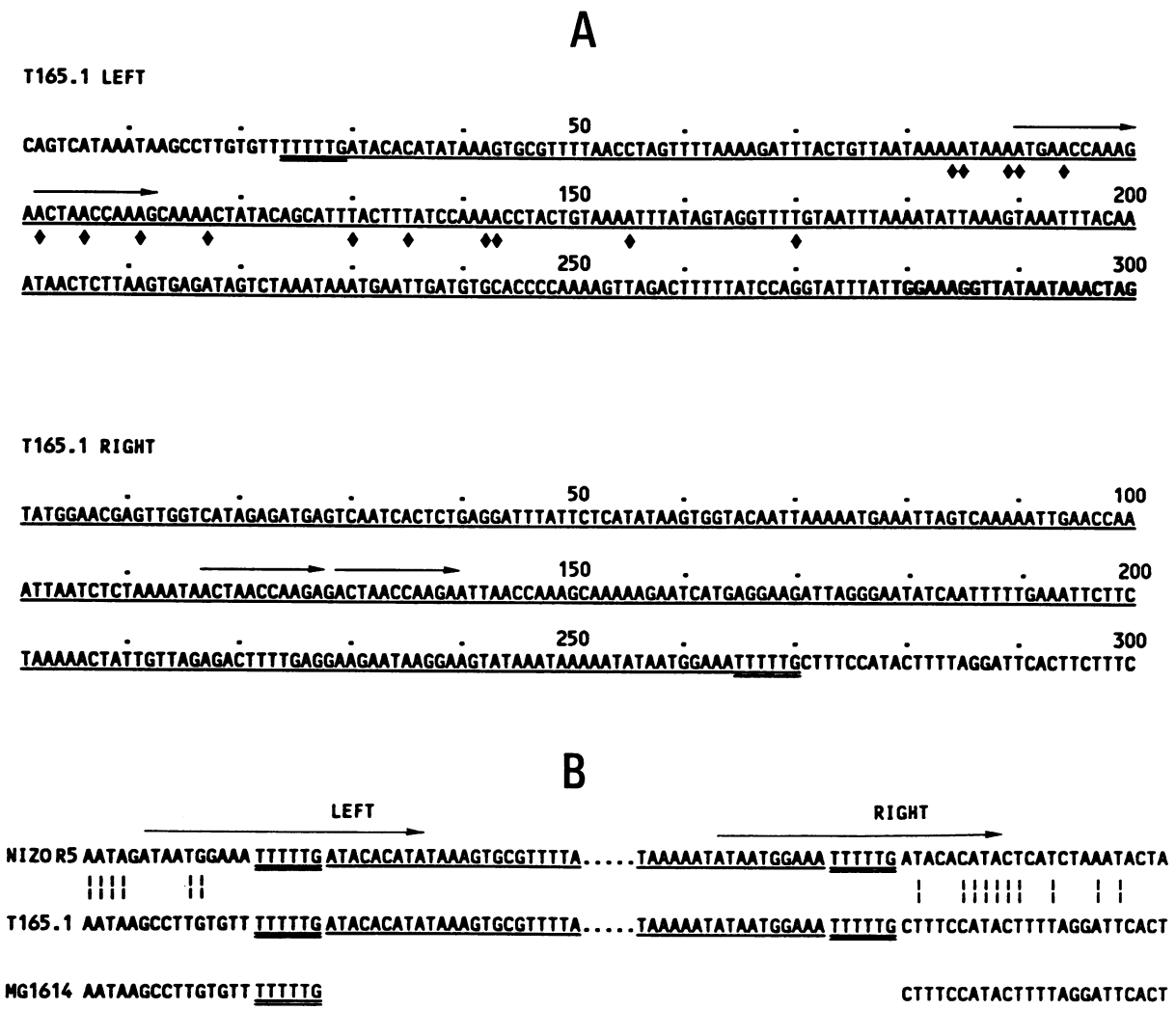

FIG. 4. Nucleotide sequences of Tn*5276* termini and junction regions in the donor and transconjugants in comparison with that of target site 1. (A) Nucleotide sequence of the left and right termini of Tn*5276* and the junction regions in T165.1. Tn*5276*-specific sequences are underlined. The direct repeated hexanucleotide sequence TTTTTG is underlined twice. The perfect and imperfect 17-bp direct repeats present in the right and left ends, respectively, are indicated by arrows. The first 22 nucleotides of *iso*-IS*904* (38) are indicated (boldface type). The nucleotide differences from the corresponding region in *L. lactis* strain FI5876 (9) (compared from position 79 in the left terminus) are indicated below the sequence (◆, absent in FI5876). (B) Comparison of Tn*5276* junction regions in NIZO R5 and T165.1 with that of target site 1. Tn*5276*-specific sequences are underlined. The direct repeated hexanucleotide sequence TTTTTG in NIZO R5 and T165.1 is underlined twice. The direct repeat spanning the Tn*5276* junctions in NIZO R5 is indicated by arrows. Sequence identity between the regions flanking Tn*5276* in strains NIZO R5 and T165.1 (not including the TTTTTG sequences) is indicated (¦).

mode of transfer of the element from NIZO R5 appears analogous to that of nisin-sucrose elements in other *L. lactis* strains that are also insensitive to DNase (20), do not involve a transducing phage (20), and are not dependent on the host-mediated homologous recombination system (46). We show here that the nisin-sucrose element from *L. lactis* NIZO R5 is a 70-kb transposon, designated Tn*5276*, that conforms to the definition of a conjugative transposon; i.e., a specific DNA segment that can repeatedly insert into a few or many sites in a genome, encodes additional functions unrelated to insertion function, and has the capacity to promote its own transfer in the absence of any plasmid or bacteriophage (4, 8). A nisin-sucrose element, Tn*5301*, showing characteristics similar to those of Tn*5276* has recently been described in *L. lactis* FI5876 (9) after submission of this manuscript.

The novel conjugative transposon Tn*5276* is chromosomally located in both the donor NIZO R5 and its transconjugants. A physical map of the 70-kb Tn*5276* was constructed, and the genes for nisin biosynthesis and sucrose utilization via a phosphotransferase system were located (Fig. 1). Insertion of Tn*5276* into at least five different chromosomal sites was found (Fig. 2), but there appears to be a preferential site for orientation-specific insertion of Tn*5276* into the chromosome of MG1614. A similar preference of Tn*5276* to insert in an orientation-specific way into a hot spot was found in at least one other, unrelated *L. lactis* strain (42). Preferential strain-dependent integration at specific sites has also been found for other transposons. Tn*554* has a strong preference for orientation-specific insertion at a single site in the *Staphylococcus aureus* chromosome (designated *att*Tn*554* [28]). In addition, the conjugative Tn*919*

inserts into a single site in the chromosome of *L. lactis* MG1363Sm, which is related to MG1614 (22), whereas it inserts at different sites in the chromosome of *L. lactis* 18-16S (23). Finally, Tn*916* and Tn*1545* integrate into sites showing some resemblance to the ends of these conjugative transposons, and two consensus sequences for integration sites have been deduced for these transposons (3, 5, 51). Similarly, we found that the regions flanking Tn*5276* in the donor and transconjugants share sequence identity (19 out of 35 bp are identical; Fig. 4B).

In various cases multiple (up to three) copies of Tn*5276* were inserted into the chromosome of colony-purified MG1614 transconjugants (Fig. 2 and 3). The presence of more than one transposon copy in the recipient genome was also reported after conjugal transfer of Tn*916* (8, 18) and for Tn*1545* (56). The occurrence of multiple integration may be explained by transposition during replication. Alternatively, those insertions may be a consequence of multiple, consecutive conjugation events. If this is the case, Tn*5276*, like Tn*916* (37), should not show transposition immunity and the efficiency of those multiple conjugation events should be high.

Tn*5276* is a large conjugative transposon of approximately 70 kb. Conjugative transposons of a similar size have been found in various streptococci and include the 67-kb Tn*3951* from *Streptococcus agalactiae* (26), the 65-kb Tn*5253* from *Streptococcus pneumoniae* (53, 54), and *Streptococcus pyogenes* Tn*3701*, which is larger than 50 kb (31). The large size of Tn*5276* is compatible with the variety of functions it should encode; i.e., transposition, conjugal transfer, nisin biosynthesis (including posttranslational modification of the precursor), nisin immunity, sucrose fermentation via a phosphotransferase system, and reduced phage sensitivity. It was reported (10) that the production of N^5-(carboxyethyl)ornithine synthase is also encoded by the nisin-sucrose element. However, several known nisin-producing *L. lactis* strains were found to produce no N^5-(carboxyethy)ornithine synthase (49), indicating that not all nisin-sucrose transposons encode production of this enzyme. Heterogeneity within the group of nisin-sucrose transposons was recently shown by analyzing the architecture of nisin-sucrose elements of several wild-type *L. lactis* strains that differed from Tn*5276* in the number and orientation of *iso*-IS*904* copies (39).

The cloning and sequence analysis of the junction fragments of Tn*5276* in the donor and recipients (Fig. 1 and 4) allows for its comparison with other known conjugative transposons that have been analyzed in detail, i.e., Tn*916* (7) and Tn*1545* (3). Similar to the ends of those transposons, the ends of Tn*5276* are highly A+T rich and contain some direct repeats, one of which (in donor NIZO R5) spans the junction regions, as also has been found for some Tn*916* insertions (7). However, in contrast to the termini of Tn*916* and Tn*1545*, which contain homologous, imperfect inverted repeats, the termini of Tn*5276* are asymmetric and do not show significant inverted repeats. The absence of inverted repeats is unusual among mobile DNA elements but has also been found in Tn*554* (35). Moreover, Tn*5276* is flanked by a direct repeat of the hexanucleotide TTTTTG in both the donor and the transconjugants. No such repeats flank Tn*916* or Tn*1545*, which are known to generate 6- or 7-bp nonidentical coupling sequences as a consequence of their unique excision-insertion mechanism (5, 51). The present sequence data do not allow us to conclude whether one (and, if so, which) of the TTTTTG copies is part of Tn*5276* or whether a target site duplication has been generated upon the transposition process. However, by analyzing a circular intermediate of Tn*5276*, we very recently found that one of the TTTTTG sequences is part of Tn*5276* and not a target repeat (42). Further studies that are presently being performed focus on the mechanism of Tn*5276* excision and insertion and the genes involved in this process.

ACKNOWLEDGMENTS

We are grateful to Marke M. Beerthuyzen and Ingrid J. van Alen-Boerrigter for skilled technical assistance, to Mike J. Gasson for sharing unpublished results, to Roland J. Siezen and Oscar P. Kuipers for critically reading the manuscript, and to Raymond A. J. van Daelen for introducing us to FIGE techniques.

This work was partially supported by grant 663/88 from the European Community.

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