The *Lactococcus lactis* Nisin-Sucrose Conjugative Transposon Tn*5276*

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The *Lactococcus lactis* Nisin-Sucrose Conjugative Transposon Tn*5276*

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus
dr. H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 26 mei 1993
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

De natuur is *beweging.*

James Hutton

Ten dage dat ik riep, hebt Gij mij geantwoord,
Gij hebt mij bemoedigd met kracht in mijn ziel.

Psalm 138 (NBG vertaling)

Aan pap en mam,
Catelijne, Marijke en Lonneke

STELLINGEN

1. De bewering van Poyart-Salmeron *et al.* dat de plaats-specifieke recombinatie-reactie van Tn*1545* zich onderscheidt van die van bacteriofaag λ doordat het Int-Tn eiwit ook functioneert in afwezigheid van Xis-Tn is onjuist.

   Poyart-Salmeron, C., P. Trieu-Cuot, C. Carlier en P. Courvalin. 1989. Molecular characterization of two proteins involved in the excision of the conjugative transposon Tn*1545*: homologies with other site-specific recombinases. EMBO J. 8:2425-2433.

   Abremski, K. en S. Gottesman. 1981. Site-specific recombination. Xis-independent excisive recombination of bacteriophage lambda. J. Mol. Biol. 153:67-78.

2. De bewering van Steen *et al.* dat het structurele nisine gen van *Lactococcus lactis* ATCC 11454 afgeschreven wordt vanuit een promoter die meer dan 4 kb stroomopwaarts ligt, is onwaarschijnlijk aangezien transcriptie van dit gen dan afhankelijk zou zijn van de plaats van insertie van het nisine-sucrose element in het chromosoom en van mogelijke IS-gemedieerde herrangschikkingen van het gebied tussen het startpunt van transcriptie en het nisine gen.

   Steen, M.T., Y.J. Chung en J.N. Hansen. 1991. Characterization of the nisin gene as part of a polycistronic operon in the chromosome of *Lactococcus lactis* ATCC 11454. Appl. Environ. Microbiol. 51:1181-1188.

   Gireesh, T., B.E. Davidson en A.J. Hillier. 1992. Conjugal transfer in *Lactococcus lactis* of a 68-kilobase-pair chromosomal fragment containing the structural gene for the peptide bacteriocin nisin. Appl. Environ. Microbiol. 58:1670-1676.

   Dit proefschrift.

3. Een vermoedelijk chromosomaal gelegen element is nog geen transposon.

   Thompson, J., N.Y. Nguyen, D.L. Sackett en J.A. Donkersloot. 1991. Transposon-encoded sucrose metabolism in *Lactococcus lactis*. Purification of sucrose-6-phosphate hydrolase and genetic linkage to N^6-(L-1-carboxyethyl)-L-ornithine synthase in strain K1. J. Biol. Chem. 266:14573-14579.

4. De meest waarschijnlijke verklaring voor de instabiliteitsverschijnselen die gevonden zijn bij pSS50 en andere *Alcaligenes* spp. 4-chlorobifenylafbraak plasmiden is de aanwezigheid van IS elementen op deze plasmiden.

   Burlage, R.S., L.A. Bemis, A.C. Layton, G.S. Salyer en F. Larimer. 1990. Comparative genetic organization in incompatibility group P degradative plasmids. J. Bacteriol. 172:6818-6825.

   Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran en G.S. Salyer. 1992. Evidence for 4-chlorobenzoic acid dehalogenation mediated by plasmids related to pSS50. Appl. Environ. Microbiol. 58:399-402.

5. De door Hayashi en Munakata bepleite akoestische methode voor het hanteren van DNA-sequenties heeft waarschijnlijk geen brede ingang gevonden als gevolg van de muzikale beperktheid ervan.

Hayashi, K. en N. Munakata. 1984. Basically musical. Nature 310:96.

6. Het belang van negatieve en/of positieve controles bij experimenten wordt vaak pas ingezien bij het weglaten ervan.

7. Als het inderdaad zo is dat, zoals uit onderzoek van het Japanse cosmeticaconcern Shiseido naar de effecten van aroma's op de hersengolven zou blijken, geuren een belangrijke rol zouden kunnen spelen bij het creëren van een bepaalde sfeer, dan is de sfeer in microbiologische laboratoria niet zo best.

Breedeveld, M. 1992. Bij de neus genomen. Intermediair 28(20):43.

8. Een betere bekendheid met de betekenis van de in een (katholieke) eucharistieviering gebruikte symbolen en uitgevoerde handelingen zou op zijn minst een gedeelte van de door sommigen als "saaie bedoening" omschreven klacht met betekking tot deze vieringen kunnen wegnemen.

9. De kritiek op *house* muziek - zij zou bijvoorbeeld niet artistiek zijn - gaat onder meer voorbij aan het feit dat muziek ook "gewoon lekker" kan zijn.

10. Hoewel het schrijven van een proefschrift wel eens wordt vergeleken met een bevalling haalt het resultaat ervan het niet bij dat van een echte bevalling.

11. De eerste is niet altijd de beste.

Stellingen behorende bij het proefschrift "The *Lactococcus lactis* Nisin-Sucrose Conjugative Transposon Tn*5276*" van Peter J.G. Rauch
Wageningen, 26 mei 1993

VOORWOORD

Wat begon als een zoektocht naar het nisine plasmide is geëindigd als de bestudering van een zeer fascinerend transposon. Zoals zo vaak bij een promotie-onderzoek was er sprake van een vertraagde start en een heftig einde. Het was dan ook moeilijk afscheid van Tn*5276* te nemen. Dat komt ook doordat het afscheid betekent van een fijne groep; een groep die ik hier graag wil bedanken:

Willem - mijn 'bekwaamheid tot het zelfstandig beoefenen van de wetenschap' leerde ik van jou. Je enthousiasme en steun waren onontbeerlijk. Marke - als stagiaire en later als analiste heb je heel wat werk verzet (werk dat lang niet allemaal in dit proefschrift staat) en dat was niet altijd even gemakkelijk (sucrose!). Ingrid - ik heb kunnen voortbouwen op jouw pionierswerk en ook later heb je me erg geholpen (genbank, vraagbaak). Léonard en Mireille - het sucrose-werk speelde ook jullie parten, maar jullie lieten je niet kisten. Alle anderen die in de kelder verbleven of daar nog steeds vertoeven - het was vreselijk gezellig! We houden contact (lees: ik kom graag nog eens 'Subben/Suppen (?)').

Alle anderen die mijn verblijf bij het NIZO veraangenaamd hebben, zowel in de werk- als in de sociale sfeer - bedankt.

Mijn collega's in Amsterdam - bedankt voor jullie geduld.

Catelijne - naar jouw licht kwam ik altijd weer terug; laat het altijd branden.

Marijke en Lonneke - We hebben heel wat in te halen. Wat zullen we het eerst gaan doen?

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

GENERAL INTRODUCTION

The bacteriocin nisin, produced by several naturally occurring *Lactococcus lactis* strains, is a promising alternative for sodium nitrate to prevent the outgrowth of clostridial spores in Gouda cheese. In the past, researchers at the Netherlands Institute for Dairy Research (NIZO) encountered two major obstacles in their efforts to design nisin-producing starter cultures. One was that the natural nisin-producers usually were bacteriophage-sensitive and lacked the appropriate industrial characteristics. The other was that bacteriophage-resistant starter strains were very sensitive to nisin. It was known that nisin production and immunity could, together with the ability to utilize sucrose, be transferred to other *L. lactis* strains in a conjugation-like process. It was therefore decided to introduce nisin production and/or nisin immunity in industrial starter strains by natural transfer of the genetic material responsible for these traits.

OUTLINE OF THE THESIS

The aim of the research described in this thesis was to identify and characterize the genetic element carrying the information for nisin biosynthesis and sucrose proficiency, the nisin-sucrose element. The thesis has the following outline. Chapter 2 describes our current knowledge of nisin genetics and the recent advances in the protein engineering of nisin. The cloning and analysis of several parts of the nisin-sucrose element of *L. lactis* strain NIZO R5 are described in the next two chapters. Chapter 3 deals with the prenisin gene, *nisA*, and its surrounding region, which includes the insertion sequence IS*1068*. In Chapter 4, the organization and transcription of the sucrose (*sac*) operon and the characterization of the gene for sucrose-6-phosphate hydrolase are described. The different parts of the nisin-sucrose element were used as DNA probes to investigate the nature and location of this element (Chapter 5). It was shown that the nisin-sucrose element of *L. lactis* NIZO R5 is a large (70 kb), conjugative transposon, designated Tn*5276*. Chapter 6 describes the isolation and analysis of the genes that encode the transposition functions of Tn*5276*. The experiments leading to a model for excision and insertion of Tn*5276* are described in Chapter 7. Chapter 8 deals with the distribution and evolution of nisin-sucrose elements in *L. lactis*. The thesis is completed with the summary and concluding remarks (Chapter 9) and a Dutch summary (Chapter 10).

CHAPTER 2

GENETICS AND PROTEIN ENGINEERING OF NISIN

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and Willem M. de Vos

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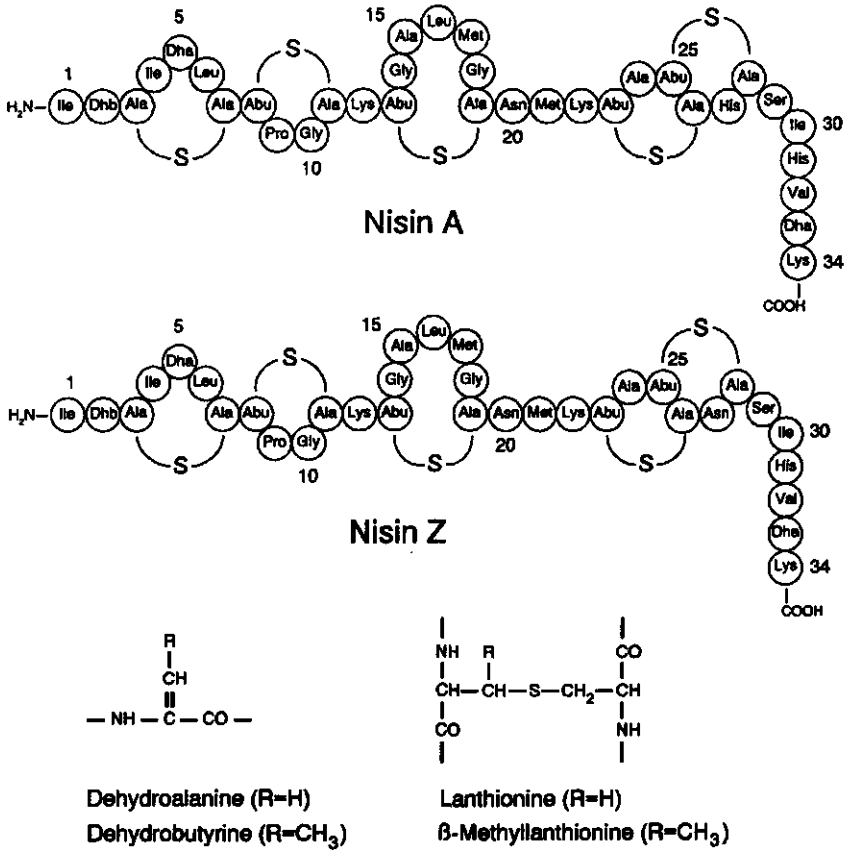

FIG. 1. The primary structures of nisin A and nisin Z. Dha, Dhb, Ala-S-Ala, and Abu-S-Ala indicate dehydroalanine, dehydrobutyrine, lanthionine, and ß-methyllanthionine residues, respectively.

1. Introduction

Of the bacteriocins produced by lactic acid bacteria, nisin (Fig. 1) is the best characterized representative. Nisin is a 34-amino acid (aa) polypeptide (Gross & Morell, 1971) produced by a number of, usually atypical, *Lactococcus lactis* subsp. *lactis* strains (Hirsch, 1953; De Vos *et al.*, 1992). Two natural variants of nisin are known, nisin A (Gross & Morell, 1971) and nisin Z (Mulders *et al.*, 1991), which differ in a single amino acid residue at position 27 (Asp in nisin A and His in nisin Z; Fig. 1). The structural genes for nisin A and nisin Z (*nisA* and *nisZ*, respectively) have been found to differ by a single mutation (see section 3.1). The two nisin

variants appear to have the same biological activities, but nisin Z seems to have other diffusion properties than nisin A (De Vos *et al.*, 1992). Nisin is the most prominent member of the group of bacteriocin-like peptides called lantibiotics (Schnell *et al.*, 1988). Lantibiotics are ribosomally synthesized antimicrobial polypeptides, produced by gram-positive bacteria, that contain the thioether amino acids lanthionine and 3-methyl-lanthionine (see Jung (1991*a*,*b*) for recent reviews). On the basis of their different types of ring structures and their differences in molecular weights, the lantibiotics have been classified into the two subgroups type A and type B, nisin being a type A lantibiotic. Other members of this group include subtilin (Gross & Kiltz, 1973) produced by *Bacillus subtilis*, epidermin (Allgaier *et al.*, 1985, 1986) and Pep5 (Kellner *et al.*, 1989), both produced by *Staphylococcus epidermidis*, and the *L. lactis* subsp. *lactis* bacteriocin lacticin 481 (Piard, 1992; Piard *et al.*, 1992*b*).

Nisin has found wide application as a food preservative owing to its antimicrobial activity against a broad range of gram-positive organisms, including food pathogens such as *Clostridium* and *Listeria* species, its non-toxicity and its instability at neutral pH and during passage through the intestine (Delves-Broughton, 1990; Molitor & Sahl, 1991). Recently, it was reported that nisin shows a bacteriocidal effect towards *Salmonella* species and other gram-negative bacteria when used in combination with chelating agents (Blackburn *et al.*, 1989; Stevens *et al.*, 1991). This is in agreement with the finding that cells of *Escherichia coli* become sensitive to nisin when their outer membrane is disrupted (Kordel & Sahl, 1986).

The primary target of nisin action is the cytoplasmic membrane and pore formation by nisin in intact cell membranes and artificial lipid bilayers has been demonstrated (Sahl *et al.*, 1987; Benz *et al.*, 1991). However, the effectivity of nisin appears to be dependent on the membrane lipid composition (Gao *et al.*, 1991). NMR structural analyses of nisin have been published (Goodman *et al.*, 1991; Lian *et al.*, 1992; Van de Ven *et al.*, 1992). These studies, in combination with structural analyses of nisin in lipophilic environments and further biophysical experiments on the interaction between nisin and membranes, may lead to elucidation of the molecular mechanism of pore formation.

This chapter is concerned with the two main subjects of nisin genetic research: firstly, the nisin gene cluster and its use in protein engineering and secondly, the conjugative transposon carrying the nisin gene cluster.

2. Nisin biosynthesis and immunity

The first indication that nisin biosynthesis occurs through the posttranslational modification of a ribosomally synthesized precursor (designated prenisin (De Vos *et al.*, 1991); see Fig. 2) came from the observations of Hurst (1966) and Ingram (1969) that inhibitors of ribosomal protein synthesis block nisin production. Ingram (1969, 1970) proposed that the formation of the lanthionines and 3-methyllanthionines occurs through dehydration of serine and threonine residues, respectively, followed by sulphide ring formation between the resulting dehydroalanine and dehydrobutyrine residues and appropriately located cysteine residues. On the basis of these mechanisms the structure of the nisin precursor polypeptide was postulated (Ingram, 1970). Twenty years later, the sequencing of the structural genes for nisin A and Z (see section 3) has now confirmed the proposed structure of the nisin precursor and therefore supports the putative mode of synthesis.

Another aspect of nisin production is self-protection or immunity of the producer. A characteristic feature of bacteriocins is the existence of specific immunity peptides, which protect the producer strains against the lethal action of their own products. Good examples are the colicins produced by certain *E. coli* strains. The lethal action of colicins E2 and E3 on the producer organism is prevented by stoichiometric complex formation with immunity proteins in the cytoplasmic membrane (Bowman *et al.*, 1971). In the case of the channel-forming colicins (A, B, E1, Ia, Ib, and N) it has been proposed that immunity at least partly arises from a specific interaction between the immunity protein and the colicin translocation apparatus that transports the bacteriocin across the outer membrane into the periplasm (Song & Cramer, 1991).

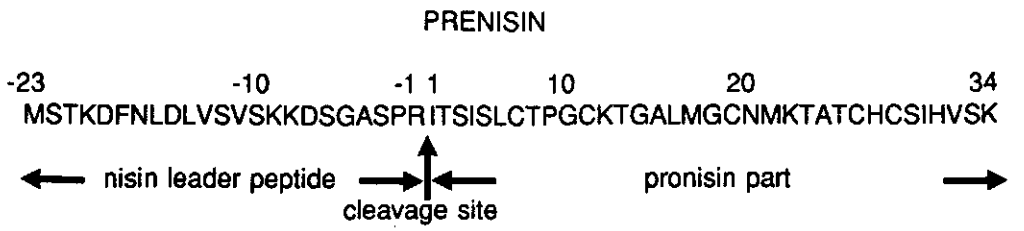

FIG. 2. The amino acid sequence of prenisin (nomenclature according to De Vos *et al.*, 1991).

Little is known about the basis of immunity to nisin. Early experiments by the group of Hurst pointed toward an active process changing the cell configuration of the producer (Hurst & Kruse, 1970). This process was initiated with the onset of nisin production, since the producer was sensitive to nisin when active nisin was not yet produced (Hurst & Kruse, 1972). There are indications that there is a relation between the expression of the *nisA* gene and the level of immunity (Rauch *et al.*, 1991). It was found that the immunity to nisin of the non-nisin producing *L. lactis* subsp. *lactis* NIZO R5 mutant NIZO R520, in which the small *nisA* transcript (see section 3) was not detected, was strongly reduced. A *L. lactis* subsp. *lactis* NIZO R5 mutant (NIZO R512) producing a low amount of nisin appeared to have an intermediate level of immunity. These results were confirmed by recent experiments that show similar effects in strains producing mutant nisins (Kuipers, O.P. *et al.*, unpublished data). Probably, the production of (pre)nisin is required for a high level of nisin immunity, e.g. directly as an immunity protein or indirectly by interaction with an immunity protein or as an effector in a regulation pathway.

The genes encoding the *E. coli* colicins and their respective immunity proteins are always in close proximity to each other (see e.g. Mankovich *et al.*, 1986). For the lactococcins produced by certain *L. lactis* strains it has been shown that the genes for production of and immunity to the bacteriocin constitute operons (Van Belkum *et al.*, 1991; Van Belkum, 1992*a*,*b*). For the lantibiotic Pep5 an open reading frame (ORF) has been identified that may encode an immunity protein (Reis & Sahl, 1991). This ORF is located immediately upstream from the Pep5 structural gene *pepA* and could encode a 69-aa polypeptide. Secondary structure predictions have indicated that the protein might be membrane-associated. No proteins with amino acid sequence similarity were found in the protein data bases. Experiments with deletion mutants suggest that immunity to Pep5 requires expression of both the putative immunity gene and the *pepA* gene. Although no comparable ORFs have been found thus far in other lantibiotic gene clusters, the dependency of immunity to the lantibiotic on the expression of the lantibiotic structural gene is analogous for Pep5 and nisin.

In view of the importance of immunity in the production of known nisin species and engineered mutants, it is essential that studies directed toward elucidation of the immunity mechanism be conducted in the near future.

3. The nisin gene cluster

3.1 Structural genes for nisin A and nisin Z

The isolation and sequence determination of the structural gene for nisin A was first reported by Buchman *et al.* (1988). Three other groups independently isolated the structural gene for nisin A (now generally designated *nisA*; De Vos *et al.*, 1991) from different nisin-producing *L. lactis* subsp. *lactis* strains (Kaletta & Entian, 1989; Dodd *et al.*, 1990; Rauch & De Vos, 1992*a*). All groups employed a similar strategy, using oligonucleotide probes based on the proposed structure of the nisin precursor (Ingram, 1970) to screen genomic libraries or isolated DNA of different nisin-producing *L. lactis* subsp. *lactis* strains. Only one nucleotide difference was encountered when the sequences of the different *nisA* genes were compared (a silent mutation in *L. lactis* subsp. *lactis* 6F3 [Kaletta & Entian, 1989]). The gene for nisin Z was also isolated and sequenced (Mulders *et al.*, 1991). The His27Asn substitution in nisin Z with respect to nisin A was accounted for by a C to A transversion in the nucleotide sequence. Nisin Z was found to be widely distributed: 14 out of 26 *L. lactis* subsp. *lactis* strains analyzed for the presence of either gene contained the *nisZ* gene (De Vos *et al.*, 1992), while the existence of another *L. lactis* subsp. *lactis* strain containing the *nisZ* gene has been independently reported (Graeffe *et al.*, 1991).

The nisin precursor derived from the *nisA* gene nucleotide sequence is a 57-aa peptide consisting of a 23-aa leader peptide segment and a C-terminal 34-aa peptide segment (Fig. 2) that is identical to the precursor proposed by Ingram (1970). This demonstrates that mature nisin is indeed synthesized by modification of a ribosomally synthesized precursor molecule. The ORF is preceded by a consensus lactococcal ribosome binding site (De Vos, 1987). The genes for several other type A lantibiotic precursors have also been cloned and sequenced. These are the genes for the precursors of epidermin (Schnell *et al.*, 1988), subtilin (Banerjee & Hansen, 1988), gallidermin (Schnell *et al.*, 1989), Pep5 (Kellner *et al.*, 1989) and lacticin 481 (Piard *et al.*, 1992*a*). A comparison of all precursors deduced from the nucleotide sequences, except that of lacticin 481, was carried out by Jung (1991*a*,*b*). The prediction profiles of the secondary structure, flexibility, hydropathy and hydrophilicity, as well as the helical-wheel plots of the precursors showed a number of similarities. All leader sequences are predicted to be very hydrophilic and strongly charged and they possibly fold into an amphiphilic α-helix in a lipophilic environment. In contrast,

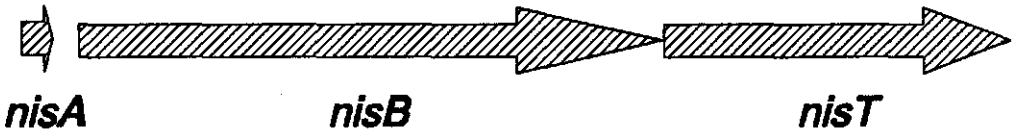

FIG. 3. Open reading frames identified thus far in the nisin gene cluster of *L. lactis* subsp. *lactis* NIZO R5 (Kuipers, O.P. *et al.*, unpublished data).

the prolantibiotic sequences are more lipophilic and their secondary structures show a preference for ß-turns and are predicted to lack α-helices. Also, the processing sites for cleavage of the leader sequence, which are located in a predicted well-accesible, hydrophilic turn, are conserved (Jung, 1991*a*,*b*). The structural conservations in the leader sequences, together with the fact that the leader sequences do not show the characteristics of prokaryotic signal peptides of secreted proteins (Von Heijne, 1985), point toward a role for the leader sequence in lantibiotic biosynthesis and/or secretion through a dedicated system (see also section 3.2).

3.2 Organization of the nisin A gene cluster

Examination of the nucleotide sequences of the regions upstream and downstream from the *nisA* gene published thus far revealed the presence of a complete ORF and the start of a third one downstream of the *nisA* gene (Buchman *et al.*, 1988; Dodd *et al.*, 1990; Rauch *et al.*, 1990; Horn *et al.*, 1991; Steen *et al.*, 1991; Rauch & De Vos, 1992*a*).

The *nisB* ORF found downstream from the *nisA* gene in *L. lactis* subsp. *lactis* ATCC 11454 (Steen *et al.*, 1991) is preceded by a potential ribosome binding site and could encode a 851-aa protein. This deduced protein has been predicted to contain many amphipathic α-helices and a C-terminal transmembrane helix. It has been reported to show homology to some membrane-associated proteins from chloroplasts (Steen *et al.*, 1991). However, nucleotide sequence data obtained in our laboratory (Kuipers, O.P. *et al.*, unpublished data) from the region downstream from the *nisA* gene (Fig. 3) show that the corresponding ORF in *L. lactis* subsp. *lactis* NIZO R5 is longer than that reported previously (Steen *et al.*, 1991). The *nisB* ORF from *L. lactis* subsp. *lactis* NIZO R5 encodes a protein with homology to the proteins encoded by the *spaB* and *epiB*

ORFs found in the subtilin gene cluster (Klein *et al.*, 1992) and epidermin gene cluster (Schnell *et al.*, 1992), respectively. The proteins that could be derived from the *spaB* and *epiB* ORFs showed no sequence similarity to already known proteins (Klein *et al.*, 1992; Schnell *et al.*, 1992), while their involvement in lantibiotic biosynthesis has been established by inactivation and complementation studies (Augustin *et al.*, 1992; Klein *et al.*, 1992). This lack of similarity to known proteins is not unexpected, since lantibiotic biosynthesis involves modification reactions performed by still unknown enzymes. In *L. lactis* subsp. *lactis* NIZO R5, the *nisB* ORF is immediately followed by another ORF, *nisT* (Kuipers, O.P. *et al.*, unpublished data; Fig. 3), which shows homology to the multi-drug-resistance (MDR)-type family of translocator proteins (Higgins *et al.*, 1986). Similar ORFs have been found in the subtilin and epidermin gene clusters (Chung *et al.*, 1992; Klein *et al.*, 1992; Schnell *et al.*, 1992). It is not clear whether the ORF in the subtilin gene cluster that corresponds to *nisT* is essential for subtilin production: Klein *et al.*(1992) reported that mutants with a disrupted ORF still produced active subtilin, although the viability of the cells was affected, possibly by the accumulation of intracellular subtilin. However, Chung *et al.* (1992) found that disruption of the ORF resulted in the loss of the ability to produce active subtilin.

It has been shown that approximately 8 kb of genetic information, containing at least six genes, is necessary to produce active epidermin in the heterologous host *S. carnosus* (Augustin *et al.*, 1992): the epidermin structural gene *epiA*, three ORFs (*epiB*, *epiC* and *epiD*) showing no homology to known proteins and two ORFs (*epiP* and *epiQ*) that, on the basis of similarity to known proteins, have been proposed to encode the leader peptidase and a regulator protein (Schnell *et al.*, 1992). The present sequence data show that all of the genes identified thus far in the subtilin and nisin gene clusters have counterparts in the epidermin gene cluster. Therefore, it may be anticipated that the gene clusters for nisin and other lantibiotics have similar sizes and numbers of genes.

3.3 Transcription of the nisin gene cluster

Although we now know that the genes involved in lantibiotic biosynthesis are arranged in clusters, it is still not clear whether these clusters constitute operons. In the case of epidermin and subtilin there are however indications that the lantibiotic genes are coordinately transcribed (Augustin *et al.*, 1992; Klein *et al.*, 1992; Schnell *et al.*, 1992).

Biologically active nisin appears in the extracellular medium of cells that are in the late logarithmic growth phase and continues to be produced during stationary phase (Hurst & Paterson, 1971; Buchman *et al.*, 1988), although low amounts of nisin may be detected in the early logarithmic phase (Hurst & Paterson, 1971). A *nisA*-specific transcript, with a size of approximately 265 nucleotides and a half-life of 7-10 min., is already present in the early logarithmic phase (Buchman *et al.*, 1988). Similar results were found by De Vuyst and Vandamme (1992), who proposed that the late appearance of active nisin is the result of the delayed expression of the maturation enzymes. This would mean that the transcription of the *nisA* gene and of the genes for the biosynthetic enzymes are regulated independently. Support for this hypothesis awaits further transcriptional studies on the nisin biosynthesis genes.

Buchman *et al.* (1988) have determined the 5'- and 3'-ends of the *nisA*-specific transcript in *L. lactis* subsp. *lactis* ATCC 11454. They found the 5'-end to be "ragged", located at two different positions. They also could not find an identifiable promoter nor a rho-independent terminator upstream from the *nisA* gene. These data were taken to indicate the processing of the small transcript from a larger polycistronic messenger (Buchman *et al.*, 1988). Subsequent primer extension studies by Steen *et al.* (1991) indicated that this polycistronic mRNA was transcribed from a promoter that is located over 4 kb upstream from the *nisA* gene in *L. lactis* subsp. *lactis* ATCC 11454. Primer extension mapping of the 5'-end of the *nisA*-specific mRNA of *L. lactis* subsp. *lactis* NIZO R5 (Kuipers, O.P., unpublished data) revealed a single position of the 5'-end corresponding to the G residue exactly between the two 5'-end nucleotides proposed by Buchman *et al.* (1988). This start site of the *nisA*-specific mRNA is preceded in the DNA by a sequence (CTGATT-N_{20}-TACAAT) that only partly resembles the consensus *L. lactis* promoter sequence (TTGACA-N_{17}-TATAAT; De Vos, 1987; Van der Vossen *et al.*, 1987). Deviation from consensus promoter sequences is not unusual among regulated genes.

Analyses of the genetic element carrying the *nisA* gene in different strains (Horn *et al.*, 1991; Gireesh *et al.*, 1992; Rauch & De Vos, 1992*a*; see section 4.2) argue strongly against the possibility that a polycistronic mRNA starts more than 4 kb upstream from the *nisA* gene, as proposed by Steen *et al.* (1991).

Transcript mapping using S1 nuclease showed that the *nisA* mRNA at its 3'-end is followed in the DNA sequence by an inverted repeat (Buchman *et al.*, 1988). This inverted repeat is not followed by the stretch of T's that is characteristic of a rho-independent terminator (Rosenberg &

Court, 1979). Therefore, the 3'-terminus of the *nisA* transcript has been proposed to be either the result of termination by a rho-dependent process, or processing of a larger transcript (Buchman *et al.*, 1988). No such inverted repeat was found upstream from the *nisA* gene, which would be in agreement with a transcription start site in the region immediately upstream from the *nisA* gene.

The *nisB* gene immediately follows the inverted repeat downstream from the *nisA* gene. It is not preceded by an obvious promoter sequence, which suggests that transcription of *nisB* is the result of read-through from the *nisA* gene. According to Steen *et al.* (1991), in *L. lactis* subsp. *lactis* ATCC 11454 *nisB* is followed by an inverted repeat and a stretch of T-residues that is characteristic of a rho-independent terminator (Rosenberg & Court, 1979). The start of the ORF downstream from *nisB* is preceded by a putative ribosome binding site and by potential promoter sequences in *L. lactis* subsp. *lactis* ATCC 11454 (Steen *et al.*, 1991). In *L. lactis* subsp. *lactis* NIZO R5 the *nisT* gene is not separated from the *nisB* gene by a terminator-like sequence nor preceded by potential promoter sequences (Kuipers, O.P. *et al.*, unpublished data).

Northern blot analyses and transcript mapping of *nisB* and of other distal genes will be needed to clarify the possible heterogeneity of the transcription of nisin gene clusters and to study their regulation.

4. The nisin-sucrose conjugative transposon

4.1 Description of nisin-sucrose conjugative elements

The first investigations concerned with the genetics of nisin biosynthesis were carried out by Kozak *et al.* (1974). They found that stable nisin-negative clones spontaneously occurred in populations of some nisin-producing strains. More importantly, they also found that the frequency of stable loss of nisin production could be elevated by applying plasmid-curing techniques. This genetic instability of nisin production was also inferred from curing experiments in other laboratories (LeBlanc *et al.*, 1980; Gasson, 1984; Gonzalez & Kunka, 1985; Steele & McKay, 1986; Tsai & Sandine, 1987).

The linkage of nisin production to sucrose fermenting ability was already suggested four decades ago when it was found that twelve nisin-producing strains tested were all able to ferment sucrose (Hirsch & Grinsted, 1951). Meanwhile, it has been shown that sucrose-proficient *L. lactis* subsp.

lactis takes up sucrose, with concomitant phosphorylation, *via* the phosphoenolpyruvate-dependent phosphotransferase system and that three sucrose-specific proteins are induced when cells are grown on sucrose: a sucrose-specific uptake protein Enzyme II, a sucrose-6-phosphate hydrolase and a fructokinase (Thompson & Chassy, 1981; Thompson *et al.*, 1991). The sucrose operon encoding these proteins has been partly characterized (Rauch & De Vos, 1992*b*). There were two strong indications for the genetic linkage of nisin production and sucrose fermenting ability. Firstly, in the curing experiments, loss of the ability to ferment sucrose was always accompanied by the loss of nisin production (LeBlanc *et al.*, 1980; Gasson, 1984; Gonzalez & Kunka, 1985; Steele & McKay, 1986; Tsai & Sandine, 1987). Secondly, the ability to ferment sucrose and nisin production could be transferred together in a conjugation-like process from a number of nisin-producing, sucrose-fermenting *L. lactis* subsp. *lactis* strains to several *L. lactis* subsp. *lactis* strains, including var. *diacetylactis* (Gasson, 1984; Gonzalez & Kunka, 1985; Steele & McKay, 1986; Broadbent & Kondo, 1991; Rauch & De Vos, 1992*a*), and to a number of *L. lactis* subsp. *cremoris* strains (Steele & McKay, 1986; Broadbent & Kondo, 1991; Rauch, P.J.G., unpublished data). Tsai and Sandine (1987) described the conjugal transfer of nisin production and sucrose fermentation to *Leuconostoc dextranicum*, but it was later reported that those results could not be reproduced (Sandine, W.E., communication at the Second Symposium on Lactic Acid Bacteria, Wageningen, The Netherlands, 22-25 September 1987).

These curing and conjugation experiments provided evidence for the existence of nisin-sucrose conjugative elements. In contrast, they did not yield conclusive information on the nature of these elements. In all cases possible nisin-sucrose plasmids were identified: a 29 MDa plasmid in *L. lactis* subsp. *lactis* ATCC 11454 (LeBlanc *et al.*, 1980; Gonzalez & Kunka, 1985; Steele & McKay, 1986), a 30 MDa unstable plasmid in transconjugants derived from several wild-type nisin-producers (Gasson, 1984), and a 17.5 MDa plasmid in *L. lactis* subsp. *lactis* 7962 (Tsai & Sandine, 1987). However, these plasmids were never established to carry the genetic information for nisin production or sucrose fermentation by subsequent transfer or gene probing studies. In addition, plasmid-free strains have been found that produce nisin (Fuchs *et al.*, 1975; Davey & Pearce, 1982). Finally, nothing is known about the effect of techniques that induce plasmid loss, such as the elevation of growth temperature, on chromosomally located genetic elements.

Steele and McKay (1986) thoroughly studied the conjugation process. Transduction and transformation were ruled out as modes of genetic transfer and it was shown that the genetic

element was capable of conjugal transfer to a recipient deficient in host-mediated homologous recombination (Rec$^-$). The latter finding pointed toward a non-chromosomal origin of the element, unless a different recombination system was used. It was not possible to transfer the capacity to produce nisin and ferment sucrose by transformation with plasmid DNA from the donor *L. lactis* subsp. *lactis* ATCC 11454. However, the nisin-sucrose element exhibited bilateral plasmid incompatibility with certain lactose plasmids. Steele and McKay (1986) interpreted their results as indicative of a plasmid nature of the nisin-sucrose genetic element, although they did not rule out the involvement of an element exhibiting both plasmid and transposon characteristics.

Curing and conjugation studies have also demonstrated the presence of genetic information for reduced bacteriophage sensitivity (Gonzalez & Kunka, 1985; Murphy *et al.*, 1988; Gireesh *et al.*, 1992; Rauch, P.J.G., unpublished data) and N^5-(carboxyethyl)ornithine (N^5-CEO) synthase (Donkersloot & Thompson, 1990) on the nisin-sucrose element. Preliminary hybridization studies were reported suggesting DNA sequence homology between the reduced bacteriophage sensitivity determinant of the nisin-sucrose element of *L. lactis* subsp. *lactis* ATCC 11454 and the reduced bacteriophage sensitivity determinant from the *L. lactis* subsp. *cremoris* UC653 plasmid pCI750 (Steele *et al.*, 1989). The physical linkage of genes involved in sucrose fermentation and N^5-CEO synthase production was confirmed by oligonucleotide hybridization studies (Thompson *et al.*, 1991). However, the gene for N^5-CEO synthase appeared not to be present on all nisin-sucrose elements (Thompson *et al.*, 1991). This is in line with the existence of natural nisin-sucrose elements with different architectures (Rauch *et al.*, 1991; see section 4.2).

4.2 Identification of nisin-sucrose transposons

With the use of DNA probes, a new strategy was adopted to locate and isolate the nisin gene locus (Buchman *et al.*, 1988; Kaletta & Entian, 1989; Dodd *et al.*, 1990, Rauch & De Vos, 1990,1992*a*). While this led to the cloning and analysis of the *nisA* gene and its surrounding region, as described in section 3, it was also the approach that ultimately led to the identification of the nisin-sucrose genetic element.

The study of Buchman *et al.* (1988), although valuable for the gaining of insight into nisin biosynthesis, did not yield any information about the nature of the nisin-sucrose genetic element, since the *nisA* gene was detected in a total genomic library of *L. lactis* subsp. *lactis* ATCC 11454.

Kaletta and Entian (1989) found their oligonucleotide probes for the *nisA* gene to hybridize only to restricted plasmid DNA of *L. lactis* subsp. *lactis* 6F3 (the undigested plasmid DNA sample was nearly immobile on agarose gels) and not to digested chromosomal DNA of this strain. Dodd *et al.* (1990) however found a strong indication for the chromosomal location of the *nisA* gene in a nisin-producing transconjugant (FI5876), derived from a mating between *L. lactis* subsp. *lactis* NCFB 894 and MG1614. They found that the *nisA* gene was preceded in this strain by an IS element, termed IS*904*. In *L. lactis* subsp. *lactis* NIZO R5, an IS element was found at the same location (Rauch *et al.*, 1990) that was almost identical to IS*904* and hence has been designated iso-IS*904* (Rauch & De Vos, 1992*a*). Part of such an IS element at a similar position had already been found in *L. lactis* subsp. *lactis* ATCC 11454 (Buchman *et al.*, 1988). Dodd *et al.* (1990) showed by hybridization that in a nisin-producing transconjugant the chromosome of the plasmid-free recipient *L. lactis* subsp. *lactis* MG1614 had gained a segment of DNA that contained the IS*904* copy and the *nisA* gene. Also, IS*904* was located at, or close to, the terminus of the sequences acquired by the transconjugant. This terminus has been designated the left end of the nisin-sucrose element. A chromosomal location of the nisin-sucrose elements in *L. lactis* subsp. *lactis* strains K1 and ATCC 11454, although without extensive examination, was also postulated (Donkersloot & Thompson, 1990; Thompson *et al.*, 1991).

Pulsed Field Gel Electrophoresis (PFGE) experiments have provided definite proof for a chromosomal location of the *nisA* gene in a wild-type strain (Hansen, 1990; Steen *et al.*, 1991), in transconjugants (Horn *et al.*, 1991) and in wild-type strains and their transconjugants (Rauch & De Vos, 1990,1992*a*; Gireesh *et al.*, 1992). It was shown that the natural nisin-producing strains *L. lactis* subsp. *lactis* ATCC 11454 (Hansen, 1990; Steen *et al.*, 1991; Gireesh *et al.*, 1992) and *L. lactis* subsp. *lactis* NIZO R5 (Rauch & De Vos, 1990,1992*a*) contained the *nisA* gene and the gene for sucrose-6-phosphate hydrolase *sacA* (in the case of NIZO R5) on large restriction fragments. After transfer of the nisin-sucrose elements to different recipients (Rauch & De Vos, 1990,1992*a*; Horn *et al.*, 1991; Gireesh *et al.*, 1992), the size of certain restriction fragments was enlarged in transconjugants when compared to the recipients. Hybridization experiments showed that this was the result of the insertion of the nisin-sucrose elements into the recipient chromosome. The estimated size of the nisin-sucrose elements derived from these experiments is 68-70 kb.

The nisin-sucrose element from *L. lactis* subsp. *lactis* NIZO R5 integrates into at least five different sites on the chromosome of *L. lactis* subsp. *lactis* MG1614 (Rauch & De Vos, 1992*a*),

although there is a strong preference for a certain site (termed site 1 or insertion hot spot): in all transconjugants studied the element had inserted into this site and insertion into secondary sites was only found in transconjugants containing multiple copies of the element. Transconjugants containing multiple copies of the nisin-sucrose element from *L. lactis* subsp. *lactis* ATCC 11454 have also been observed (Gireesh *et al.*, 1992). This element was found to move to other sites in the chromosome upon transconjugant subculturing. For the nisin-sucrose element from *L. lactis* subsp. *lactis* NIZO R5, the only instability phenomenon observed is its loss from transconjugants containing multiple copies, resulting in single-copy-containing transconjugants (Rauch, P.J.G., unpublished data). Integration of nisin-sucrose elements into different sites, but not in multiple copies, was also reported by Horn *et al.* (1991).

A chromosomal location of the *nisA* gene (Rauch & De Vos, 1990,1992*a*; Horn *et al.*, 1991; Steen *et al.*, 1991; Gireesh *et al.*, 1992) and of the gene for sucrose-6-phosphate hydrolase *sacA* (Rauch & De Vos, 1992*a,b*) left open two possibilities for the nature of the nisin-sucrose genetic elements: they could be plasmids that preferably integrate into the chromosome (episomes) or transposons. There are two known mechanisms for integration of plasmids (episomes) into the chromosome. One mechanism is host-mediated homologous recombination between sites on the plasmid and on the chromosome. It appears that this is also the mechanism by which the conjugative transposon Tn*916* integrates into the chromosome of *L. lactis* subsp. *lactis* MG1363 during intraspecific conjugation (Bringel *et al.*, 1991). The other possible mode of plasmid integration is a mechanism, in which site-specific recombination occurs between attachment (*att*) sites on the plasmid and on the chromosome, mediated by plasmid-encoded proteins. This mechanism is used by several plasmids from actinomycetes (see e.g. Brown *et al.*, 1990). Several observations argue against an integrative plasmid nature of the nisin-sucrose element and are in favor of a transposon nature. Firstly, transfer of nisin-sucrose elements has been shown to be independent of host-mediated homologous recombination (Steele & McKay, 1986; Rauch & De Vos, 1992*a*). In addition, as mentioned above, nisin-sucrose elements integrate into a number of sites on the recipient chromosome. Both mechanisms of plasmid integration would result in insertion at a single site or at a very limited number of sites, while integration at several sites by definition is a characteristic of transposons (Campbell *et al.*, 1979). Further evidence came from the comparison of the nucleotide sequences of the left and right junctions of the element in the *L. lactis* subsp. *lactis* MG1614 insertion hot spot to the nucleotide sequence of this site before

insertion of the element (Fig. 4; Horn *et al.*, 1991; Rauch & De Vos, 1992*a*). The TTTTTG hexanucleotide found in the insertion hot spot prior to integration and found flanking the nisin-sucrose element after integration is too short to be used in host-mediated homologous recombination or to be considered a plasmid attachment site. Finally, the observation that no hybridization signals have been obtained with element-specific probes in total DNA separated by PFGE (Hansen, 1990; Rauch & De Vos, 1990,1992*a*; Horn *et al.*, 1991; Gireesh *et al.*, 1992), rules out the existence of replicative, episomal intermediates.

The analyses of the ends of the nisin-sucrose elements also questioned the *nisA* transcription data reported by Steen *et al.* (1991) for *L. lactis* subsp. *lactis* ATCC 11454. A mRNA starting more than 4.5 kb upstream from the *nisA* gene would start from outside the nisin-sucrose element, making expression of nisin production dependent on the location of the element in the genome. Furthermore, with the exception of the putative transposase ORFs of IS*904*/iso-IS*904* (see section 4.3), no ORFs are present upstream from the *nisA* gene in the region sequenced thus far. Possibly, this region has a completely different organization in *L. lactis* subsp. *lactis* ATCC 11454 than the nisin-sucrose elements described by Horn *et al.* (1991) and Rauch & De Vos (1992*a*). This would however be in contradiction with the fact that the *nisA* gene has been found to be also located close to one end of the nisin-sucrose element of *L. lactis* subsp. *lactis* ATCC 11454 (Gireesh *et al.*, 1992).

It has been shown that the transfer of nisin-sucrose elements is insensitive to DNase (Gonzalez & Kunka, 1985; Steele & McKay, 1986; Rauch & De Vos, 1992*a*), does not involve a transducing phage (Gonzalez & Kunka, 1985; Steele & McKay, 1986) and can occur in the absence of any plasmid DNA (Steele & McKay, 1986; Rauch & De Vos, 1992*a*). Together with the results described above this shows that the nisin-sucrose elements conform 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 (Campbell *et al.*, 1979; Clewell & Gawron-Burke, 1986). The *L. lactis* subsp. *lactis* NIZO R5 transposon has been designated Tn*5276* (Rauch *et al.*, 1990; Rauch & De Vos, 1992*a*) and the transposon described in *L. lactis* subsp. *lactis* FI5876, Tn*5301* (Horn *et al.*, 1991). The nisin-sucrose elements in *L. lactis* subsp. *lactis* K1 and ATCC 11454, although at that time not characterized as transposons, have been designated Tn*5306* and Tn*5307*, respectively (Thompson *et al.*, 1991).

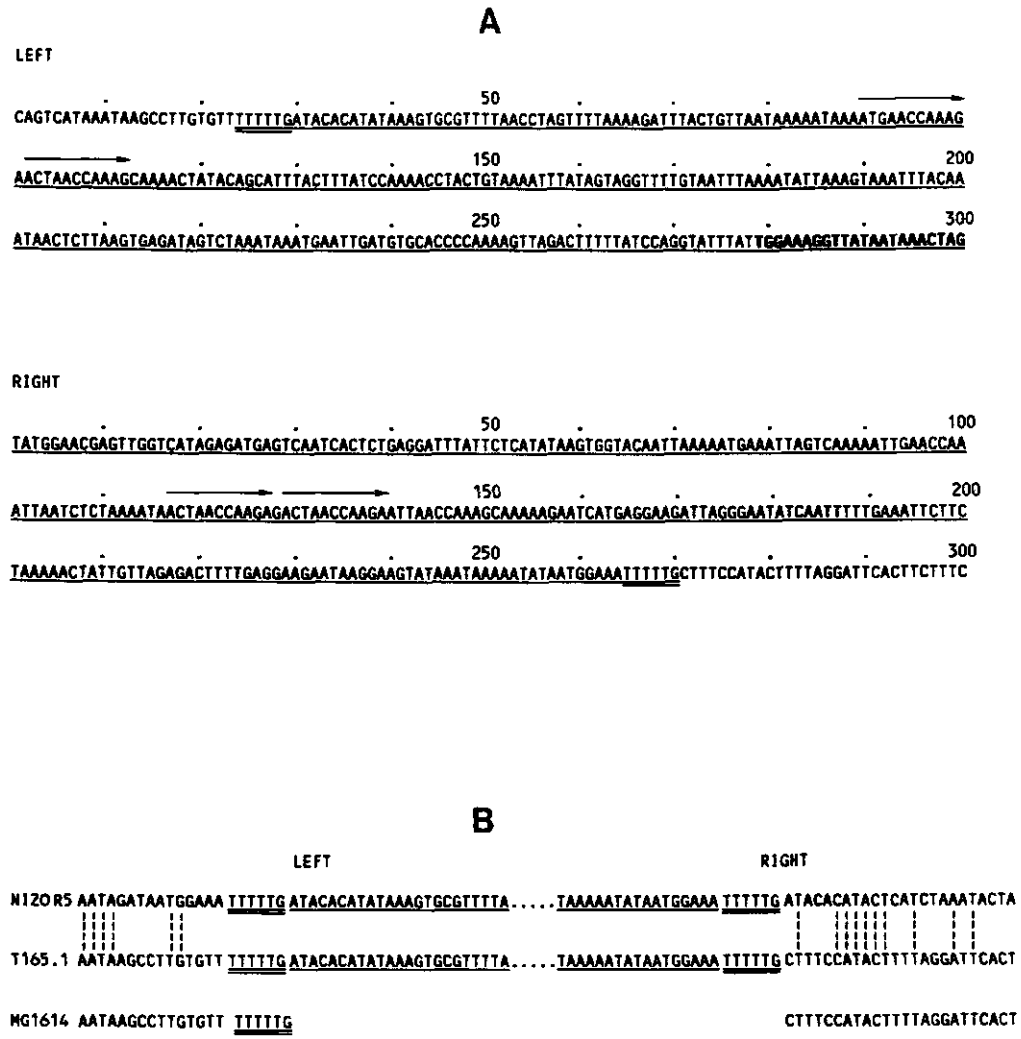

FIG. 4. Nucleotide sequences of the termini of the nisin-sucrose element from *L. lactis* subsp. *lactis* NIZO R5 and junction regions in the donor and transconjugants in comparison with that of the insertion hot spot (taken from Rauch & De Vos, 1992*a*). **A:** Nucleotide sequence of the left and right termini of the nisin-sucrose element from *L. lactis* subsp. *lactis* NIZO R5 inserted into the insertion hot spot of *L. lactis* subsp. *lactis* MG1614. Element-specific sequences are underlined. The hexanucleotide direct repeat TTTTTG is underlined twice. Perfect and imperfect 17-bp direct repeats present in the left and right ends, respectively, are represented by arrows. The first 22 nucleotides of iso-IS*904* (Rauch *et al.*, 1990) are indicated in bold face. **B:** Comparison of element junctions in NIZO R5 and a transconjugant (T165.1) hot spot with that of the hot spot in *L. lactis* subsp. *lactis* MG1614. See (A), for indications of specific sequences. Sequence identity between the regions flanking the element in the different strains is indicated (|).

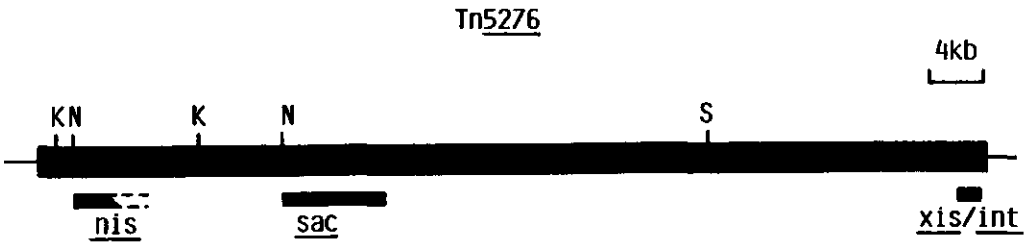

FIG. 5. Physical and genetic map of Tn5276. Tn*5276* is represented by a black bar. Smaller bars indicate the positions of the nisin gene cluster (*nis*), the sucrose regulon (*sac*) and the transposition genes (*xis int*). Restriction enzyme abbreviations: K, *Kpn*I; N, *Nci*I; S, *Sst*II.

The spontaneous and induced loss of nisin production by nisin-producing strains described in early studies possibly is a result of excision and loss of the transposon. Such spontaneous loss has also been described for the conjugative transposon Tn*916* from *Enterococcus faecalis* (Gawron-Burke & Clewell, 1982). Low-frequency excision of Tn*916* in a circular form has been proposed to be part of its mechanism of conjugative transfer (Gawron-Burke & Clewell, 1982; see also section 4.3). The incompatibility phenomenon described by Steele and McKay (1986) cannot be easily explained. It has been suggested that the element combines transposon and plasmid features (Steele and McKay, 1986). One could think of a mechanism that is similar to that described for the *Streptomyces coelicolor* element SLP1^{int}, where a genetic locus (*imp* for *i*nhibitor of *m*aintenance of SLP1 *p*lasmids) inhibits maintenance of the element as an extrachromosomal replicon but also the maintenance of SLP1-derived plasmids (Grant *et al.*, 1989). Alternatively, the transposon could contain plasmid-derived replication regions that are inactive but still show incompatibility.

4.3 Characterization of the nisin-sucrose transposon Tn*5276* and its transposition mechanism

The ends of Tn*5276* (Rauch & De Vos, 1992*a*) and Tn*5301* (Horn *et al.*, 1991) are identical for over 250 bp and since Tn*5276* has been studied most extensively, we will focus on this element. A physical map of Tn*5276* has been constructed on which the nisin and sucrose gene clusters have been located (Fig. 5; Rauch & De Vos, 1992*a*). A physical map of the *L. lactis* subsp. *lactis*

ATCC 11454 nisin-sucrose element has also been published (Gireesh *et al.*, 1992). The maps of the two elements appears to be identical for the restriction enzymes used in both studies. However, the map of the *L. lactis* subsp. *lactis* ATCC 11454 element clearly differs from the physical map of this region published by Steen *et al.* (1992). A possible movement of the element to another location in the chromosome in the Prt$^-$ derivative of *L. lactis* subsp. *lactis* ATCC 11454 used by Gireesh *et al.* (1992) cannot completely account for this difference. The mapping of the nisin and sucrose gene clusters within the left one third of Tn*5276* (Rauch & De Vos, 1992*a*) has left approximately 50 kb for the genes encoding its remaining established properties: conjugal transfer, transposition (excision and insertion) and reduced phage sensitivity. Thompson *et al.* (1991) have located the gene for N^5-CEO synthase (*ceo*) within 7 kb from the sucrose genes on Tn*5306*.

The *nisA* gene is preceded by almost identical copies of an IS element (IS*904*) in several strains. IS*904* belongs to the IS*3* family of insertion sequences (see Dodd *et al.* (1990), Rauch *et al.* (1990) and Prère *et al.* (1990) for recent compilations). From the location of IS*904*, at or close to the left end of the nisin-sucrose element, Dodd *et al.* (1990) initially inferred a role for the IS element in transposition of the nisin-sucrose element. Indeed, the presence of the putative transposase ORF upstream from the *nisA* gene has tempted more researchers to speculate on a role for it in transposition of the nisin-sucrose element (Buchman *et al.*, 1988; Thompson *et al.*, 1991). However, the possibility that this IS element is part of a composite transposon was excluded by the later finding that it is separated from the left end of the transposon by approximately 250 bp (Horn *et al.*, 1991; Rauch & De Vos, 1992*a*) and by the fact that a second IS*904* copy could not be found near the right end of Tn*5301* (Horn *et al.*, 1991) and not at all in Tn*5276* (Rauch & De Vos, 1992*a*). A comparative study of a large number of the left end regions of nisin-sucrose elements in our laboratory (Rauch *et al.*, 1991) has excluded the role of a IS*904* copy at the position upstream from *nisA* in transposition since nisin-sucrose elements were found in several wild-type strains that are capable of conjugative transfer while not containing an IS*904* copy upstream from *nisA*. This study also showed that the presence of a sequence identical or similar to the left end of Tn*5276* upstream from the *nisA* gene was essential for conjugative transposition. Rearrangements involving homologous recombination between copies of IS*904* and resulting in the disappearance of the left end from its original location are possibly responsible for rendering some nisin-sucrose elements non-transposable, thereby fixing them at the position taken in the last transposition event (Rauch *et al.*, 1991).

When trying to elucidate the mechanism of excision and insertion of a transposon, a first step is to analyze the nucleotide sequence of its ends. Like the ends of the known conjugative transposons Tn*916* (Clewell *et al.*, 1988) and Tn*1545* (Caillaud & Courvalin, 1987), the extremities of Tn*5276* are AT-rich and contain several direct repeats (Fig. 4; Rauch & De Vos, 1992*a*). In contrast to the ends of Tn*916* and Tn*1545*, which contain homologous, inverted repeats, the termini of Tn*5276* are asymmetric. The ends do not show any similarity to the ends of known transposons, although they share their asymmetry with the ends of Tn*554* from *Staphylococcus aureus* (Murphy *et al.*, 1985).

When examining the ends of Tn*5276* in different transconjugants, small sequence diversities in the junction regions were found (Rauch, P.J.G., unpublished results) reminiscent of the variability of the ends of Tn*916* and Tn*1545* generated by their unique transposition mechanism (Caparon & Scott, 1989; Poyart-Salmeron *et al.*, 1990). This pointed toward a similar mechanism of transposition for Tn*5276* and Tn*916/1545*. Since in the latter transposons the genes for the transposition proteins are present near one end of the element, corresponding genes were looked for near the right end of Tn*5276*. Indeed, two ORFs were found the deduced proteins of which, in analogy to related systems termed Xis and Int, showed homology in relevant parts to the transposition proteins described for Tn*916* and Tn*1545* (Fig. 5; Rauch, P.J.G., unpublished results). Both ORFs have been shown to be functional genes in *E. coli* and a covalently closed circular form of Tn*5276*, which probably serves as a transposition intermediate, has been detected *in vivo* in *L. lactis* (Rauch, P.J.G., unpublished results). Thus, while the nature of the nisin-sucrose genetic element remained unknown for decades, recent progress has shown that this element is the first conjugative transposon described in *L. lactis*, which uses a mechanism of transposition that is similar to that of the Tn*916* family.

5. Protein engineering of nisin

Major goals in nisin research are the elucidation of the mechanism of biosynthesis of this peptide and the functional role and structure of the uncommon amino acid residues and the improvement of the antimicrobial and physico-chemical properties of nisin. Insight in these topics is required to allow for tailoring nisin for specific uses. One might think of nisin species with a higher antimicrobial activity against food pathogens such as certain *Listeria* and *Clostridium* strains or of

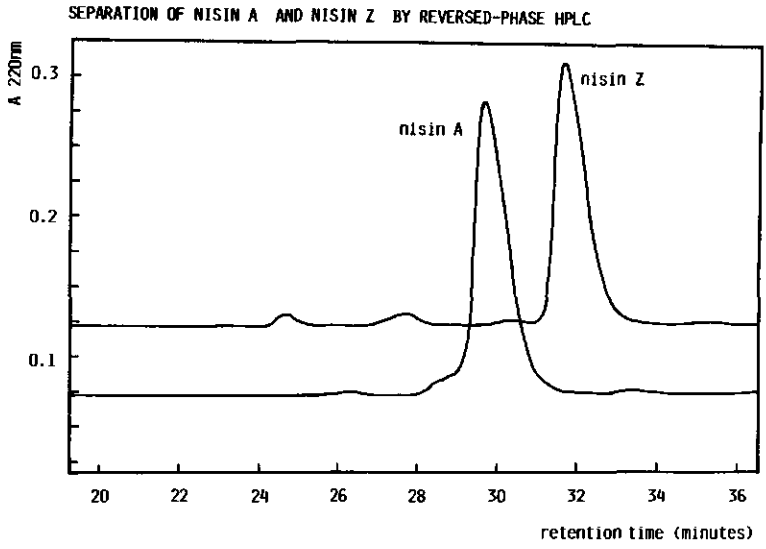

FIG. 6. Separation of nisin A and nisin Z by reversed-phase HPLC.

nisins with increased solubility and stability at elevated pH values that enlarge the possibilities for applications.

One of the most powerful approaches for the study of the topics mentioned is the use of protein engineering. Protein engineering of nisin and other lantibiotics enables the study of features in the primary translation product that are required for the cascade of post-translational modification reactions and subsequent translocation and cleaving processes. Moreover, it enables the tailoring of nisin to specific applications.

5.1 Development of an expression system for nisin genes

To be able to apply the protein engineering strategy in the case of nisin it is a necessity to develop expression systems for structural (mutated) nisin genes. Systems for (over)expression of the *nisA* gene have recently been developed in *E.coli* and resulted in the production of inactive prenisin (Mulders, J.W.M. *et al.*, unpublished observations; De Vuyst *et al.*, 1992). To produce the fully matured nisin species, the presence is required of genes encoding the necessary proteins for biosynthesis, immunity and processing. It has now been shown for epidermin biosynthesis that at

least six genes present in a putative operon structure, comprising about 6.5 kb, are required for these processes (Augustin *et al.*, 1992; Schnell *et al.*, 1992). For nisin biosynthesis it was shown that a 9.0-kb *Pst*I fragment, including the *nisA* gene and several downstream ORFs, was able to complement the non-nisin-producing transconjugant *L. lactis* subsp. *lactis* NIZO T520 (Kuipers *et al.*, 1991*b*). The most straightforward way, however, to express *nis* genes was to use a plasmid-free nisin A-producing *L. lactis* subsp. *lactis* strain that was transformed with a small multi-copy plasmid containing the *L. lactis lac* promoter followed by the *nisZ* gene (Kuipers *et al.*, 1991*b*). The engineered lactococci were found to secrete both nisin A and nisin Z, which could be fully separated by reversed-phase HPLC (Fig. 6). By using this system and a PCR-based mutagenesis approach (Kuipers et al., 1991*a*) several *nisZ* genes that were altered by site-directed mutagenesis, could be expressed and subsequently the maturated products were purified and characterized.

5.2 First engineered nisin species

One of the most intriguing features of nisin and other lantibiotics is the presence of dehydrated and lanthionine residues. Some of the unique properties of nisin have been attributed to these features. It has been found for instance that dehydrated residues *in vitro* can react with sulfhydryl containing reagents and it has been postulated that *in vivo* they might react with the sulfhydryl groups of membrane proteins of germinated bacterial spores (Morris *et al.*, 1984; Buchman and Hansen, 1987). This would point to a direct functional role of dehydrated residues in the mechanism of nisin action. Moreover, it has been shown by several groups studying lantibiotic degradation products (Chan *et al.*, 1989; Hansen *et al.*, 1991; Rollema *et al.*, 1991), or chemically synthesized fragments (Wakamiya *et al.*, 1991) that residue Dha-5 in nisin and subtilin is critical for antimicrobial activity. In contrast, residue Dha-33 in nisin has been shown not to be essential for activity, since the degradation products [Ser^{33}]nisin A and (1-32)nisin A both retain almost full antimicrobial activities (Rollema *et al.*, 1991; Chan *et al.*, 1992; Lian *et al.*, 1992). By making synthetic nisin A analogues it has been shown that Dhb-2 is also important for the maintenance of antimicrobial activity (Wakamiya *et al.*, 1991).

To study the role of residue Dha-5 in more detail, the effect of a mutation at this position was investigated recently. By site-directed mutagenesis a Thr-codon was introduced in the *nisZ* gene instead of the Ser-codon at position 5 (Kuipers *et al.*, 1992), to see whether dehydration would take

place and, if so, what would be the effect on further biosynthesis and on antimicrobial activity. NMR studies showed that the Thr present at position 5 in nisin Z was fully converted to Dhb, since no nisin Z species could be found that still contained the unmodified Thr residue. The [Dhb-5]nisin Z displayed a two- to ten-fold lower bacteriocidal activity than nisin Z on the indicator organisms *Micrococcus flavus*, *Streptococcus salivarius* subsp. *thermophilus* and *Bacillus cereus* (Kuipers *et al.*, 1992). This shows that a dehydrated residue at position 5 indeed contributes to antimicrobial activity, since in nisin degradation products or synthetic analogues in which residue 5 was saturated the activity was decreased at least fifty-fold (Chan *et al.*, 1989; Rollema *et al*, 1991; Wakamiya *et al.*, 1991; Lian *et al.*, 1992). It can not be ruled out that the unsaturated residue at position 5 is directly involved in the bacteriocidal mechanism of nisin and subtilin by reacting with free sulfhydryl groups of cell-wall proteins, since the less reactive Dhb residue would then indeed be expected to cause lower antagonistic activity. The Ala-5 substitution in nisin Z affected the biosynthesis of the mutant, because no nisin Z species containing this mutation could be isolated from the supernatant of the expression strain (Kuipers *et al.*, 1992). Possibly, mutant precursor nisin Z in some stage of modification accumulates in the cell and further modification is hampered. It can be postulated that a double bond between Cα and Cß at position 5 induces a conformation that is required to allow lanthionine formation between residues 3 and 7.

In another study using site-directed mutagenesis, it was attempted to introduce the third 3-methyl-lanthionine ring of subtilin (Gross, 1975) into nisin Z to investigate the possibility and effect of introducing a fourth dehydrated residue in nisin Z (Kuipers *et al.*, 1991*b*; Kuipers *et al.*, 1992). For this purpose, the codons for Met-17 and Gly-18 in *nisZ* were replaced by codons for Gln-17 and Thr-18. Following expression of the mutated gene in *L. lactis* subsp. *lactis*, it was found during RP-HPLC purification that two new species were present in the supernatant, both displaying antimicrobial activities. Characterization of these two species by ^{1}H-NMR spectroscopy showed that the main mutant produced (~ 90%) contained a Dhb residue at position 18, whereas a smaller amount (~ 10%) of mutant nisin contained a Thr residue at this position. This result demonstrates that, although some of the mutant nisin molecules escape dehydration at position 18, this does not prevent their further maturation and secretion. Although nisin A-producing *L. lactis* subsp. *lactis* strains are sensitive to subtilin, the introduction of the third ring of subtilin into nisin Z obviously does not inhibit the producer strain. The antimicrobial activity of subtilin against nisin-producing *L. lactis* subsp. *lactis* strains must therefore reside in some other part of subtilin. The Dhb-18-

containing species showed similar activities as nisin Z towards three different indicator strains (Kuipers *et al.*, 1992), indicating that an additional dehydrated residue at position 18 in nisin Z does not enhance or reduce antimicrobial activity. On the other hand the nisin mutant containing Thr-18 (and Gln-17) was about fourfold less active against two of the indicator strains and twice as active against *Micrococcus flavus* than wild-type nisin Z. The change in relative sensitivities of these strains to the Thr-18 containing mutant could be due to either differences in membrane phospholipid composition of the three indicator strains, or to different membrane potentials, which is known to affect the pore forming capacity of nisin (Gao *et al.*, 1991). These results show that it is possible to overproduce wild-type and mutant nisins in *L. lactis* subsp. *lactis*, to introduce or exchange dehydrated residues in nisin by protein engineering and to construct modified nisins with altered antimicrobial activity and host selectivity.

Other mutations that can be considered in nisin Z are the introduction of polar and charged residues at different positions in an attempt to increase the solubility at neutral and higher pH values. Furthermore, the introduction of a fluorescent probe, i.e. a Trp-residue, at different positions in nisin Z, will enable the study of the behaviour of nisin interacting with phospholipids at the lipid-water interface. It might also be possible to engineer additional lanthionines in nisin or, in contrast, to remove one or more lanthionines from nisin, to gain insight in the biosynthetic requirements and to enlarge the understanding of structure-function relationships in lantibiotics. Moreover, protein engineering of nisin offers the perspective of introducing uncommon amino acid residues in other lantibiotics than nisin and possibly even in unrelated peptides. The purpose of this would be either to introduce desired properties in these molecules, such as increased stability by substituting disulphide bridges for lanthionines in a known peptide, or to create completely new peptides, which might have desirable properties for application in the agro-food or pharmaceutical industry.

6. Concluding remarks

The nisin-sucrose elements have proven to be very intriguing genetic elements: large conjugative transposons that do not confer antibiotic resistances, but encode seemingly non-related properties like nisin production, sucrose fermentation, reduced bacteriophage sensitivity and in some cases the production of unusual amino acids. Despite these unique properties, Tn*5276* has been shown

to encode proteins belonging to a group of enzymes (Argos *et al.*, 1986) involved in the excision and integration of several classes of mobile genetic elements. These elements range from integrative plasmids (found in actinomycetes; Brown *et al.*, 1990), to transposons (e.g. Tn*554*; Murphy *et al.*, 1985), conjugative transposons and bacteriophages (including bacteriophage lambda; Hoess *et al.*, 1980). Campbell and Botstein (1983) have speculated that bacteriophages like lambda evolved by the joining together of different modules, DNA fragments encoding specific functions. This would be an attractive hypothesis for the origin of Tn*5276* and other nisin-sucrose elements.

Among the challenges for future nisin research (De Vos, 1992) is the elucidation of the biosynthesis at the molecular level. Investigations of the genes involved in the biosynthetic processes is already being carried out in several laboratories and is expected to yield insights in the requirements for and regulation of nisin biosynthesis. Overexpression of biosynthetic genes in *E. coli*, *L. lactis* and other hosts, and subsequent purification of the gene products, will enable the biochemical characterization of proteins and enzymes involved in biosynthesis. These proteins may include dehydratases, translocator proteins, immunity proteins, regulatory proteins, leader proteases and possibly enzymes involved in lanthionine formation. These proteins might find application also outside the field of lantibiotic biosynthesis, e.g. for increasing the stability of other proteins and peptides by introducing uncommon amino acids, or for the development of dedicated secretion systems for homologous and heterologous proteins.

Protein engineering can be used for the study of biosynthetic processes (e.g. mutations in the leader peptide, mutations affecting dehydrated residues and lanthionine-forming residues), and, in addition, for the improvement of certain properties of nisin, such as chemical stability and solubility at neutral pH values. Moreover, it can be attempted to improve the antimcrobial activity and specificity of nisin by random and site-directed mutagenesis. For the latter purpose a better understanding of the mode of action of nisin in relation to the properties of target organisms is crucial.

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CHAPTER 3

CLONING AND CHARACTERIZATION OF THE STRUCTURAL GENE FOR NISIN A, *NISA*, AND IS*1068* FROM *LACTOCOCCUS LACTIS* NIZO R5

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Abstract The structural gene for the precursor of nisin A, *nisA*, of *Lactococcus lactis* NIZO R5 was detected in a λEMBL3 library of total DNA in *Escherichia coli*. The *nisA* gene was subcloned and its nucleotide sequence was determined. The nisin A precursor deduced from the *nisA* gene nucleotide sequence is a 57-amino acid peptide consisting of a 23-amino acid leader peptide segment and a C-terminal 34-amino acids peptide segment. This demonstrates that mature nisin A is synthesized in *L. lactis* NIZO R5 by modification of a ribosomally synthesized precursor molecule. Upstream from the *nisA* gene a region was found that exhibited all the characteristics of a member of the IS*3* group of insertion sequences. Three copies of this element, termed IS*1068*, or strongly related elements are present in the genome of *L. lactis* NIZO R5. The organization and coding capacity of IS*1068* and other members of the IS*3* family are discussed.

INTRODUCTION

Nisin, produced by some *Lactococcus lactis* strains, is the most prominent member of the group of bacteriocins called lantibiotics (36); some of the other members are subtilin from *Bacillus subtilis* (14) and epidermin from *Staphylococcus epidermis* (1). Lantibiotics are ribosomally synthesized by gram-positive bacteria and contain the thioether amino acids lanthionine and 3-methyllanthionine (see Fig. 1 in Chapter 2). Ingram (17, 18) proposed that the formation of these lanthionine rings in nisin A (15) occurs through dehydration of serine and threonine residues followed by sulphide ring formation between the resulting dehydroalanine and dehydrobutyrine residues and appropriately located cysteine residues. On the basis of these mechanisms, the structure of the ribosomally synthesized nisin A precursor was proposed (18).

The cloning of the gene encoding the nisin A precursor could be the first step toward the localization and identification of the genetic element encoding and transferring nisin A production. Furthermore, it was expected that the genes encoding the proteins involved in nisin A biosynthesis and immunity would be clustered near the gene for the nisin A precursor. Thus, cloning of the gene for the nisin A precursor (or nisin A structural gene) was considered to be essential for the elucidation of nisin biosynthesis and nisin protein engineering. Using a 'reversed genetics' approach, the nisin A structural gene (*nisA*) was detected in a gene library of *L. lactis* subsp. *lactis* strain NIZO R5, a nisin A-producing and sucrose-fermenting wild-type strain from the strain collection of the Netherlands Institute for Dairy Research (NIZO). *L. lactis* NIZO R5 had been isolated from raw milk and used for evaluating the potential of nisin in industrial cheese making

(11). A similar approach has been used by others to clone the nisin genes of several other nisin A-producing *L. lactis* strains (3, 5, 20). The *nisA* gene of *L. lactis* NIZO R5 has been used to isolate the *nisZ* gene encoding a variant nisin, nisin Z, which differs from nisin A in a single amino acid position (27). Next to the cloning and characterization of the *L. lactis* NIZO R5 *nisA* gene, the identification and characterization of a copy of an iso-IS*904* element (5) is described in this chapter. This IS element, designated IS*1068*, was found to preceed the *nisA* gene of *L. lactis* NIZO R5. IS*1068* belongs to the IS*3* group of insertion sequences, a family of IS elements that is widely spread in both gram-positive and gram-negative bacterial genera.

MATERIALS AND METHODS

Strains, plasmids, and bacteriophages. The nisin A-producing and non-nisin-producing *L. lactis* strains used were NIZO R5 (11) and MG1614 (12), respectively. *Escherichia coli* strains TG1 (13) and MB406 (obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden) were used as hosts for M13mp18 and M13mp19 (47) and bacteriophage λEMBL3 (9), respectively.

Growth and culture conditions. *E. coli* strains were grown in L broth-based medium as described previously (34). *L. lactis* strains were grown at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5% sucrose (NIZO R5) or glucose (MG1614).

DNA manipulations and genomic library construction. Plasmid and bacteriophage DNAs were isolated from *E. coli* cells or lysates essentially by using established protocols (34). For the isolation of plasmid DNA from *L. lactis* NIZO R5 the methods of Anderson and McKay (2) and Vos *et al.* (45) were used. Total DNA was isolated from *L. lactis* strains 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 (45) followed by phenol-chloroform extraction and ethanol precipitation. DNA fragments were recovered from agarose gels with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). Standard cloning procedures were used throughout (34). The library of *L. lactis* NIZO R5 DNA was prepared in λEMBL3 by using the Packagene Lambda DNA packaging system (Pharmacia LKB). The NIZO R5 DNA cloned was a 15- to 25-kb fraction of partially *Sau*3A-digested total DNA recovered from an agarose gel.

Agarose gel electrophoresis, DNA transfer, and hybridization. Agarose gel electrophoresis was carried out using established protocols (34). GeneScreen Plus nylon membranes (Du Pont,

NEN Research Products, Wilmington, Del.) were used in all DNA transfers. Transfer, hybridization, and washing conditions were as recommended by the membrane manufacturer.

DNA sequencing. Nucleotide sequences were determined by using the dideoxy-chain termination method (35) adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) on fragments cloned into M13mp18 and M13mp19. The universal M13 primer and specifically synthesized primers were used and the sequence of both strands was determined. The sequence data were assembled with the use of the PC/Gene program version 5.01 (Genofit, Geneva, Switzerland).

RESULTS AND DISCUSSION

Cloning and nucleotide sequence analysis of the *nisA* gene. A region of low codon degeneracy (MGCNMKT, extending from position 17 to 23) in the proposed nisin precursor sequence (18) was chosen and, based on *L. lactis* codon usage (calculated from a compilation of all known *L. lactis* nucleotide sequences; W. M. de Vos, unpublished data), an oligonucleotide was designed and synthesized to be used in DNA hybridizations to detect the *nisA* gene. This oligonucleotide (5'-ATGGGTTGTAATATGAAAAC) was found to yield single hybridizing bands in restriction digests of total *L. lactis* NIZO R5 DNA at a maximum temperature of 45°C. At this temperature, the oligonucleotide neither hybridized to the five plasmids present in *L. lactis* NIZO R5 nor to total DNA from the non-nisin-producing laboratory strain *L. lactis* MG1614 (12; data not shown). The hybridization of the *nisA* probe to total but not plasmid DNA isolated from *L. lactis* NIZO R5 indicated that the genetic element containing the *nisA* gene was located in the *L. lactis* NIZO R5 chromosome or was an extrachromosomal element that could not be isolated in conventional plasmid isolation procedures. A *L. lactis* NIZO R5 genomic library in λEMBL3 was screened by hybridization at 45°C to the *nisA* probe. One of 16 hybridizing clones (λNZ700) was characterized in detail and was found to carry a 20-kb insert (data not shown). A 1.7-kb *Sau*3A fragment hybridizing to the oligonucleotide probe was subcloned into M13mp18 and mp19 and the nucleotide sequence of the *nisA* gene of *L. lactis* NIZO R5 was determined. The nucleotide sequence (Fig. 1) contained an open reading frame (ORF) of which the 32-amino acids C-terminal part was identical to the nisin precursor postulated by Ingram (18). This demonstrates that mature nisin is indeed synthesized in *L. lactis* NIZO R5 by modification of a ribosomally synthesized

precursor molecule. The ORF is preceded by a consensus lactococcal ribosome binding site (4) and by a sequence (CTGATT-N_{20}-TACAAT) that partly resembles the consensus *L. lactis* promoter sequence (TTGACA-N_{17}-TATAAT [4, 43]). The deviation from the consensus promoter sequence could point toward regulated transcription. The sequence of the *nisA* gene of *L. lactis* NIZO R5 was found to be identical to those published just previously (3) or later (5, 20), except for a silent mutation in the *nisA* gene of *L. lactis* 6F3 (20). For further results of the analysis of this region in different *L. lactis* strains the reader is referred to Chapter 2.

```
                                    -35
          .         .         .    ------    .         .         -
TTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTCTGAAGTTTGTTAGAT   60

 -10
------    .         .         .          ******    .         .
ACAATGATTTCGTTCGAAGGAACTACAAAATAAATTATAAGGAGGCACTCAAAATGAGTA  120
                                                 nisA → M  S      2

          .         .         .         .         .         .
CAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCAC  180
T  K  D  F  N  L  D  L  V  S  V  S  K  K  D  S  G  A  S  P     22

          .         .         .         .         .         .
GCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATGGGTTGTA  240
R  I  T  S  I  S  L  C  T  P  G  C  K  T  G  A  L  M  G  C     42

 T        .         .         .         .         .         .
ACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAACCAAATCAAAGGA  300
N  M  K  T  A  T  C  H  C  S  I  H  V  S  K  -                57
```

FIG. 1. Nucleotide sequence of the *nisA* gene of *L. lactis* NIZO R5. The deduced amino acid sequence of the nisin precursor is given below the nucleotide sequence; the amino acids of the leader sequence are in italics and residues that are modified in mature nisin are in bold face. In the nucleotide sequence, a potential promoter is overlined and a potential ribosome binding site is indicated by stars over the residues that are complementary to the 3' end of *L. lactis* 16S rRNA (23). The nucleotides in bold face represent the oligonucleotide used to detect the *nisA* gene.

Nucleotide sequence analysis of IS*1068*. Because the presence of an incomplete ORF upstream from the *nisA* gene of *L. lactis* ATCC 11454 had been reported (3), we determined the nucleotide sequence of part of the region upstream from the *nisA* gene of *L. lactis* NIZO R5 (Fig. 2). This sequence was found to contain a region that exhibited characteristics of an IS element. The element is 1245 basepairs (bp) long. Its termini are 39-bp imperfect inverted repeats. A 4-bp direct target

repeat (TTAT) was found. The element contains two large open reading frames of 96 (ORFA) and 299 (ORFB) amino acids. The NIZO R5 IS-like element differs in 8 bp from the 1241-bp IS*904* found upstream from the *nisA* gene of *L. lactis* strain NCFB894 (5). Therefore, we initially referred to the *L. lactis* NIZO R5 IS-like element as an (iso-)IS*904* element (31). However, in view of the differences in coding capacity to IS*904* (see below) the IS-like element found upstream from the *nisA* gene of *L. lactis* NIZO R5 has now been designated IS*1068* (registered with the Plasmid Reference Centre [22]). Recently a *L. lactis* IS element (IS*1076*) similar to IS*904* (24 scattered mismatches) and IS*1068* (22 scattered mismatches) has been described (16). The nucleotide sequence of IS*1076* gives rise to one ORF comprising both IS*1068* ORFs.

Hybridization analyses using IS*1068*-specific oligonucleotides have shown that the genomes of *L. lactis* strains NIZO R5 and MG1614 contain three and seven copies, respectively, of IS*1068* or very related IS elements (data not shown; see Chapter 8 for more data on the presence of IS*1068* in the genomes of *L. lactis* strains).

IS*1068* belongs to the IS*3* family of insertion sequences. On the basis of homology between ORFs and inverted repeats (Fig. 3) IS*904*, IS*1068*, and IS*1076* belong to the IS*3* family of insertion sequences. This family further includes IS*2* (32), IS*3* (41), IS*150* (37), and IS*3411* (19) from *E. coli*, IS*600* (25) and IS*911* (29) from *Shigella dysenteriae*, IS*629* (26) from *S. sonnei*, IS*51* (46) from *Pseudomonas savastanoi*, IS*136* (or IS*426*; 42) from *Agrobacterium tumefaciens*, ISL*1* (39) from *Lactobacillus casei*, IS*476* (21) from *Xanthomonas campestris* pv. *vesicatoria*, IS*861* (33) from group B *Streptococcus* type III, IS*6110* (40) from *Mycobacterium tuberculosis*, and ISR*1* (30) from *Rhizobium* class IV.

At least ten members of the IS*3* family have been found to carry two major ORFs: a small upstream frame (ORFA) and a longer downstream frame (ORFB) shifted 1 nucleotide to the 5' end with respect to ORFA (-1 frameshift [8, 29]). In comparisons of the IS*3* group members, both ORFAs and ORFBs were found to show significant similarities to each other. However, ORFB was found to be the better conserved of the two, especially in its carboxy-terminal region (8, 29). All ORFAs were found to contain a sequence (29) that could potentially form a helix-turn-helix DNA-binding motif with significantly high scores calculated with the weight matrix of Dodd and Egan (5). ORFA of IS*1068* also contains such a motif (Fig. 2); its calculated score is 1196, while the scores reported for other ORFAs (29) range from 782 (IS*861*) to 1961 (ISR*1*). This segment is

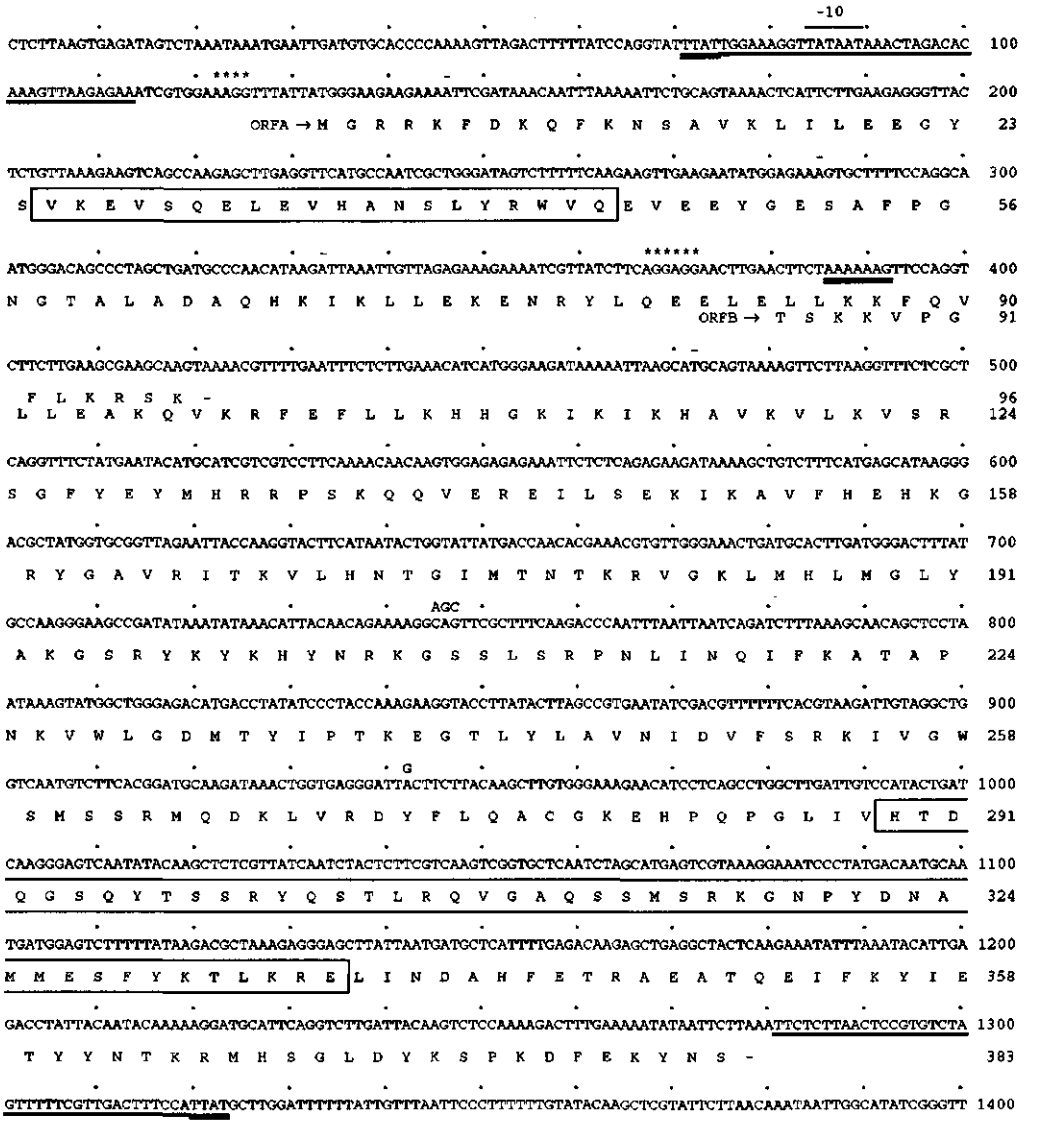

FIG. 2. Nucleotide sequence of IS*1068*. The amino acid sequences deduced from ORFs A and B are given below the nucleotide sequence. The stretches of residues that correspond to the helix-turn-helix motif detected in the ORFAs of IS*3* group members (29) and the domain in ORFB that shows similarity to retroviral integrases (8) are boxed. Amino acid numbering follows ORFAB as predicted by using the results obtained with IS*911* (28). In the nucleotide sequence, potential ribosome binding sites are denoted by stars over the bases that are complementary to the 3' end of *L. lactis* 16S rRNA (23). The 32-bp imperfect repeat is underlined. The 4-bp target repeat and the frameshift motif (29) are doubly underlined. Nucleotide sequence differences to IS*904* (5) are indicated above the nucleotide sequence (-: nucleotide deletion). A possible -10 sequence is overlined.

A:

```
IS629       HSDKGSQYVSLAYTQRLKEAGLLASTGSTGDSYDNAMAESINGLYKAK
ISL1        HSDRGSQYTAKEVTKLVNQYHWQRSFSALGKPGDNAWSESFFAIMKKE
IS1068      HTDQGSQYTSSRYQSTLRQVGAQSSMSRKGNPYDNAMMESFYKTLKRE

Consensus HSDNGSQY-S--Y---L-E-GIR-SMSA-GNSYDNA--ESFFG-LK-E
           T Q GEF A  F     Q  LK AVGS  DPWQ G   T W  VR
              T   T        D      LT        S
```

B:

```
IS150       L  TGTACTGCACCCATTTTGTTGGACG
            R  TGTACTGACCCCAAAAAGTTGGACA
IS3411      L  TGAACCGCCCCGGGAATCCTGGAGA
            R  TGAACCGCCCCGGGTTTCCTGGAGA
IS911       L  TGAAGTGGTCAACAAAAACTGGCCA
            R  TGAAGTGGCACACTGAATTTGGCCA
IS600       L  TGAGGTAGCCTGAGTTTAACGGACA
            R  TGAGGTGTACTGGCAATAGCGGACA
IS476       L  TGACCTGCCCCCACTGAGCCGTACC
            R  TGACCTGCCCCCATCGT-CCGTACC
IS861       L  TGAACTGCACCCCAAAAGTTAGACA
            R  TGTACTGACCCCCAAAAGTTGGACA
IS629       L  TGAACCGCCCCGGGAATCCTGGAGA
            R  TGAACCGCCCCGGGTTTCCTGGAGA
IS51        L  TGAACCGCCCCGGGTTTCTCGGAGA
            R  TGAACCGCCCCGGGTTTCTCGGAGA
IS6110      L  TGAACCGCCCCGGCATGTCCGGAGA
            R  TGAACCGCCCCGGTGAGTCCGGAGA
ISL1        L  tGGGTCTGTACTAGAATTTCGGACA
            R  tGGGAAGTCAACACTTTTTCGGACA
IS1068      L  TGGAAAGGTTATAATAAACTAGACA
            R  TGGAAAGTCAACGA-AAACTAGACA

Consensus   TGRRYYR-YYY--------YRGACA
                                  g
```

```
IS2         L  TGGATTTGCCCC-T-ATATT-TC-CAGACA
            R  TAGACTGGCCCCCTGA-AT-CTC-CAGACA
IS3         L  TGATCTTACCCAGC-AATAG----TGGACA
            R  TGATCCTACCCACGTAATA-----TGGACA
IS426       L  tgaACTGCCCCCCATTTCGAC---CGGACA
(=IS136)    R  tggAGTGCACCCCATTTC-AC---CGGACA
ISR1        L  TGACGTGACCCCCTGAAACTCCTCCAGGAA
            R  TGACGTGACCCCC-GTTTCTCATCCAGCCA

Consensus   TGRRYYR-YYY----------YRGACA
```

FIG. 3. The IS*3* group of insertion sequences. **A:** Alignment of the most highly conserved C-terminal segments of several ORFBs. An alignment of the complete ORFBs of all other group members has been published (8). The segment shown is similar to a domain conserved in retroviral integrases (8). The consensus sequence has been deduced from a compilation of ten IS*3* group members (8). Amino acid residues fitting the consensus sequence are in bold face. **B:** Alignment of terminal inverted repeats. The consensus sequences (differing in gap length) were designed to fit the majority of inverted repeats of the IS*3* group members. Nucleotides corresponding to the consensus sequences are shown in bold face. The small case nucleotides in the ISL*1* and IS*426* sequences were reported not to be part of the inverted repeats (39, 42), but have been added here to maximize similarity to the consensus.

thought to be involved in the sequence-specific binding to the terminal inverted repeats of the IS element (29). A highly conserved region of the ORFBs was also found to exhibit a significant similarity to the integrase domain of retroviruses (8; Fig. 3). Additional characteristics that members of the IS*3* group were found to share with retroviruses are the sequences of the ends (the important conserved terminal dinucleotides 5'-TG...CA-3' [28]) and the presence of a motif in the overlap region of ORFA and ORFB that possibly promotes translational frameshifting (29). For

IS*911* it has been shown that translational frameshifting at this motif results in the production of an ORFAB fusion protein in a T7 expression system (28), while the production of IS*911* ORFA and ORFB products was also observed (28). *Trans*-complementation assays strongly suggested that the ORFAB fusion protein is the IS*911* transposase (28). Production of a transposase fusion protein has also been observed for IS*150* (44), and for the unrelated IS*1* (7, 24, 38) and it may be a relatively common way of controlling transposition activity. In IS*1068*, ORFB is potentially coupled to ORFA by a -1 frameshift and ORFA contains a helix-turn-helix motif at a similar position as in other ORFAs (Fig. 2; 29). Furthermore, the nucleotide sequence around the potential frameshift point contains the frameshift-promoting motif mentioned above (Fig. 2). ORFA is present in IS*1068*, but absent in IS*904* due to mutations at positions 146 and 285 (Fig. 2), which may have rendered IS*904* inactive. Regulation of transposition of IS*1076* (16) apparently does not take place *via* expression of different proteins switched by translational frameshifting as possibly is the case for other members of the IS*3* group, since this IS element contains only one large ORF.

Transcription of the IS*1068* coding regions may be initiated outside the element. No consensus lactococcal transcription initiation signals could be found at appropriate positions in the nucleotide sequence of IS*1068*, except for a consensus TATAAT box upstream from ORFA in the left inverted repeat. Possibly, transcription of the IS*1068* ORFs is initiated from outside the element. This would mean that transcription would only be possible in certain locations in which a -35 sequence would be provided at the appropriate spacing. This could be considered to function as a mechanism for modulating the IS copy number in the genome. In the case of IS*1076*R it has been proposed that a promoter for transcription of the transposase ORF was created by duplication of one terminal repeat (16). Alternatively, the sequence promoting initiation of transcription of the IS*1068* ORFs could show low similarity to consensus sequences. This would be in agreement with the low level of transcription often found for ORFs of IS elements and with the fact that consensus promoter sequences are rarely found upstream from coding regions in IS elements (10).

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CHAPTER 4

TRANSCRIPTIONAL REGULATION OF THE TN*5276*-LOCATED *LACTOCOCCUS LACTIS* SUCROSE OPERON AND CHARACTERIZATION OF THE *SACA* GENE ENCODING SUCROSE-6-PHOSPHATE HYDROLASE

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Transcriptional regulation of the Tn*5276*-located *Lactococcus lactis* sucrose operon and characterization of the *sacA* gene encoding sucrose-6-phosphate hydrolase

(Recombinant DNA; lactic acid bacteria; sucrose utilization; phosphotransferase system; transcription initiation; helix-turn-helix motif; operator)

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SUMMARY

The *Lactococcus lactis* sucrose operon was located on the conjugative transposon Tn*5276* and the nucleotide sequence of the *sacA* gene, encoding sucrose-6-phosphate hydrolase, and its surrounding regions was determined. Northern blot analysis showed that the sucrose operon contains two divergent transcriptional units of 3.2 and 3.6 kb, the expression of which is considerably higher in cells grown on sucrose than in cells grown on glucose. This was confirmed by primer extension studies which demonstrated that transcription is initiated at two sucrose-inducible promoters with a back-to-back organization. The 3.2-kb transcriptional unit includes the *sacB* gene which most probably encodes the sucrose-specific enzyme II of the phosphotransferase system, and may contain the gene encoding fructokinase. The 3.6-kb transcriptional unit includes genes *sacA* and *sacR*. The protein encoded by the *sacR* gene is likely to be involved in the regulation of the *sac* operon expression, since its deduced N terminus is homologous to helix-turn-helix DNA-binding domains found in several regulatory proteins.

INTRODUCTION

In addition to their normal growth on lactose, some *Lactococcus lactis* strains can also utilize sucrose (LeBlanc et al., 1980). This ability can be conjugally transferred to other *L. lactis* strains, together with production of and immunity to the lantibiotic nisin (Gasson, 1984). Recent investigations have shown that in *L. lactis* NIZO R5 those traits are encoded by a 70-kb conjugative transposon, designated Tn*5276* (Rauch and De Vos, 1992).

L. lactis imports sucrose by means of a phosphoenolpyruvate-dependent PTS (Thompson and Chassy, 1981). The product of this translocation is sucrose 6-phosphate, which is subsequently cleaved by a specific S6PH to glucose 6-phosphate and fructose. Fructose is then converted to fructose 6-phosphate by an ATP-dependent fructokinase. The sucrose-PTS and S6PH activities have been shown to be coordinately induced during growth on sucrose (Thompson and Chassy, 1981), and a sucrose-inducible fructokinase has recently been reported (Thompson et al., 1991b).

Sucrose PTS and hydrolase genes have been cloned from several bacteria. In the Gram$^-$ bacteria, strongly related

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Abbreviations: aa, amino acid(s); *B.*, *Bacillus*; bp, base pair(s); EII^{suc}, sucrose-specific enzyme II; kb, kilobase(s) or 1000 bp; *L.*, *Lactococcus*; oligo, oligodeoxyribonucleotide; nt, nucleotide(s); ORF, open reading frame; PTS, phosphotransferase system (see INTRODUCTION); RBS, ribosome-binding site; *S.*, *Streptococcus*; *sacA*, gene encoding S6PH; *sacB*, gene encoding the putative EII^{suc}; *sacR*, gene encoding a putative regulator of the sucrose system; S6PH, sucrose-6-phosphate hydrolase; *tsp*, transcription start point(s); *V.*, *Vibrio*.

sucrose gene clusters have been found in *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Salmonella thompson* (Cowan et al., 1991), while *Vibrio alginolyticus* appeared to contain differently organized sucrose genes (Blatch and Woods, 1991). Regulation of transcription has only been studied in the *Salmonella thompson* system (Cowan et al., 1991), although (putative) repressor genes have been identified in the above-mentioned systems (Blatch and Woods, 1991; Cowan et al., 1991). For Gram$^+$ bacteria, sucrose uptake and utilization genes have only been isolated from *Bacillus subtilis* (Fouet et al., 1986; 1987; Débarbouillé et al., 1990) and *Streptococcus mutans* (Hayakawa et al., 1986; Lunsford and Macrina, 1986; Sato and Kuramitsu, 1988; Sato et al., 1989). The regulation of the *B. subtilis* sucrose system is complex, involving two control circuits that probably act via transcription antitermination (Steinmetz et al., 1989; Débarbouillé et al., 1990; Zukowski et al., 1990). In contrast, the regulation of the *S. mutans* sucrose genes has not been extensively characterized (Sato et al., 1991).

We have studied the transcriptional organization of the Tn*5276*-located sucrose operon in *L. lactis* NIZO R5 and characterized the S6PH gene, *sacA*. The results show that this sucrose operon includes two divergent transcriptional units and that transcription initiates at two back-to-back oriented, sucrose-dependent promoters.

RESULTS AND DISCUSSION

(a) Cloning and nt sequencing of the *sacA* gene and its flanking regions

Since there is considerable aa sequence conservation between the sucrose hydrolases from different organisms (Gunasekaran et al., 1990), a DNA fragment containing the S6PH gene of *S. mutans* was used as a probe to detect the corresponding gene in *L. lactis*. A 0.8-kb *Eco*RV-*Hin*dIII fragment from pVA1343 (Lunsford and Macrina, 1986), containing the *S. mutans* S6PH gene, was hybridized (at 50°C) to Southern blots of digested total DNA from *L. lactis* strain NIZO R5 containing the nisin-sucrose transposon Tn*5276* (Rauch and De Vos, 1992). Single hybridizing bands were found only with DNA of strain NIZO R5 and not with DNA of the sucrose-deficient *L. lactis* strain MG1614 (Gasson, 1983). A hybridizing 6.6-kb *Bam*HI fragment was isolated from the chromosomal DNA of strain NIZO R5 and cloned in *Escherichia coli* JM83 (Vieira and Messing, 1982) by using the low-copy-number vector pSHG576 (Takeshita et al., 1987). This resulted in plasmid pNZ755. A physical map of the *Bam*HI fragment was constructed and, based on hybridization and preliminary sequence analysis, the putative S6PH-encoding gene was located on a *Bcl*I-*Tha*I fragment (Fig. 1). The complete sequence of 1800 nt including the *Bcl*I-*Tha*I fragment was determined on both strands by sequencing overlapping restriction fragments subcloned into M13mp18 and M13mp19 (Yanish-Perron et al., 1985), except for the region 5′ from the putative S6PH-encoding gene, which could not be stably maintained in M13-derived vectors. Therefore, the nt sequence of this region was determined using pNZ755 as a template. Similar instability was encountered when we tried to isolate clones containing the sucrose region from a λEMBL3 library (Rauch and De Vos, 1992).

The nt sequence (Fig. 2) contains an ORF that could encode a 473-aa protein. The ORF is preceded by a potential RBS (Fig. 2) with a calculated free energy (Tinoco et al., 1973) for binding to the 3′ end of *L. lactis* 16S rRNA

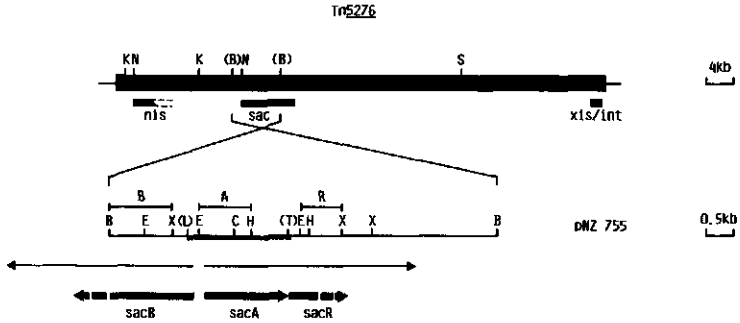

Fig. 1. Physical and genetic map of Tn*5276* and the 6.6-kb *Bam*HI fragment containing part of the sucrose genetic region of *L. lactis* cloned in pNZ755. The Tn*5276* map was taken from Rauch and De Vos (1992). The solid bars below Tn*5276* indicate the positions of the *nis* (nisin), *sac* (sucrose) and *xis/int* (transposition) gene clusters (Rauch and De Vos, 1992 and unpublished data). For convenience, the Tn*5276* and pNZ755 maps are drawn in opposite orientations. The fragments used for the Northern hybridizations are indicated above the pNZ755 map (in parentheses). The position of the sequenced 1.8-kb *Bcl*I-*Tha*I fragment is indicated in bold lettering. The thin arrows represent the location and size of the detected sucrose-specific transcripts of 3.2 kb (left) and 3.6 kb (right). The other arrows represent the sucrose-regulated genes (partly based on unpublished sequencing results). Restriction enzyme abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; L, *Bcl*I; N, *Nci*I; S, *Sst*II; T, *Tha*I; X, *Xba*I. Complete maps are shown for each contiguous DNA fragment except for the sites that are in parentheses.

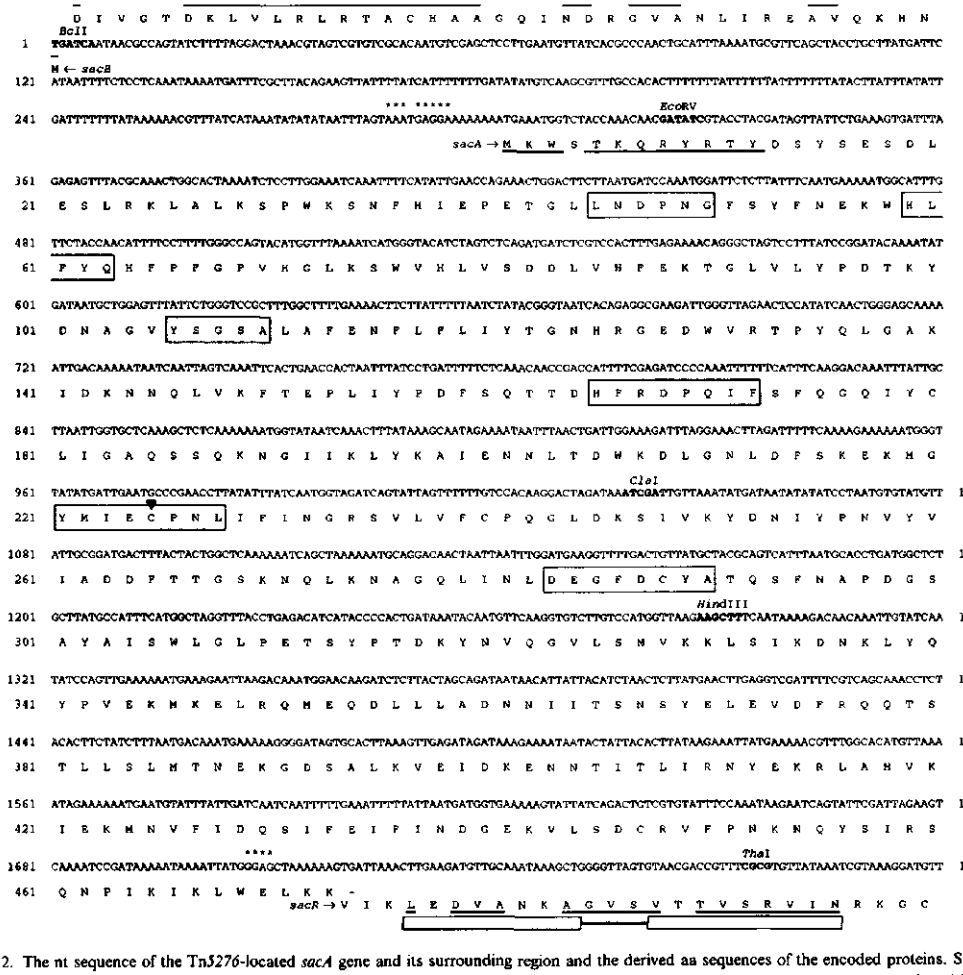

Fig. 2. The nt sequence of the Tn*5276*-located *sacA* gene and its surrounding region and the derived aa sequences of the encoded proteins. Sequencing of both strands was performed following the Sequenase protocol of Tabor and Richardson (1987) on fragments cloned into M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) with universal and insert-specific primers and using the double-stranded sequencing protocol of Hsiao (1991) on pNZ755 and with synthesized primers. The aa corresponding to the N terminus of *L. lactis* K1 S6PH (Thompson et al., 1991a) are underlined, as are the aa deduced from the start of the *sacR* gene that are identical to aa in the N terminus of the *V. alginolyticus scr* repressor (Blatch and Woods, 1991). The position of the helix-turn-helix motif is indicated by connected bars. The aa deduced from the start of the *sacB* gene that are identical to aa in the N terminus of *S. mutans* EII^{suc} (Sato et al., 1989) are overlined. The restriction sites from the map in Fig. 1 are indicated in bold face. Potential RBS are indicated by asterisks. The regions found to be conserved in seven β-fructosidases (Gunasekaran et al., 1990) are boxed. The Cys of the putative active site, as proposed by Martin et al. (1987), is marked with a black triangle. The nt sequence has been given the GenBank accession No. M96669.

(Ludwig et al., 1985) of −9.8 kcal/mol, which is in the range (−8 to −14) usually found for *L. lactis* (De Vos, 1987). The identity of the putative S6PH-encoding gene of *L. lactis* NIZO R5 was established by comparing the deduced aa sequence to the recently published N-terminal 12 aa of S6PH purified from *L. lactis* strain K1 (Thompson et al., 1991a; Fig. 2). There appeared to be only one difference between the two sequences (position 4: Q in strain K1, S in strain NIZO R5). The deduced aa sequence predicts a molecular size for S6PH of *L. lactis* strain NIZO R5 of 54624 Da, a value that is close to that estimated for S6PH of *L. lactis* strain K1 (approx. 52 kDa; Thompson et al., 1991a). The S6PH-encoding gene of *L. lactis* NIZO R5 was designated *sacA*. So far, all attempts have failed to

(over)express the *sacA* gene using several expression systems, including the T7 system (Studier et al., 1990), in order to visualize the expression product or determine the S6PH activity (Chassy and Porter, 1982).

A comparison of the deduced aa sequence of the *L. lactis* NIZO R5 S6PH to that of the *S. mutans* S6PH showed an overall aa identity of 51%, while the corresponding genes showed 57% identity in nt sequence. The S6PH enzymes belong to the group of β-fructosidases, for which an extensive homology comparison has recently been published (Fig. 2; Gunasekaran et al., 1990).

In the nt sequence 5′ and 3′ from *sacA* the starts of two ORFs were found (Fig. 2) with start codons that were preceded by potential RBS with calculated free energies for binding to *L. lactis* 16S rRNA of −11.6 and −9.4 kcal/mol, respectively. The ORF located 5′ from and in opposite orientation to *sacA* was designated *sacB* and the ORF following *sacA*, with a GTG start codon overlapping the *sacA* stop codon, was designated *sacR* (see sections b and c).

(b) Analysis of transcription of the sucrose operon and its regulation

The activities of S6PH and the sucrose PTS are strongly induced during growth on sucrose (Thompson and Chassy, 1981). To study the transcriptional organization of the *sac* genes and to analyze the control of their expression in *L. lactis*, Northern blot analysis and primer extension studies were performed using total RNA from cells grown on glucose and on sucrose.

Northern blot analysis (Fig. 3) showed higher amounts of *sacA*-, *sacB*-, and *sacR*-specific mRNA in cells grown on sucrose than in glucose-grown cells. However, the difference in mRNA-levels between cells grown on sucrose and cells grown on glucose was larger for the *sacB*- and *sacR*-specific mRNA than for the *sacA*-specific mRNA. Although some degradation of mRNAs was visible, as is often observed in bacterial systems (Newbury et al., 1987), the size of the major transcripts could be determined easily. The *sacA* probe and the *sacR* probe both hybridized to a

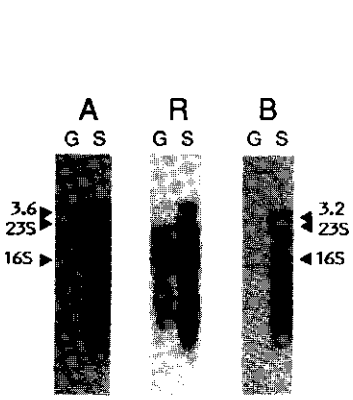

Fig. 3.

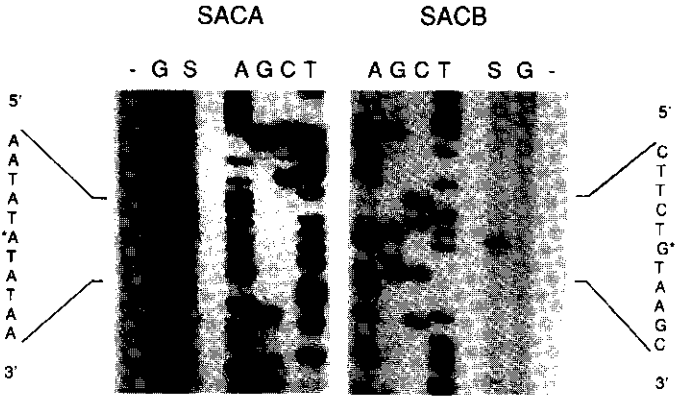

Fig. 4.

Fig. 3. Northern blot analysis of *sacA* (panel A), *sacB* (panel B) and *sacR* (panel R) gene expression in *L. lactis* NIZO R5. Cells were grown to logarithmic phase on glucose (G) or sucrose (S) and 20 μg of RNA, isolated as described (Van Rooijen and De Vos, 1990), glyoxalated and separated on a denaturing 1% agarose gel. The gel was either stained with ethidium bromide or, after blotting to a GeneScreen Plus membrane (NEN, Du Pont de Nemours), hybridized to a 1.0-kb *Eco*RV-*Hin*dIII fragment specific for *sacA* (A), a 0.6-kb *Eco*RV-*Xba*I fragment specific for *sac*R, a 1.1-kb *Bam*HI-*Xba*I fragment specific for *sacB* (B; see Fig. 1). Blotting, hybridization and washing conditions were as recommended by the membrane manufacturer. Restriction fragments were labeled by nick translation (Sambrook et al., 1989). As a size marker, the 0.24–9.5-kb RNA ladder from Gibco BRL was used. The positions of the 23S (2.8-kb) and 16S (1.5-kb) rRNAs, and the estimated sizes of the *sacA*- and *sacB*- and *sacR*-specific transcripts (3.6 and 3.2 kb, respectively) are indicated.

Fig. 4. Primer extension products of RNA transcribed from the *sacA* and *sacB* genes. Panels **SACA** and **SACB** show the primer extension products obtained for *sacA* and *sacB*, respectively. The relevant nt sequences are indicated and the determined *tsp* are marked by asterisks. Lanes: A, G, C, T, sequencing reactions; G, primer extension using RNA from cells grown on glucose; S, primer extension using RNA from cells grown on sucrose; dash, RNA from cells grown on sucrose and no primer added to the primer extension reaction mixture. Primer extension (Van Rooijen and De Vos, 1990) was carried out using 15 μg of total RNA, isolated from cells harvested in the logarithmic phase as described by Van Rooijen and De Vos (1990), and an amount of primer (5 pmol) that was in excess over the amount of specific messenger. The sequences of the oligos used are 5′–TTTCCAAGGAGATTTTAGTGCCAGTTTGCG–3′ (complementary to nt 370–399 in Fig. 2) and 5′–CGCCAGTATCTTTTAGG–3′ (nt 11–27 in Fig. 2) for *sacA* and *sacB*, respectively. Extension products were electrophoresed on a denaturing 6% polyacrylamide gel together with a sequencing reaction obtained using the same oligo primers on pNZ755 template DNA.

3.6-kb mRNA, which establishes the cotranscription of both genes. Transcription of the *sacB* gene was shown by the detection of a 3.2-kb *sacB*-specific transcript (Fig. 3).

The *tsp* of the *sacA* and *sacB* genes, as determined by primer extension mapping (Fig. 4), appeared to be located in close proximity to each other (nt 155 and nt 34, respectively; Fig. 5). For both the *sacA* and the *sacB* gene sequences corresponding to consensus *L. lactis* promoters (De Vos, 1987; Van der Vossen et al., 1987) were identified (Fig. 5). The divergent promoters are arranged back-to-back (Beck and Warren, 1988). The intergenic region has an A+T-content (82%) that is considerably higher than that of the *sacA* gene (68%). It contains an extremely A+T-rich (97%) sequence between the −35 regions of both promoters (nt 80–117; Fig. 5).

Since the primer extension experiments were carried out with excess primer (Fig. 4), the differences in transcription levels of *sacA* and *sacB* between glucose- and sucrose-grown cells should be reflected by the band intensities of the cDNA-products. Comparison of the cDNA-levels showed an increased level of transcription of the *sac* genes in cells grown on sucrose in comparison with that in cells grown on glucose and with the *sacB*-specific primer even no primer extension product was detected. These results confirm the Northern blot experiments, which indicate that the sucrose operon is controlled at the transcriptional level.

(c) Homology analysis of the *sacA*-flanking regions, including the sucrose operon promoter region

The N-terminal part of the *sacB* gene product deduced from the nt sequence was found to show homology to the *S. mutans* sucrose-specific enzyme II (Sato et al., 1989; 56% identical aa in the first 40 residues and 59% identical nt; Fig. 2). The latter protein in turn has been shown (Sato et al., 1989) to share homology with sucrose-specificenzyme II from *B. subtilis* (Fouet et al., 1987) and that encoded by the *S. typhimurium*/*E. coli* pUR400 plasmid (Ebner and Lengeler, 1988). Thus, it is likely that the *sacB* gene encodes the EII^{suc} of the *L. lactis* PTS. Considering the homology between the *L. lactis* and the *S. mutans* sucrose systems, the size of the *sacB* gene for the EII^{suc} of *L. lactis* is expected to be approx. 2 kb. Recently, Thompson et al. (1991b) have proposed that the genes for fructokinase and EII^{suc} of *L. lactis* strain K1 are located close to each other. The size of the fructokinase gene, as calculated from the molecular size of the sucrose-inducible fructokinase purified from *L. lactis* strain K1 (33 kDa; Thompson et al., 1991b), is approx. 0.9 kb. These estimated sizes of the genes for EII^{suc} and fructokinase would fit the size of the mRNA transcribed from the *sacB* promoter (approx. 3.2 kb) as a polycistronic messenger. Future gene probing and sequencing studies will have to show if indeed the *sacB* gene is followed by the gene for fructokinase.

The sequence of 82 nt determined 3′ from *sacA*, containing the start of the *sacR* gene, shows 70% identity to the sequence 3′ from the *S. mutans* S6PH-encoding gene (Sato and Kuramitsu, 1988). The N terminus deduced from the *sacR* gene shows homology (up to 50% identity in the first 27 aa) to the N-terminal parts of a group of regulatory proteins, the best known members of which are the *E. coli* GalR and LacI repressors (von Wilcken-Bergmann and Müller-Hill, 1982) and the most recently described members of which are the repressor protein of the *V. alginolyticus* sucrose utilization system (Blatch and Woods, 1991), the CcpA protein involved in catabolite repression in *B. subtilis* (Henkin et al., 1991) and the MalI repressor protein of the *E. coli* maltose regulon (Reidl et al., 1989). These proteins contain an N-terminal helix-turn-helix motif, which in a number of regulatory proteins has been shown to be involved in the binding of operator DNA (Brennan and Mat-

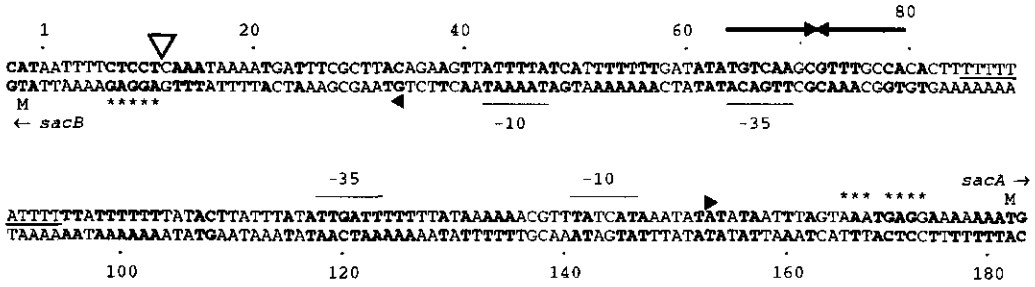

Fig. 5. The nt sequence of the *L. lactis* sucrose operon intergenic region and comparison to the corresponding *S. mutans* region (Sato and Kuramitsu, 1988). Relevant features of the *L. lactis* sequence are indicated: the RBS (asterisks), the promoter boxes (overlined), the *tsp* (black arrowheads) and the imperfect inverted repeat (facing arrows). Identical nt in the *S. mutans* sequence are in bold face. In the comparison, gaps have been introduced for optimal alignment and major gaps are shown (open triangle, 18-bp deletion in *L. lactis* sequence; underlined, 10-bp deletion in *S. mutans* sequence).

thews, 1989). Interestingly, a 16-nt sequence with an imperfect dyad symmetry was found in the promoter region of the *L. lactis* sucrose operon (Fig. 5). This sequence is related to the (putative) operators recognized by the helix-turn-helix motifs of this group of regulatory proteins (44–69% identity to five operators and two putative operators compared by Rolfes and Zalkin, 1988). Similar putative operators were found in the upstream region of the *V. alginolyticus scrR* gene (Blatch and Woods, 1991). The finding of a helix-turn-helix motif in the proposed *sacR* gene product in conjunction with a suitable putative operator in the promoter region of the sucrose operon suggests that the *sacR* gene product is involved in the regulation of the *L. lactis* sucrose system. The simultaneous transcription of the *sacA* and *sacR* genes, that is induced in sucrose-grown cells, points toward an activator function of the *sacR* gene product, which is also compatible with the position of the putative operator with respect to both the *sacA* and the *sacB* promoter (Collado-Vides et al., 1991). Future expression and binding studies are aimed at elucidating the role of the SacR protein in the transcriptional regulation of the *L. lactis* sucrose utilization system.

When comparing the nt sequences of the promoter regions of the sucrose operons of *L. lactis* and *S. mutans* (Fig. 5), regions of considerable nt similarity were found (overall 52% identical nt between the 180-bp *L. lactis* region and the 193-bp *S. mutans* region). Strikingly, these regions comprised all promoter sequences and the putative operator. Together with the high percentages of nt sequence identity found thus far between the sucrose genes of both organisms (57–70%), this suggests that both systems have a common ancestor and that the sucrose genes in *S. mutans* are similarly regulated as reported here for the *L. lactis* sucrose genes.

(d) Conclusions

(*1*) A 6.6-kb *Bam*HI-fragment containing a large part of the *sac* operon from the *L. lactis* conjugative transposon Tn*5276* has been cloned in *E. coli* and partly sequenced.

(*2*) The *L. lactis* sucrose operon includes two divergently oriented transcriptional units: one unit (3.6 kb) contains at least the *sacA* and the *sacR* genes, the latter probably involved in regulation of the system; the other unit (3.2 kb) includes the *sacB* gene, which most probably encodes the sucrose-transport protein EII^{suc}.

(*3*) Transcription of the sucrose genes is induced in cells grown on sucrose when compared with that in glucose-grown cells.

(*4*) The two sucrose-dependent promoters have been mapped and are located, in a back-to-back orientation, within a 180-nt region that also contains a region of dyad symmetry to which a regulatory protein (possibly the *sacR* gene product) could bind.

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CHAPTER 5

CHARACTERIZATION OF THE NOVEL NISIN-SUCROSE CONJUGATIVE TRANSPOSON TN*5276* AND ITS INSERTION IN *LACTOCOCCUS LACTIS*

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

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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*

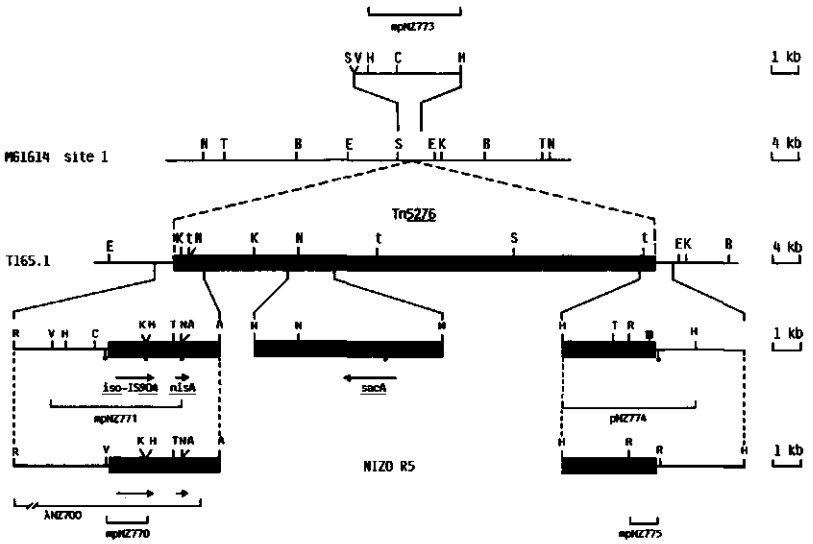

FIG. 1. Physical and genetic map of Tn*5276* in MG1614 transconjugant T165.1 and donor NIZO R5. Tn*5276* is represented by a black bar, MG1614 DNA is represented by a thin line, and NIZO R5 DNA is indicated by a thick line. Some regions are enlarged to show more detail. Arrows indicate the positions 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.

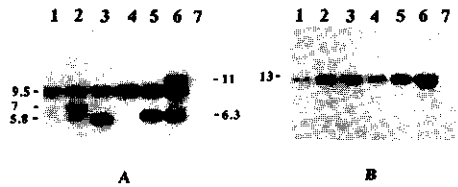

FIG. 2. Presence of *nisA*- and *sacA*-specific sequences in *L. lactis* transconjugants. Equal amounts of total DNA from six transconjugants from the mating between NIZO R5 and MG1614 and from strain MG1614 were digested with *Eco*RI and separated by agarose gel electrophoresis. A Southern blot was 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-

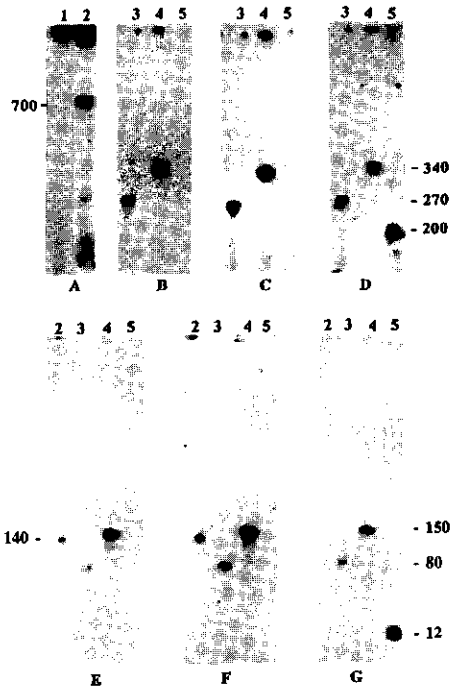

FIG. 3. Determination of size and location of the nisin-sucrose element. Undigested DNA from NIZO R5 (A, lane 1) and *Sma*I digests (A, lane 2; B through D) and *Eag*I digests (E through G) of DNA from strains NIZO R5 (lanes 2), T165.5 (lanes 3), T165.6 (lanes 4), and MG1614 (lanes 5) were separated 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

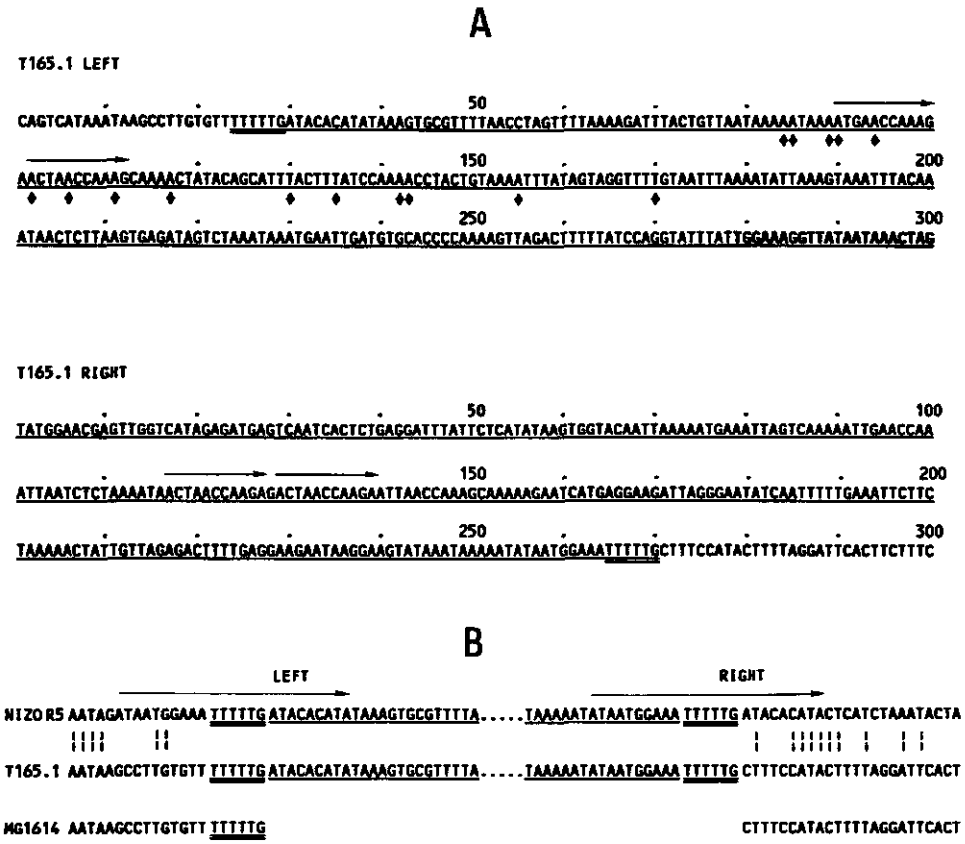

FIG. 4. Nucleotide sequences of Tn*5276* termini and junction regions in the donor and transconjugants in comparison with that of target site 1. (A) Nucleotide sequence of the left and right termini of Tn*5276* and the junction regions in T165.1. Tn*5276*-specific sequences are underlined. The direct 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.

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CHAPTER 6

IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN EXCISION OF THE *LACTOCOCCUS LACTIS* CONJUGATIVE TRANSPOSON TN*5276*

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Abstract The 70-kb transposon Tn*5276*, originally detected in *Lactococcus lactis* NIZO R5 and carrying the genes for nisin production and sucrose fermentation, can be conjugally transferred to other *L. lactis* strains. Sequence analysis and complementation studies showed that the right end of Tn*5276* contains two genes, designated *xis* and *int*, that are involved in excision. The deduced 379 amino acids *int* gene product shows high (up to 50%) similarity to various integrases, including that of the Tn*916*-related conjugative transposons. The putative *xis* gene product, like almost all known excisionase (Xis) proteins, is a small (68 residues), basic protein. Expression of the Tn*5276* *int* gene resulted in the excision of the ends of Tn*5276* in *Escherichia coli*, while expression of the Tn**5276** *xis* gene strongly stimulated this activity. The Tn*5276* ends appeared to be circularized in the excision process and the excision site was reconstituted.

INTRODUCTION

The transposon Tn*5276*, originally detected in the chromosome of *Lactococcus lactis* NIZO R5 (35, 36), can be conjugally transferred to other *L. lactis* strains. This 70-kb element carries the genetic determinants for a number of seemingly unrelated traits, including production of the lantibiotic nisin, sucrose fermenting ability and reduced bacteriophage sensitivity (39). It has been shown to integrate in one orientation into a preferred site (designated site 1) on the chromosome of *L. lactis* strain MG1614, a derivative of the plasmid-free strain MG1363, but also into several secondary sites (36). The ends of Tn*5276* are asymmetric and do not show any homology to the ends of known transposons. No target site duplication was found upon insertion of Tn*5276* (34,36). A similar element, Tn*5301*, has been described in a transconjugant derived from a mating between the wild-type nisin-producer *L. lactis* NCFB 894 and *L. lactis* MG1614 (19, 24).

Before the identification of the *L. lactis* transposons Tn*5276* and Tn*5301*, conjugative transposons were known as carriers of antibiotic resistance genes and they were originally identified in clinical isolates of several groups of streptococci (28). The 16.4-kb Tn*916* and the 25.3-kb Tn*1545* were the first conjugative transposons to be described and they are the best-studied representatives of this group (for reviews see references 15 and 42). These two related elements possess nearly identical ends, which contain a terminal imperfect inverted repeat sequence (10, 16). Unlike most transposons, they do not generate a target duplication upon insertion. The transposition system of these transposons is related to the excision-insertion system of lambdoid phages (12, 32, 33). Excision and integration occur by reciprocal site-specific recombination between

nonhomologous DNA sequences of 5 or 6 bp (Tn*916*; 12, 42) or 6 or 7 bp (Tn*1545*; 32, 33). Excision results in the appearance of a free, nonreplicative, covalently closed circular (ccc) molecule, which may be the intermediate that is transferred during conjugation (43). After conjugal transfer to the recipient, the transposon inserts into a new target. Excision and integration of Tn*1545* have been shown to be mediated by the transposon-encoded integrase (Int) protein, which is stimulated by the excisionase (Xis) protein in excision (32,33). The *xis* and *int* genes are located near the right end of Tn*1545* (32). Tn*916* contains essentially identical genes in the same location, but in Tn*916* this has been termed the left end (17). The excisive and integrative activities of Tn*916* Int have been shown to be required for conjugative transposition of Tn*916* (44). Although it was at first reported that for conjugative transposition an active *int* gene was required in both donor and recipient (44), it has recently been shown that a functional *int* gene is required only in the donor (6). From these results it was concluded that Tn*916* Int is not required for insertion of Tn*916* into the recipient genome or that the Int protein is transferred from the donor to the recipient during conjugation. There are indications that at least one host factor is involved in excision of Tn*916*, that is absent in *L. lactis* MG1363 (5, 7).

Here we report the sequencing of two genes, designated *xis* and *int*, that are located near the right end of Tn*5276*. Expression of these genes in *Escherichia coli* resulted in the excision of the ends of Tn*5276* from a plasmid vector. A rejoined excision site and a circular molecule consisting of the excised Tn*5276* termini were formed. The experiments also showed that expression of the *int* gene alone can promote excision of the Tn*5276* ends in *E. coli*, but that this activity is strongly stimulated by simultaneous expression of the *xis* gene. The deduced amino acid sequence of the *int* gene shows overall similarity to the Int protein encoded by Tn*1545* and to other proteins belonging to the group of site-specific recombinases.

MATERIALS AND METHODS

Strains, plasmids and growth conditions. The following *E. coli* hosts were used to propagate plasmids and bacteriophages: strain MC1061 (13) for pNZ774 (36), strain TG1 (22) for M13mp18 and mp19 (50), and strain HB101 (4) for all other plasmids. Growth conditions were as described elsewhere (41). Antibiotics were added at the following concentrations: ampicillin, 50 μg/μl; chloramphenicol, 35 μg/μl.

DNA techniques. Standard procedures were used for DNA isolation and manipulations (41), agarose gel electrophoresis (41), and Southern blot analysis (36).

Hybridization probe. As probe for the left end of Tn*5276* an oligonucleotide with the sequences 5'-GCTGTATAGTTTTGCTTTGG, designated Tn*5276*-L and complementary to position 107-126 in the nucleotide sequence of the left end of Tn*5276* (36), was used at a hybridization temperature of 52°C.

Nucleotide sequencing. Subclones of pNZ774 in M13mp18 and mp19 were sequenced by the dideoxy chain termination method adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with either the M13 universal primer or synthesized primers. The sequence data were assembled and analyzed using the PC/Gene program version 6.6 (Genofit, Geneva, Switzerland).

Plasmid constructions. The following plasmids were constructed to study the action of the *xis* and *int* gene products in *E. coli* (see Fig. 1).

(i) Construction of pNZ780. A 1.7-kb *Sca*I-*Hin*dIII fragment containing the left junction of Tn*5276* in site 1 (0.6 kb of site 1 and 1.1 kb of the Tn*5276* left end) was isolated from *L. lactis* T165.6 (36) and cloned into *Sma*I- and *Hin*dIII-digested M13mp19, resulting in mpNZ776. The insert of mpNZ776 was isolated by digestion with *Hin*dIII, followed by filling in of the 3'-recessed ends with Large Fragment of DNA Polymerase I, deproteinization, and digestion with *Eco*RI. The right end of Tn*5276* was isolated as a 0.9-kb *Pvu*II-*Hin*dIII fragment from mpNZ775/2 (36), which contains the right junction of Tn*5276* from *L. lactis* NIZO R5 cloned as a 1.0-kb *Eco*RI fragment in *Eco*RI-linearized M13mp19 (0.9 kb of the Tn*5276* right end and 0.1 kb of NIZO R5 DNA). The 1.7-kb *Eco*RI-blunt fragment (containing 1.1 kb of the left end) and the 0.9-kb *Pvu*II-*Hin*dIII fragment (containing 0.8 kb of the right end) were ligated into pUC19 (50) digested with *Eco*RI and *Hin*dIII (Fig. 1).

(ii) Construction of pNZ781 and pNZ782. A 1.9-kb *Bsp*HI-fragment containing the *xis* and *int* genes (Fig. 1) was isolated from pNZ774 (36) and ligated into *Bsp*HI-digested pACYC184 (14). One of the resulting plasmids, designated pNZ781, contains the *xis* and *int* genes in a clockwise orientation with respect to the promoter of the Tc^R gene, while in the plasmid designated pNZ782 these genes are in a counterclockwise orientation.

(iii) Construction of pNZ783 and pNZ784. The *xis* gene was inactivated in pNZ781 by digestion with *Afl*III followed by filling-in of 3'-recessed ends with Large Fragment of DNA

Polymerase I and religation, resulting in pNZ783. The *int* gene was inactivated in pNZ781 by partial digestion with *Eco*RI followed by filling in of recessed ends with Large Fragment of DNA Polymerase I and religation, resulting in pNZ784.

Database searches. The EMBL (release 31.0), Swiss-Prot (release 22.0), and NBRF/PIR (release 27.0) databases were searched using the program FASTA (31) through the facilities of the CAOS/CAMM Center, Nijmegen, The Netherlands.

RESULTS

The nucleotide sequence of the *xis* and *int* genes located in the right end region of Tn*5276*. The homologous *xis* and *int* genes of the conjugative transposons Tn*916* and Tn*1545* are located at a comparable position near one of the ends (17, 32). When the recently reported nucleotide sequence of the right end of Tn*5276* (36) was analyzed for the presence of open reading frames

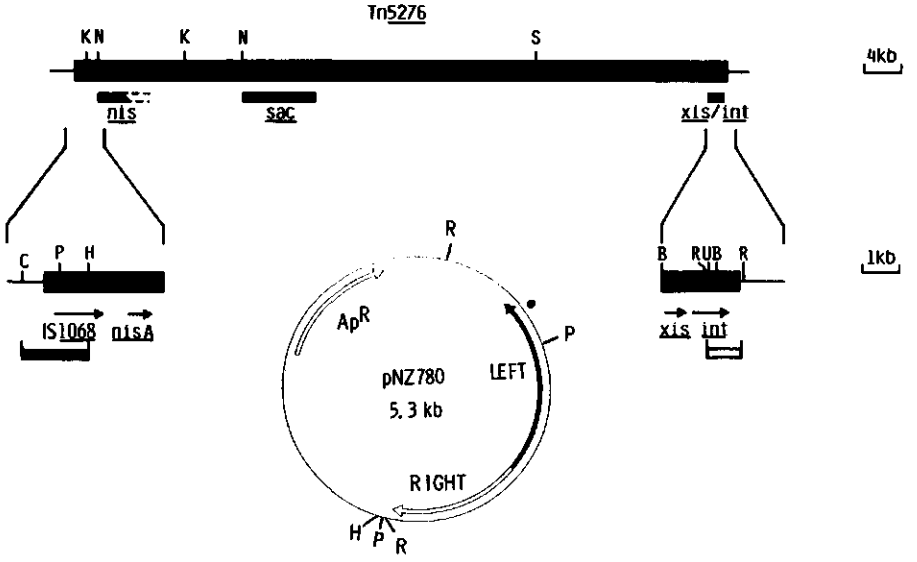

FIG. 1. Construction of pNZ780 consisting of pUC19 (50) containing the left (black) and right ends of Tn*5276* (map was taken from 36). The solid bars below Tn*5276* indicate the positions of the nisin (*nis*; 36, 39), sucrose (*sac*; 37) and transposition (*xis/int*) gene clusters. Arrows indicate the positions and the directions of the putative transposase of the iso-IS*904* element, IS*1068* (34, 35), *nisA* (36), and *sacA* (37). The location of the Tn*5276*-L probe is indicated by the dot. Restriction enzyme abbreviations: B, *Bsp*HI; C, *Sca*I; H, *Hin*dIII; K, *Kpn*I; N, *Nci*I; P, *Pst*I; R, *Eco*RI; S, *Sst*II; U, *Pvu*II.

(ORFs), we found a 1.9-kb *Bsp*HI-fragment to contain the 3' end of an ORF that stopped 152 nucleotides upstream from the hexanucleotide TTTTTG located at the terminus. The amino acid sequence deduced from this ORF contained a stretch of residues that fitted the consensus sequence of domain II of the integrase family of site-specific recombinases (2). Therefore, the complete nucleotide sequence of the 1.9-kb *Bsp*HI-fragment was determined (Fig. 2) and found to contain two complete genes, designated *xis* and *int* (see below). Both genes start with ATG initiation codons that are preceded by potential lactococcal ribosome binding sites (18). A promoter-like sequence (18, 49) was found upstream from the *xis* gene and is located in the 3' part of another ORF, designated ORF1 (Fig. 2). The amino acid sequences deduced from ORF1 and the *xis* and *int* genes are shown in Fig. 2. The *xis* gene could encode a polypeptide of 68 amino acids with a predicted molecular weight of 8,292 (18, 49). The *int* gene could encode a polypeptide of 379 amino acids with a predicted molecular weight of 44,621. An alternative GTG start codon for the *int* gene is located at position 648-650, but it is not preceded by a consensus lactococcal ribosome binding site. The *int* gene is not followed by an inverted repeat that could serve as a terminator of transcription. The region between the *xis* and *int* genes is almost identical in size (85 bp) to the corresponding 84-bp regions of Tn*916* and Tn*1545*, which have identical sequences (17, 32).

Both the *xis* and the *int* gene are required for efficient excision of Tn*5276* ends in *E. coli*. To assess the functionality of the Tn*5276* *xis* and *int* genes, we determined whether their expression would result in excision of the ends of Tn*5276* in *E. coli*. For this purpose the 1.29-kb *Bsp*HI-fragment containing both genes was cloned in two orientations in the low-copy-plasmid pACYC184, resulting in plasmid pNZ781 and pNZ782. Each of these plasmids was introduced into the *recA E. coli* strain HB101 harboring pNZ780, a compatible plasmid containing the left and right ends of Tn*5276* (Fig. 1). Excision promoted by members of the integrase family is a strand exchange process in which the excision site is resealed and the excised DNA is circularized (40). Since pNZ780 contains 1.1 kb of the left end of Tn*5276* and 0.8 kb of its right end, Int-promoted excision of the termini of Tn*5276* from pNZ780 would generate a 3.4-kb derivative of pNZ780 lacking the Tn*5276* ends and a 1.9-kb non-replicative covalently closed circular form of the Tn*5276* ends. Analysis of total plasmid DNA indicated that all of the transformants (12 from each transformation) contained the expected 3.4-kb plasmid and low amounts of a 1.9-kb plasmid consisting of the Tn*5276* ends, in addition to a low amount of intact pNZ780 (data not shown). Restriction enzyme digestions and hybridization analysis of the multiplasmid DNA from repre-

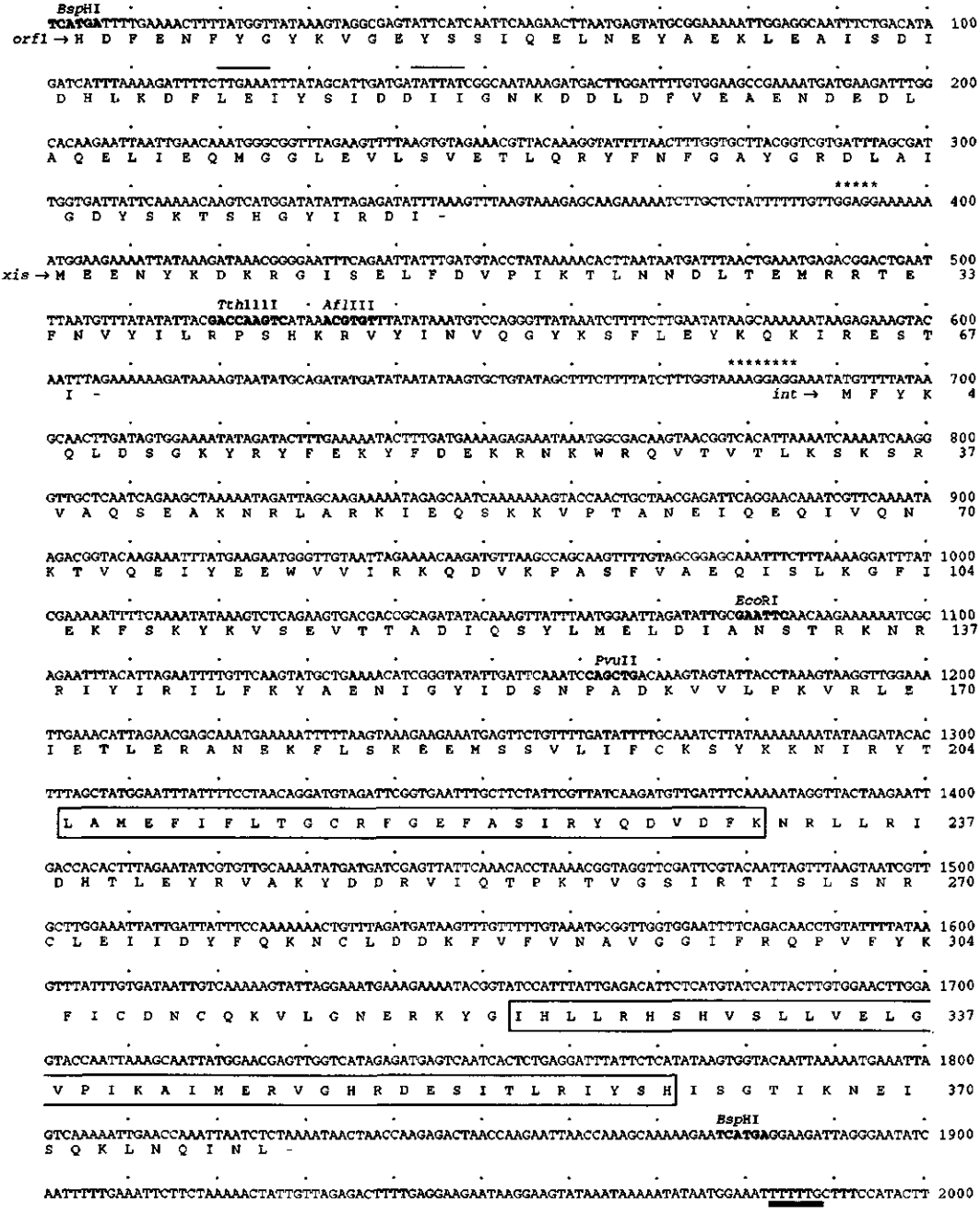

FIG. 2. Nucleotide sequence of the right junction of Tn*5276* in *L. lactis* T165.1 site 1. The amino acid sequences deduced from the ORFs are given below the nucleotide sequence. The amino acid stretches in the Int sequence that correspond to the consensus domains I and II of the Int-family of site-specific recombinases (2) are boxed. Potential ribosome binding sites are denoted by stars over the bases that are complementary to the 3' end of *L. lactis* 16S rRNA (27). Restriction sites are in bold face. A putative promoter sequence is overlined. The TTTTTG hexanucleotide flanking the right end of Tn*5276* (26) is doubly underlined.

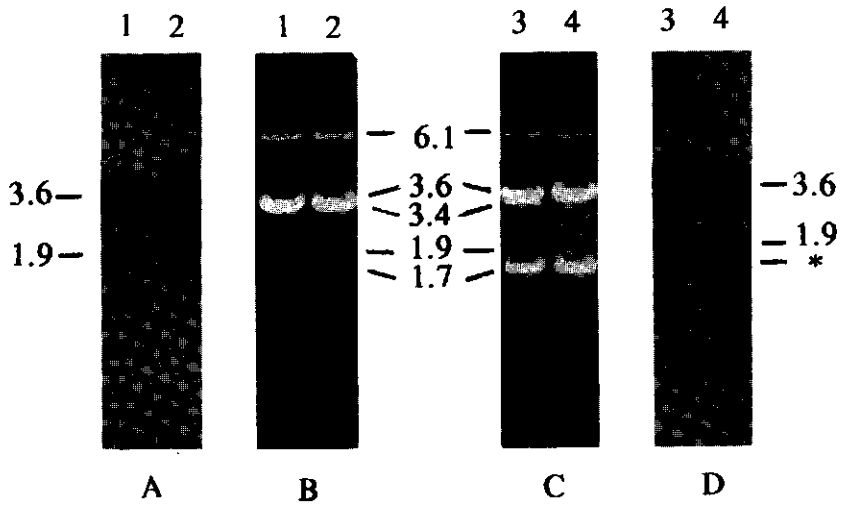

FIG. 3. Detection of excision and circularization of Tn*5276* ends in *E. coli* HB101 containing pNZ780 and transformed with different plasmids containing the *xis* and *int* genes of Tn*5276*. **A,B:** Transforming plasmids were pNZ781 (lane 1) and pNZ782 (lane 2). **C,D:** Transforming plasmids were pNZ783 (lane 3) and pNZ784 (lane 4). Plasmid DNA was isolated from overnight cultures of transformants, digested with *Eco*RV and *Pst*I, and electrophoresed on a 1.0 % agarose gel (panels B and C). Major bands are indicated in between the panels and include: the 6.1-kb *Eco*RV-linearized pNZ781, pNZ782, pNZ783, or pNZ784, the 3.6-kb and 1.7-kb *Pst*I-fragments from pNZ780 containing the Tn*5276* ends, the 3.4-kb *Pst*I-fragment resulting from pNZ780 without Tn*5276* ends, the *Pst*I-linearized 1.9-kb circle consisting of the excised Tn*5276* ends. Subsequently, Southerns blot of the gels were hybridized to the oligonucleotide Tn*5276*-L specific for the left end of Tn*5276* (panels A and D, respectively). Autoradiography of the blot shown in A was approximately five times longer than that of the blot shown in D. The probe hybridized to the 3.6-kb *Pst*I-fragment of pNZ780 containing the Tn*5276* left end and to the 1.9-kb circle consisting of the Tn*5276* ends. Background hybridization to the 1.7-kb *Pst*I-fragment from pNZ780 is indicated by the star.

sentative transformants confirmed this (Fig. 3A,B). Total plasmid DNA was digested with *Pst*I that generates two fragments of 1.7 and 3.6 kb from pNZ780 (see Fig. 1) and with *Eco*RV to linearize pNZ781 or pNZ782. Upon staining of agarose gels on which the digested DNA was separated (Fig. 3B) the linearized 6.1-kb *Eco*RV fragments of pNZ781 (lane 1) or pNZ782 (lane 2) were readily detectable but only small amounts of the 1.7-kb and 3.6-kb *Pst*I fragments of pNZ780 were found. In contrast, large amounts of a 3.4-kb *Pst*I fragment representing the linearized derivative of pNZ780 lacking the Tn*5276* ends were present, indicating efficient excision. Finally, the excised and circularized ends of Tn*5276* appeared in a *Pst*I-digest as a 1.9-kb fragment. However, this 1.9-kb *Pst*I fragment was present in low amounts and could only be detected after autoradiography of

blots hybridized to the left end-specific oligonucleotide probe Tn*5276*-L that also hybridized to the 3.6-kb *Pst*I fragment of pNZ780 (Fig. 3A). The low amount of the 1.9-kb plasmid found in the transformants can be attributed to the non-replicative nature of this molecule. Since the same results were obtained with either pNZ781 or pNZ782 (Fig. 3B, lanes 1 and 2), the vector-located promoter is not involved in the expression of the expression of *xis* and/or *int* gene(s).

To investigate if the *xis* and *int* genes are both required for excision of the Tn*5276* ends in *E. coli* plasmids pNZ783 and pNZ784 were constructed that contained mutations in either of the genes. Each of the plasmids was introduced into *E. coli* HB101 harboring pNZ780 and the efficiency of excision was analyzed. Representative results are shown in Fig. 3C. When the *int* gene was disrupted, large amounts of the expected 3.6-kb and 1.7-kb *Pst*I fragments of pNZ780 were found in addition to linearized pNZ784 (Fig. 3C, lane 4). Moreover, no 1.9-kb *Pst*I fragment generated by excision and recircularization of the ends from pNZ780 was found after hybridizing the blot with the left-end probe Tn*5276*-L (Fig. 3D, lane 4). Disruption of the *xis* gene, as is the case in pNZ783, resulted in a considerable decrease in excision efficiency as compared with that in cells harboring pNZ781 or pNZ782 (Fig. 3B), since the majority of the pNZ780 molecules still contained the Tn*5276* ends (Fig. 3C, lane 3). However, in the presence of pNZ783, a considerable amount of the 1.7-kb *Pst*I fragment could be detected, even in the stained digest. Thus, in the presence of the *int* gene alone (as in pNZ783) the ends of Tn*5276* in pNZ780 are excized and recircularized, but the additional presence of a functional *xis* gene (as in pNZ781 or pNZ782) strongly enhances the excision efficiency.

When the *xis* gene was inactivated as in pNZ783, a higher amount of circular intermediate was found than with pNZ781 or pNZ782 containing intact *xis* and *int* genes (compare the autoradiographs in Fig. 3A and 3D and note the longer period of exposure in Fig. 3A). This could be explained by the inefficiency of the excision process in absence of functional Xis. In this case, pNZ780 molecules containing the Tn*5276* ends will continuously be present and excision of the 1.9-kb circle persists. In contrast, efficient Int-promoted excision in the presence of functional Xis (Fig. 3A,B) results in a rapid loss of the substrate (intact pNZ780).

Since Int promotes insertion in the absence of Xis in other Xis-Int systems, the possible insertion of the 1.9-kb circle into new sites on the *E. coli* genome was investigated in transformants containing pNZ780 and pNZ783. Insertion into new sites was not observed, but reinsertion of the 1.9-kb circle into the site of excision can not be excluded.

Features of Int and Xis amino acid sequences. Databases were searched for the presence of protein sequences that were similar to those deduced from ORF1 and the *xis* and *int* genes. No proteins with significant similarity to the amino acid sequence deduced from ORF1 were found. In addition, no proteins with significant similarity to the small Tn*5276* Xis were found. However, Tn*5276* Xis, like most known excisionases, is a short, basic protein (calculated pI of 9.71).

Five proteins showing overall similarity to Int were detected in the databases (Fig. 4). The highest percentages of similar and identical residues were encountered in the C-terminal regions. An alignment of the C-terminal regions of these proteins and Tn*5276* Int is given in Fig. 4. As the five other integrases, Tn*5276* Int is a basic protein with a calculated pI of 9.99.

FIG. 4. Amino acid sequence alignments of the C-terminal regions of, from top to bottom, the Int proteins of Tn*5276*, bacteriophage L54a (51), Tn*1545* (32), bacteriophage ϕ11 (52), and pSAM2 (32). Domains I and II (2) are in bold face. Asterisks indicate residues present in at least four sequences and similar residues (I-L-V-M, D-E, R-K, Q-N, S-T, F-Y) present in at least four sequences are indicated by points. Triangles indicate the residues conserved in all known integrases (except in pSAM2 Int).

DISCUSSION

Excision of the conjugative transposon Tn*5276* from the donor replicon would be the first step of the transposition process, in analogy with the conjugative transposons Tn*916* and Tn*1545*. Here we show that Tn*5276* contains two juxtaposed genes, *xis* and *int*, at its extreme right terminus. The functionality of the *xis* and *int* genes was established by the observation that their expression resulted in excision of the Tn*5276* ends in *E. coli*. Expression of only the *int* gene appears to result in excision of the Tn*5276* ends, but simultaneous expression of a functional *xis* gene enhances the excision efficiency. Two products are formed in the excision reaction: a deleted plasmid containing a religated excision site and a 1.9-kb covalently closed circular molecule consisting of the ends of Tn*5276*. Recently, we found that the nucleotide sequence of the joint of this 1.9-kb plasmid was identical to that found for the circular intermediate of Tn*5276* in *L. lactis* (34, 38). These results strongly suggest that the *xis* and *int* genes code for proteins that are required for excision of Tn*5276* in *L. lactis*.

Tn*5276* Xis and Int, each in different degrees, are similar to other proteins involved in excision and insertion of genetic elements. The Int-related family (2) is one of the two main groups of site-specific recombinases. The homologous domains I and II (2) in the C-terminal regions of a large number of proteins that belong to the Int-related family have been aligned (1, 2, 3, 8, 32). These alignments showed that a histidine, a tyrosine and two arginine residues are conserved in all integrases, except for that of pSAM2. It has been shown that Tyr-342 in domain II of bacteriophage λ Int is the residue that forms an *O*-phosphotyrosine bond with the 3'-phosphate of *att* at the site(s) of strand exchange (30). Thus, it is very likely that the C-terminal region of Tn*5276* Int participates in strand transfer and that the active site is at the corresponding Tyr-359.

A variety of excisionases have been identified thus far from different mobile elements. These elements are the lambdoid phages (26), several *Streptomyces* integrative plasmids (3, 8), the streptococcal conjugative transposons Tn*916* (17) and Tn*1545* (32), and the bacteriophages L54a (51) and ϕ11 (52), present as prophages in *Staphylococcus aureus*. The Tn*5276* Xis protein shares with almost all of these excisionases its small size and high pI. The excisionases of bacteriophages L54a and ϕ11 are acidic and represent a different class of excisionases (52). The basic character of the excisionases from the first class is thought to reflect their interaction with DNA. In the case of bacteriophage λ, it has been shown that Xis in conjunction with the *E. coli* protein factor for

inversion stimulation (FIS) binds to sites in *attR* and enhances excision (9, 29, 47). When λ Xis binds the DNA at its recognition sites in λ *attR* the DNA is bent (45). The formation or stabilization of this bend is thought to be required for excisive recombination of λ. The involvement of FIS in λ excision couples recombination to the growth phase of the host cell (47). Another host factor involved in λ site-specific recombination is Integration Host Factor (IHF; 46). We cannot exclude the involvement of host factors in excision of Tn*5276*, since the possibility exists that *E. coli* IHF and FIS can substitute for similar lactococcal host factors. Several subsequences conforming to the proposed consensus *E. coli* IHF and FIS binding sites (23, 25) can be found in the ends of Tn*5276* (34). However, excision experiments in *E. coli* strains deficient in the production of IHF and FIS should be carried out to investigate the involvement of *E. coli* host factors in excision of the Tn*5276* ends from pNZ780. It was recently suggested that a host factor that is absent from *L. lactis* MG1363 is required for the conjugative transposition of Tn*916* (5). This putative host factor is evidently not involved in transposition of Tn*5276*, since we have shown that insertion of Tn*5276* into the genome of a derivative of *L. lactis* MG1363 occurs *via* site-specific recombination (36, 38) and not, as for Tn*916* in *L. lactis* MG1363, through homologous recombination (5, 7).

The requirement of host factors and Xis in the site-specific recombination reactions (excision and insertion) of the lambdoid phages serves as a means of controlling these reactions. Next to the control exercised by the host cell *via* the necessary host factors, the phages themselves control the direction of site-specific recombination (excision versus insertion) by the differential expression of the *xis* and *int* genes (for a review see reference 21). The organization of the *xis* and *int* genes of Tn*5276* is similar to that of the corresponding genes in Tn*1545* (32) and Tn*916* (17). In all cases the genes are located near one end of the element and the *xis* and *int* genes are separated by a region of 84 (Tn*916* and Tn*1545*) or 85 bp (Tn*5276*). The significance of this similarity in the organization of the *xis* and *int* genes is not yet known. Since the efficiency of excision is independent of the orientation of the *xis* and *int* genes in pACYC184 (Fig. 3B) it is possible that the promoter-like sequence found upstream from the *xis* gene (Fig. 2) is functional in *E. coli*. It remains to be elucidated whether this is also the case in *L. lactis*, whether differential expression of the *xis* and *int* genes occurs in *L. lactis* and, if so, how this is effected.

The amino acid sequence of Tn*5276* Int shows an overall (up to 50%) similarity to the Int proteins of conjugative transposons from *Streptococcus* (Tn*1545* and Tn*916*), an integrative plasmid

from *S. ambofaciens* (pSAM2), and bacteriophages carried as a prophage in *S. aureus* (L54a and ϕ11). In contrast to the overall similarity of the Int proteins (which is highest in their C-terminal regions), other features of these elements, like the structure of their ends, the amino acid sequence of their Xis proteins, their insertional behavior, and the requirements of host factors for their excision and insertion, differ strongly from each other. The question arises how these elements have acquired the genes for the homologous Int proteins. The joining together of distinct modules from different sources as proposed for the evolution of lambdoid phages (11) would be an attractive model for the evolution of Tn*5276* and related transposons, since they carry the genes for a number of very different traits, like nisin production (36), sucrose fermentation (37), and in some cases the synthesis of N^5-carboxyethyl-ornithine (20). Further investigation of Tn*5276* could contribute to our knowledge of its origin and its evolutionary relationships with conjugative transposons, bacteriophages and plasmids.

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CHAPTER 7

TRANSPOSITION OF THE *LACTOCOCCUS LACTIS* CONJUGATIVE TRANSPOSON TN*5276*: IDENTIFICATION OF A CIRCULAR INTERMEDIATE AND ANALYSIS OF ITS EXCISION AND INSERTION SITES

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Submitted for publication

Abstract Excision and insertion of the *Lactococcus lactis* nisin-sucrose conjugative transposon Tn*5276* occur by reciprocal site-specific recombination. Based on the nucleotide sequences of site-specific recombination products in both *L. lactis* and *Escherichia coli*, we propose a model for excision and insertion of Tn*5276* in conjugal transposition. Low levels of covalently closed, circular molecules were detected in different Tn*5276*-containing *L. lactis* strains and, since the nucleotide sequences of their joints were in agreement with the proposed model, they probably represent the intermediates transferred in conjugal matings. In contrast to the conjugative transposons Tn*916* and Tn*1545*, the regions involved in reciprocal strand exchange (overlap regions) in Tn*5276* insertion and excision show a high degree of nucleotide sequence identity. Since in this respect Tn*5276* resembles temperate bacteriophages, the recombining sites have been termed attachment sites. However, a single nucleotide difference in the overlap regions does not prevent efficient excision and insertion, as observed for conjugal transposition of Tn*5276* to site 1 (now termed *attB1*) of *L. lactis* MG1614. The deduced Tn*5276* *attB* site in *L. lactis* NIZO R5 was found to share a 25 bp region of sequence identity (termed core region) with *attP* in the Tn*5276* circular form. Besides the known nucleotide sequence of *L. lactis* MG1614 *attB1*, the sequence of a second Tn*5276* insertion site (*attB2*) could be deduced. These *attB* sites show only limited (52-60%) similarity to the core region.

INTRODUCTION

The 70 kb conjugative transposon Tn*5276*, originally detected in *Lactococcus lactis* NIZO R5 (Rauch *et al.*, 1990), encodes a variety of metabolic properties including the biosynthesis of the lantibiotic nisin (Rauch and De Vos, 1992a) and the transport and hydrolysis of sucrose (Rauch and De Vos, 1992b). Tn*5276* has been shown to insert into several sites in the chromosome of the plasmid-free strain *L. lactis* MG1614 after conjugal transfer and insertion of multiple copies has been observed (Rauch and De Vos, 1992a). A preferential site, designated site 1, for Tn*5276* insertion into the *L. lactis* MG1614 chromosome has been identified (Rauch and De Vos, 1992a). A *L. lactis* element, termed Tn*5301*, with similar features has been described in *L. lactis* FI5876 (Horn *et al.*, 1991).

We recently located two genes, *xis* and *int*, near the right end of Tn*5276* that are involved in excision of the element (Rauch and De Vos, 1993). The deduced 379 amino acids *int* gene product (Int) shows significant (up to 50%) similarity to various proteins belonging to the integrase family of site-specific recombinases. The putative *xis* gene product (Xis), like most other excisionases, is

a small (68 residues), basic protein. We found that in *Escherichia coli* excision of the Tn*5276* ends required a functional *int* gene and resulted in the appearance of a circular molecule consisting of the Tn*5276* ends. Additional presence of a functional *xis* gene resulted in a more efficient excision of the Tn*5276* ends. This is in agreement with the stimulating role of other Xis proteins in the excision reactions mediated by the corresponding Int proteins (Leong *et al.*, 1986; Poyart-Salmeron *et al.*, 1989; Brown *et al.*, 1990; Ye *et al.*, 1990).

Site-specific recombinases promote reciprocal recombination between two limited DNA sequences. The recombination reaction involves a strand exchange mechanism that does not involve any net synthesis or loss of DNA and it has therefore been called conservative (Campbell, 1981). In almost all cases the recombining sites contain regions of perfect homology in which reciprocal strand exchange occurs. A well-studied example is bacteriophage λ insertion, involving the bacteriophage attachment site *attP* and the chromosomal attachment site *attB*, which contain core regions of 15 bp with identical nucleotide sequences. Reciprocal strand exchange involves staggered cuts within this core region that generate a 7 bp overlap region (Mizuuchi *et al.*, 1981). Sequence identity between overlap regions appears to be important for efficient recombination. Recombination between sites with non-identical overlap regions is inefficient (Hoess *et al.*, 1982; Weisberg *et al.*, 1983). However, the integrases of the streptococcal conjugative transposons Tn*916* and Tn*1545*, which are identical to each other, have been shown to promote efficient site-specific recombination between non-homologous overlap regions in the excision and insertion of these elements (Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1989, 1990).

A circular form of Tn*916* has been detected in an *Escherichia coli* strain transformed with a cosmid clone containing the transposon (Scott *et al.*, 1988). This molecule was able to transform *B. subtilis* and the resulting strain could subsequently transfer tetracycline resistance to *Streptococcus pyogenes*. Therefore, this supercoiled molecule was considered to be the Tn*916* intermediate that is transferred in conjugal matings and analysis of recombination products of the circular form from *E. coli* was used to design a model for Tn*916* transposition (see Scott (1992) for a recent review). The trigger for the (yet unobserved) appearance of the circular intermediate in the conjugal donor has been proposed to be cell-to-cell contact (Scott, 1992). The first conjugative transposon of which a circular form has been detected in its original host is the Tn*916*-related, tetracyline resistance-conferring Tn*5381* from *Enterococcus faecalis* (Rice *et al.*, 1992). Hybridization analyses showed that this 19 kb transposon, next to being present in the chromosome,

exists as a free, closed circle. The amount of circular form present in alkaline lysis preparations of *E. faecalis* strains containing the transposon could be elevated by exposure of these strains to subinhibitory levels of tetracycline. Exposure to tetracycline also resulted in an increase in the conjugal transfer frequency of Tn*5381*. This suggests that the trigger for the appearance and transfer of the circular form of Tn*5381*, which has not yet been shown to be the conjugal transposition intermediate, is exposure to tetracyline.

Previous analysis of the nucleotide sequences of left and right junctions of Tn*5276* in *L. lactis* NIZO R5 and in site 1 of a *L. lactis* MG1614-derived transconjugant showed that the ends of the transposon are asymmetric, do not contain a clear inverted repeat and are flanked by a hexanucleotide (TTTTTG) repeat in both sites. One half of this repeat was already present in site 1 before insertion (Rauch and De Vos, 1992a).

In this report we show the presence of a circular form of Tn*5276* in *L. lactis*. In addition, recombination products of Tn*5276* in *E. coli* and *L. lactis* are analyzed and used to deduce the region in which strand exchange occurs in Tn*5276* transposition. The results indicate that heteroduplexes can be formed in the recombination process and that the circular form of Tn*5276* detected in *L. lactis* is the conjugal transposition intermediate.

RESULTS

Analysis of different Tn*5276* insertions: indications for a recombination event different from that in insertion of other conjugative transposons. A TTTTTG hexanucleotide repeat is present in the junction regions of Tn*5276* analyzed thus far, i.e. in its location in the wild-type strain *L. lactis* NIZO R5 and in site 1 of the *L. lactis* MG1614-derived transconjugant T165.1 (Rauch and De Vos, 1992a). Furthermore, the TTTTTG hexanucleotide is present in site 1 before insertion. We have recently shown that Tn*5276* excises through a site-specific recombination reaction mediated by a protein related to λ Int (Rauch and De Vos, 1993). Thus, the halfs of the TTTTTG repeat possibly constitute the core regions of what might be called the left and right attachment sites of Tn*5276* in its inserted state (*attL* and *attR*, respectively). Such presence of invariable core regions in Tn*5276* site-specific recombination would distinguish this transposon from the conjugative transposons Tn*916* and Tn*1545*. In the excision and insertion of the latter

transposons the recombining sites do not share common core regions (Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1989; 1990).

Transconjugant T165.4, which was isolated in an earlier study from a mating between *L. lactis* strains NIZO R5 and MG1614 (Rauch and De Vos, 1992a), contains two copies of Tn*5276*, one in site 1, which we would now rather term *attB1*, and one in another location (designated *attB2*). We analyzed the junctions of the latter copy of Tn*5276* after their amplification by different polymerase chain reaction (PCR) methods. This showed that also in this case a TTTTTG hexanucleotide repeat was present (Fig. 1).

If the TTTTTG hexanucleotides found in the Tn*5276* junctions would constitute the core regions of Tn*5276* *attL* and *attR*, the junction sequences of all insertions of Tn*5276* in *attB1* of *L. lactis* MG1614 would be expected to be identical. However, when we determined the nucleotide sequence of the left junction of Tn*5276* in *attB1* of the *L. lactis* MG1614-derived transconjugant T165.6 isolated in an earlier study (Rauch and De Vos, 1992a), we found that it differed from the corresponding sequence in transconjugant T165.1 (Rauch and De Vos, 1992a) in one position: the nucleotide 3' from the TTTTTG hexanucleotide was a C in T165.6 and an A in T165.1. Sequence variability of transposon ends is a feature of Tn*916* and Tn*1545* insertion. The single nucleotide variation found in the left junction of Tn*5276* in *attB1* could, like the sequence variability of Tn*916* and Tn*1545* junctions, be the result of the fact that the base at the variable position was part of the overlap region generated by the strand exchange reaction in insertion of Tn*5276*. This would mean that a heteroduplex with one mismatch was formed during this reaction.

Tn*5276*

...TTTCAACTTGTCAAATTTTTGATACA...GGAAATTTTTGATATTCAATAATTCA...

FIG. 1. Nucleotide sequence of Tn*5276* junctions after its insertion into *attB2* of *L. lactis* MG1614. The nucleotide sequence was determined from PCR amplification products containing the junctions of the copy of Tn*5276* present in *attB2* in a derivative of transconjugant T165.4 (Rauch and De Vos, 1992a; see 'experimental procedures'). The TTTTTG hexanucleotide repeat is doubly underlined.

During insertion of Tn*5276* into *L. lactis* MG1614 *attB1* heteroduplexes are formed. The transposition intermediates of Tn*916* and Tn*1545* very probably are non-replicating circles (Scott *et al.*, 1988; Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1989, 1990). We have shown that in excision of the Tn*5276* ends also a circular molecule is formed (Rauch and De Vos, 1993).

Based on the one-nucleotide variation found in the left junction of different Tn*5276* insertions in *attB1*, a model for excision of Tn*5276* from its location in *L. lactis* NIZO R5 and insertion of the resulting circular intermediate into *attB1* of *L. lactis* MG1614 can be proposed (Fig. 2). The heteroduplexes formed in the overlap regions after insertion could either be resolved in the following replication round or be corrected by a mismatch repair system. If these heteroduplexes would be resolved in the replication round following the insertion of Tn*5276*, this would lead to the appearance of two types of progeny: one type with *attL* originating from the donor (*attP*; with an A at the mismatch position) and *attR* originating from the recipient (*attB1*; with a C at the mismatch position) and one type containing *attL* originating from the recipient (*attB1*) and *attR*

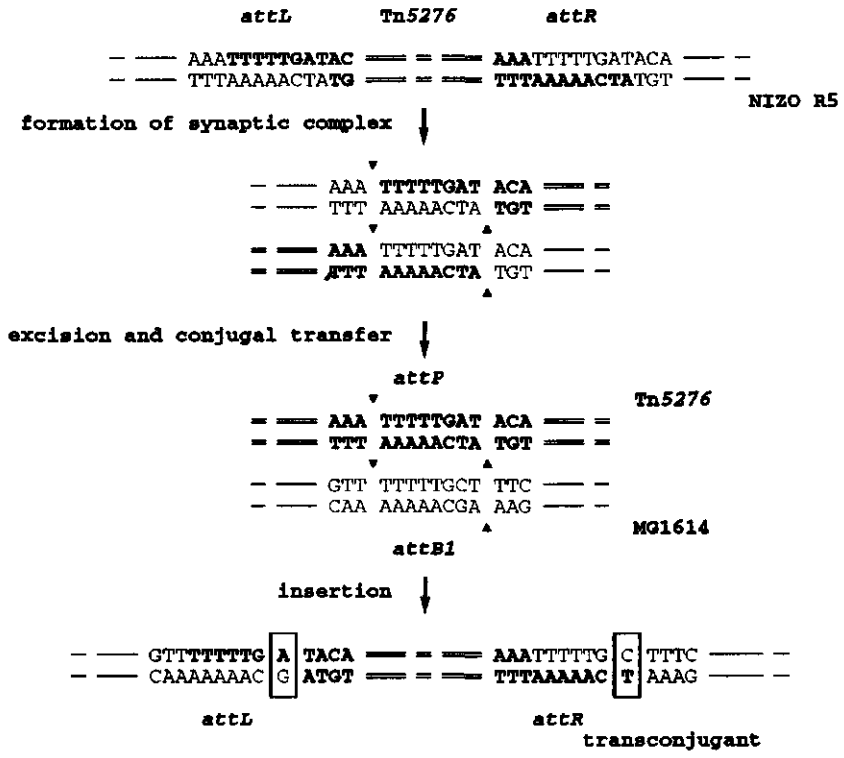

FIG 2. A model for excision and insertion of Tn*5276* in conjugal transfer from *L. lactis* NIZO R5 to *L. lactis* MG1614 (*attB1*). The triangles indicate the positions of the cuts that generate the overhanging ends. The exact positions of the cuts cannot be deduced from the nucleotide sequence data obtained thus far, but the positions generating the largest possible overlap region are shown. Also, the nucleotide sequence data do not allow us to determine whether 5'- or 3'-protruding ends are generated, but 5'- protruding ends have been chosen in analogy to other Int-related site-specific recombinases (Sadowski, 1986). The Tn*5276*-specific nucleotides (based on the assumed positions of the cuts) are in bold face. The heteroduplexes resulting from the insertion of the Tn*5276* circle into *attB1* of *L. lactis* MG1614 are boxed.

originating from the donor (*attP*). Mismatch repair however would lead to the appearance of one type of progeny with unique junction sequences. Assuming that the repair mechanism would have no preference for certain mismatches, all four possible junction sequence combinations (containing an A or a C at the left and right end mismatch positions) would be expected to occur.

To test the model proposed for insertion of Tn*5276* into *L. lactis* MG1614 *attB1* after its excision and transfer from *L. lactis* NIZO R5 (Fig. 2), the left and right junctions of the transposon inserted into *attB1* were analyzed in a number of transconjugants. Since we wanted to analyze the appearance of different junction sequences in the progeny of an original transconjugant cell, strains NIZO R5 and MG1614 were mated according to the method of Broadbent and Kondo (1991), in which conjugation is performed directly on the selective plates. Cultures derived from the transconjugant colonies would contain all types of progeny, which could be individually analyzed in isolated colonies. First we analyzed the location of Tn*5276* in the DNAs isolated from liquid cultures of twelve original transconjugant colonies by hybridizing *Eco*RI-digests to an oligonucleotide probe specific for the left end of Tn*5276* (Fig. 3A). In all original transconjugant DNAs, Tn*5276* was found in a number of locations. Three sites were occupied in all cases. The 9.5 kb hybridizing *Eco*RI fragment in Fig. 3A corresponds to the insertion of Tn*5276* in *attB1* (Rauch and De Vos, 1992a). The 5.8 kb hybridizing *Eco*RI fragment corresponds to the insertion of Tn*5276* in *attB2* (Fig. 1; Rauch and De Vos [1992a]). The hybridization signals were not of equal intensity within each sample, the *attB1* insertion signal always showing the highest intensity. A possible explanation for these hybridization results would be the presence of different, unequally represented populations of cells within each culture, each population containing Tn*5276* in one or more different locations (with a majority of the cells containing Tn*5276* in *attB1*). The presence of several populations with different Tn*5276* insertion profiles could be explained in two ways. One explanation would be that the different populations originated from different mating events (within one colony). Alternatively, the original transconjugant cell contained multiple copies of Tn*5276*, but during the growth in culture, descendants of the original cell lost copies of Tn*5276*.

We determined the nucleotide sequences of the left and right junctions of Tn*5276* in *attB1* of six of these transconjugants by direct sequencing of PCR amplification products containing these regions. This approach eliminated the interference of PCR errors in the nucleotide sequence, which may occur when amplification products are cloned prior to sequence analysis. In all cases, the

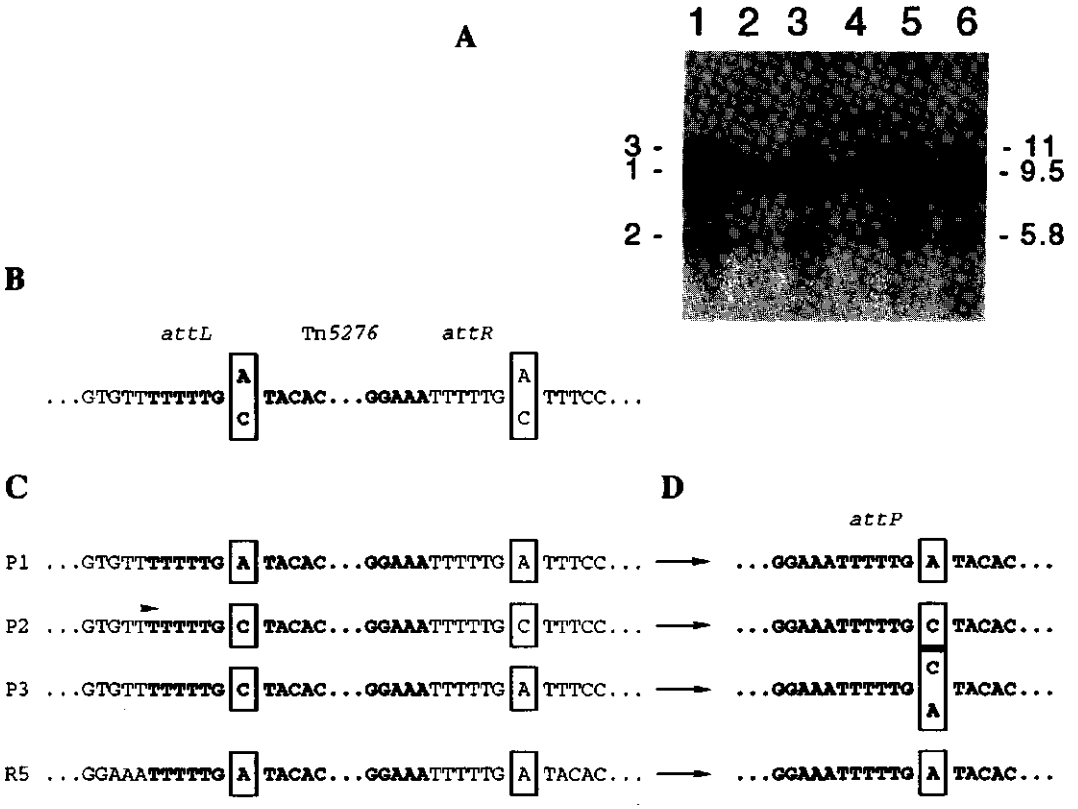

FIG. 3. Analysis of Tn*5276* insertion into the chromosome of *L. lactis* MG1614 after conjugal transfer from strain NIZO R5 and of Tn*5276* circular forms in different *L. lactis* strains. **A:** Tn*5276* insertion profiles in *L. lactis* MG1614-derived transconjugants. Six representative transconjugants from a series of twelve (derived from a mating between *L. lactis* NIZO R5 and MG1614 using the method of Broadbent and Kondo [1992]) are shown. Transconjugant colonies picked directly from the mating plate were used to inoculate liquid cultures, which were grown overnight. Total DNAs were isolated from these cultures, digested with *Eco*RI and separated by agarose gel electrophoresis. A Southern blot was then hybridized to a probe specific for the left end of Tn*5276*. The estimated sizes of hybridizing fragments are indicated in kilobases. The insertion sites corresponding to the hybridizing *Eco*RI fragments have been numbered in accordance with the assigned *attB* site numbers. **B:** Nucleotide sequences of Tn*5276* junctions after insertion into *L. lactis* MG1614 *attB1*. The junctions were PCR amplified using the total DNAs of the six transconjugants from panel A as templates. The nucleotide sequences shown here were found for all six transconjugants. Only one strand is shown. **C:** Nucleotide sequences of Tn*5276* junctions in transconjugant progeny. Total DNAs were isolated from liquid cultures inoculated with progeny colonies derived from the first transconjugant in panel A. Three of these DNAs (that by hybridization analysis were shown to contain Tn*5276* in *attB1* and were termed P1, P2, and P3) were used to PCR-amplify the Tn*5276* junctions and the nucleotide sequences of the amplification products were determined. The NIZO R5 junction sequences are form Rauch and De Vos (1992a). **D:** Nucleotide sequences of Tn*5276* circle joints in different *L. lactis* strains. The total DNAs from panel C and total DNA from *L. lactis* NIZO R5 were used as templates for PCR amplification of the joints of the Tn*5276* circular form. The nucleotide sequences of the amplification products were determined. Nucleotides at the variable positions in the overlap regions are boxed and Tn*5276*-specific nucleotides (according to the model in Fig. 2) are in bold face in panels B, C, and D.

sequences of the left and right amplification products contained two different nucleotides (A and C) at the positions 3' from the TTTTTG hexanucleotides (Fig. 3B). The nucleotide sequence variation found in the Tn*5276* junctions concurs with the model in Fig. 2.

There are two possible explanations for the fact that both an A and a C appear in the nucleotide sequences derived from single transconjugant colonies: firstly, the presence of two types of progeny resulting from resolution of the heteroduplexes through replication and secondly, the presence of transconjugants derived from different mating events (see above). Analysis of junction sequences in progeny colonies could discriminate between these possibilities. The left and right junctions of three progeny colonies containing Tn*5276* in *attB1* (T165P1 through P3) were PCR-amplified and their nucleotide sequences were determined (Fig. 3C). Two of the three combinations of *attL* and *attR* sequences found (mismatch nucleotides A left / A right in T165P1 and C left / C right in T165P2) were not compatible with resolution of the heteroduplexes through replication. Thus, mismatch repair rather than replication was responsible for the correction of the mismatches. This would mean that the original transconjugant colonies contained progeny from different mating events.

Mismatches in the overlap regions are also allowed in Tn*5276* excision. Elsewhere, we have described how the expression of the *xis* and *int* genes of Tn*5276* *in trans* in *E. coli* resulted in the excision of the Tn*5276* ends contained in plasmid pNZ780 (Rauch and De Vos, 1993). When both a functional *xis* gene and *int* genes were present on the complementing plasmid (plasmid pNZ781), excision was very efficient. When only a functional *int* gene was present (plasmid pNZ783), excision still occurred but less efficiently. The left end of Tn*5276* in pNZ780 was derived from the *attB1* insertion of Tn*5276* in transconjugant T165.6 (with a C following the TTTTTG hexanucleotide) and the right end from strain NIZO R5 (with an A following the TTTTTG hexanucleotide). Therefore, heteroduplexes identical to those found to occur in Tn*5276* insertion into strain MG1614 *attB1* should be formed in excision of the Tn*5276* ends from pNZ780.

The nucleotide sequences of excision sites were determined by using pNZ780 molecules that had lost the Tn*5276* ends through Int-mediated excision as templates. These pNZ780 molecules were derived from plasmid DNA preparations of two cultures each of *E. coli* HB101 harboring pNZ780 and either pNZ781 or pNZ783. These plasmid preparations contained four kinds of molecules: (i) pNZ81 or pNZ783 (5.4 kb), (ii) pNZ780 still containing the Tn*5276* ends (5.3 kb), (iii) circularized Tn*5276* ends (1.9 kb), and (iv) pNZ780 no longer containing the Tn*5276* ends (3.4

kb). The latter class of plasmids was separated from the other plasmids present in the preparation by isolating them as 3.4 kb *Bam*HI-linearized molecules. The 3.4 kb *Bam*HI fragments were used directly as templates for nucleotide sequencing. In all cases both an A and a C residue (Fig. 4) were found to be evenly distributed at the position 3' from the TTTTTG hexanucleotide, as judged from band intensities in the nucleotide sequence ladders (data not shown).

Joints of the circularized Tn*5276* ends present in the plasmid DNA preparation of *E. coli* HB101 containing pNZ780 and pNZ781 were cloned as 1.3 kb *Bgl*II-*Pst*I fragments into M13mp19 digested with *Bam*HI and *Pst*I and their nucleotide sequences were determined (Fig. 4). The

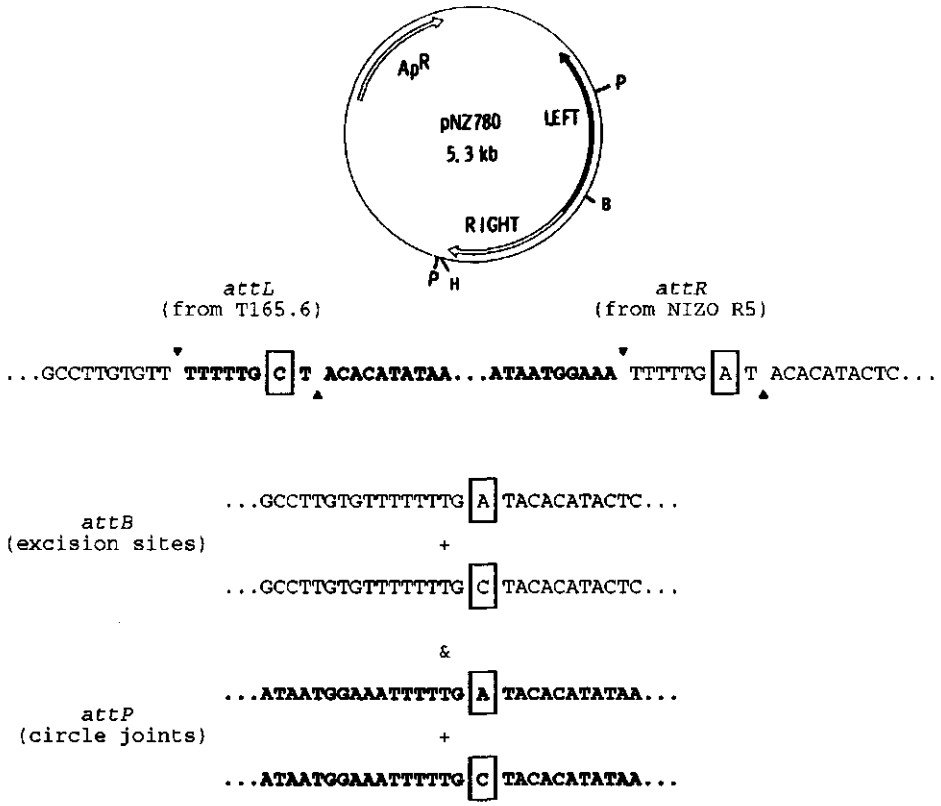

FIG. 4. Nucleotide sequence analysis of the products of excision of the Tn*5276* ends in *E. coli*. A physical map of pNZ780 and the nucleotide sequences of the junctions of Tn*5276* present in pNZ780 are shown. Below these sequences, the nucleotide sequences determined from M13mp19 clones containing Tn*5276* circle joints (*attP*) and the sequences of excision sites (*attB*) determined from pNZ780 derivatives no longer containing the Tn*5276* ends. The triangles indicate the positions of the cuts generated in the Int-mediated excision as in Fig. 2. Tn*5276*-specific nucleotides (according to the model in Fig. 2) are in bold face and the variable nucleotide is boxed. Abbreviations in the physical map: B, *Bgl*II; H, *Bam*HI; P, *Pst*I.

TTTTTG hexanucleotide was present in all joints and was followed by either the C residue from *attL* (3 out of 6) or the A residue from *attR* (3 out of 6). Thus, the C and A residues were also evenly distributed over these excision products. Since the circle formed by the Tn*5276* ends could not replicate, the presence of joints either containing the C residue or the A residue in the M13 clones indicated that the heteroduplex proposed to be formed in the excision process was corrected by an *E. coli* mismatch repair system, either immediately after excision or after cloning of the joint.

These results show that Tn*5276* excision can be described by the model proposed above (Fig. 2) and indicate that also in Tn*5276* excision (in *E. coli*) formation of a heteroduplex with a 1 bp mismatch is tolerated.

Detection and analysis of circular forms of Tn*5276* in *L. lactis*. Pulsed Field Gel Electrophoresis experiments have shown that Tn*5276* is present as an integral part of the chromosome in *L. lactis* (Rauch and De Vos, 1992a). In hybridization experiments the circular form of Tn*5276* could not be detected in *L. lactis* (Rauch and De Vos, 1992a). However, using PCR amplification of the circle joint, we were able to detect Tn*5276* circular forms in *L. lactis*. Two amplification rounds (the second with the product from the first round as a template) were needed to obtain sufficient product for nucleotide sequencing. The nucleotide sequences of the amplification products were determined for strains NIZO R5, T165P1, T165P2, and T165P3 (Fig. 3D) and were found to agree with the proposed model for Tn*5276* excision and insertion (Fig. 2).

DISCUSSION

This paper describes the analysis of Tn*5276* site-specific recombination through the analysis of the nucleotide sequences of its products both *in vivo* in *L. lactis* and in an artificial system in *E. coli*.

The sequence variability of the Tn*5276* site-specific recombination products showed that heteroduplexes are probably allowed in the overlap regions in Tn*5276* site-specific recombination. Heteroduplexes in the regions of strand exchange are a prominent feature of the models for excision and insertion of Tn*916* and Tn*1545* (Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1990). In site-specific recombination between sites with non-identical overlap regions mediated by the integrase of bacteriophages λ, the partial heteroduplexes in the overlap region can limit or prevent branch migration from the first to the second exchange site (De Massy *et al.*, 1989). As

a consequence, the location of the second single-strand exchange point is probably altered and formation of heteroduplex products is prevented. This results in the unequal segregation of the mismatched bases into the two recombination products (Weisberg *et al.*, 1983, Bauer *et al.*, 1989). In contrast, the Tn*5276* Int-mediated excision reaction in *E. coli* appeared to proceed efficiently in the presence of functional Tn*5276* Xis (Rauch and De Vos, 1993). In addition, the mismatched bases were found to be evenly distributed over the recombination products in Tn*5276* excision and insertion. Both observations strongly suggest that the generation of a single basepair heteroduplex, as found in the Tn*5276* overlap regions after its insertion into *L. lactis* MG1614 *attB1*, is no barrier for Tn*5276* site-specific recombination.

After insertion of Tn*5276* into *attB1* of strain MG1614 heteroduplexes can be resolved through replication or corrected by a mismatch repair system. The results obtained here indicate that mismatch repair is responsible for correction of the mismatches. The appearance of different combinations of *attL* and *attR* sequences and of heterogeneous Tn*5276* insertion patterns in the original transconjugant colonies indicate that transconjugant colonies appear containing progeny from separate mating events. It is conceivable that mating complexes (possibly mediated by aggregation proteins) are formed in which a number of donor and recipient cells are joined and different transfer events take place. In this respect one should bear in mind that *L. lactis* grows in chains and that chain-chain interaction may occur.

In analogy to lambdoid phages and other genetic elements that site-specifically recombine with the bacterial genome, we termed Tn*5276* insertion sites *attB* sites and the joint of the circular intermediate part of the Tn*5276* *attP* site. We do not know the size of the Tn*5276* attachment sites; in analogy to λ these sites should contain all regions involved in binding of proteins involved in the recombination process. The insertion profiles of different transconjugants (Fig. 3A; Rauch and De Vos, 1992a) show that Tn*5276* can insert into at least six sites in the genome of *L. lactis* MG1614, of which three sites (*attB1*, *B2*, and *B3*; 1, 2, and 3 in Fig. 3A) are used most often. Of these three sites, *attB1* is preferred. This insertional behavior probably reflects the need for the presence of specific sequences with sufficient similarity to the putative *attP* core region for Tn*5276* insertion to occur. Using the model for excision of Tn*5276* (Fig. 2), two Tn*5276* *attB* sites, NIZO R5 *attB* and MG1614 *attB2*, can be deduced from the *attL* and *attR* sequences in *L. lactis* NIZO R5 (Rauch and De Vos, 1992a; Fig. 2) and the second site in *L. lactis* T165.4 (Fig. 1), respectively, and added to *attB1* of *L. lactis* MG1614 (Fig. 5). Remarkably, the Tn*5276* insertion site found in

the wild-type strain *L. lactis* NIZO R5 shares a stretch of 25 bp with the Tn*5276 attP* site. In analogy to λ, these regions in Tn*5276 attP* and *L. lactis* NIZO R5 *attB* could be termed the core regions of these attachment sites. The two *L. lactis* MG1614 *attB* sites *attB1* and *attB2* share only 13 and 15 bp with the *attP* core region, respectively. Apparently, regions outside the proposed core region are of importance for Tn*5276* insertion, since *L. lactis* MG1614 *attB1* is preferred for insertion over *attB2*, despite its lower percentage of identity to *attP* and the presence of a mismatch in the overlap region.

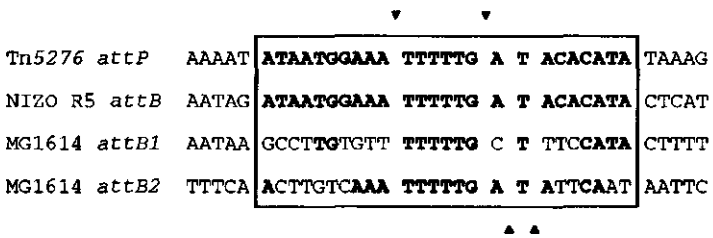

FIG. 5. Comparison of determined and deduced Tn*5276 attP* and *attB* sites. The sizes of the *attP* and *attB* sites (defined as the regions in which proteins involved in recombination bind) are unknown and only the parts relevant to the comparison are shown. The *attP* sequence is that present in the circular form of Tn*5276* after excision from its location in *L. lactis* NIZO R5. NIZO R5 *attB* and MG1614 *attB2* have been derived from the Tn*5276* junction sequences in *L. lactis* NIZO R5 and the secondary insertion in *L. lactis* T165.4 (see Fig. 1), respectively, using the model in Fig. 2. MG1614 *attB1* is *L. lactis* MG1614 site 1 (Rauch and De Vos, 1992a). The 'core region', defined as the region of sequence identity between *attP* and NIZO R5 *attB*, is boxed. Nucleotides identical to *attP* in this region are in bold face. The outer and inner triangles indicate the maximal and minimal overlap regions, repsectively, generated by the Int-mediated single-strand cuts as based on the nucleotide sequences of Tn*5276* site-specific recombination products.

When the insertional behavior of Tn*5276* is compared to that of other genetic elements that use Int-mediated site-specific recombination for insertion and excision into bacterial genomes, Tn*5276* appears to combine the properties of two different groups of mobile elements. Like Tn*916* and Tn*1545*, Tn*5276* can insert into several sites, which show only limited sequence similarity to the inserting transposon. However, in the cases examined thus far for Tn*5276*, the recombining sites contain overlap regions of (near) sequence identity, like in the case of the temperate bacteriophages and streptomycete integrative plasmids (Hoess *et al.*, 1982; Weisberg *et al.*, 1983; Brown *et al.*, 1990).

Excision and insertion of Tn*5276* are mediated by Tn*5276* Int. In site-specific recombination reactions, a pair of single-stranded cuts is made and single-stranded (5' or 3') overhanging ends are generated, creating an overlap region. The members of the integrase family of site-specific

recombinases of which strand exchange has been examined generate 5' protruding ends of 6-8 bases (Sadowski, 1986). For Tn*916* and Tn*1545*, nucleotide sequencing data suggest overlap regions of 5 to 8 basepairs (Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1989, 1990). As was done for Tn*916* and Tn*1545*, possible positions of the cuts generated by Tn*5276* Int can be postulated, based on the nucleotide sequences of recombination products (Fig. 2 and 5). The resulting overlap region could be 1 to 8 bp long (Fig. 5). Comparable to the experiments with Tn*916* and Tn*1545*, we presently cannot distinguish between the generation of 5' or 3' protruding ends in the overlap region.

Using PCR amplification, we were able to show the presence of circular forms of Tn*5276* in the wild-type strain *L. lactis* NIZO R5 and several *L. lactis* MG1614-derived transconjugants. Apparently, the circular forms were present in very low amounts, since they were not visible in hybridization analyses (Rauch and De Vos, 1992a) and two PCR amplification rounds were needed to obtain sufficient amounts of product for nucleotide sequencing. The nucleotide sequences of the amplification products were as predicted by the model for Int-mediated excision. This was also the case for the joints of the circles formed by the excised Tn*5276* ends in the presence of a functional *int* gene (Fig. 4). Therefore, it is very likely that the circular forms of Tn*5276* detected in *L. lactis* are the products of Int-mediated excision of Tn*5276* and are the intermediates that are transferred in conjugal matings. It could not be determined whether the heteroduplex present in the circular form immediately after excision from one site was maintained or repaired. Low-level excision of Tn*5276* might be responsible for the instability of nisin production reported for some strains (Kozak *et al.*, 1974). Possibly, only the small fraction of cells containing Tn*5276* in the free, circular form is 'conjugally competent'. For Tn*5381* it has been shown that exposure to tetracycline raises both the level of free, circular form of the transposon and the frequency of conjugal transfer (Rice *et al.*, 1992). This phenomenon is in line with the assumption that gram-positive, conjugative transposons can only be transferred from cells that contain their free, circular forms. Contact between a 'competent' donor cell and a recipient cell might then trigger the transfer of Tn*5276* between these cells (see also Scott, 1992), and possibly also between other cells in the strands containing them. The elucidation of the cell-to-cell transfer mechanism(s) of conjugative transposons will provide a challenge for future genetic research in gram-positive bacteria.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and bacteriophages; microbiological techniques. The lactococcal strains used are referenced in the text or below. For PCR amplification of the Tn*5276* junctions in *attB2* in *L. lactis* T165.4 (Rauch and De Vos, 1992a), a derivative (designated T165.4S2) was used that was obtained after repeated subculturing and that no longer contained the copy inserted into *attB1*. *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) was used as a host for pNZ780, pNZ781, and pNZ783 (Rauch and De Vos, 1993) and *E. coli* TG1 (Gibson, 1984) as a host for M13mp19 (Yanisch-Perron *et al.*, 1985).

Growth and culture conditions were as described (Rauch and De Vos, 1992a). The conjugal mating between *L. lactis* strains NIZO R5 (Galesloot and Pette, 1957) and MG1614 (Gasson, 1983) was performed as described by Broadbent and Kondo (1991).

DNA amplification methods. All PCR reactions were carried out in reaction mixtures composed as described previously (Kuipers *et al.*, 1991) and, unless indicated otherwise, with approximately 100 ng of *L. lactis* total DNA as a template. The oligonucleotide primers used are listed in Table 1. Except in the cases indicated, PCR was performed in 30 cycles, each cycle consisting of a denaturing step at 93°C for 1 min, a primer annealing step at a temperature dependent on the primers used (see below) for 1.5 min, and an extension step at 72°C for 2.5 min using a Biomed Thermocycler 60 (ICN Biomedicals, Inc., Amsterdam, The Netherlands). For purification, the amplification products were electrophoresed on 2% agarose gels and recovered as

TABLE 1. Oligonucleotides used in this study

Code	Nucleotide sequence	Location
PL1	5'-GCTGTATAGTTTTGCTTTGG	Tn*5276* left end; complementary to nucleotides (nt) 107-126 in Rauch and De Vos, 1992a)
PL2	5'-GTATGAACTAGGGCTG	left hand region of *attB1*; 5'-end 156 nt to the left of the TTTTTG hexanucleotide
PR1	5'-CTAACCAAGAGACTAACC	Tn*5276* right end; nt 118-135 in Rauch and De Vos (1992a)
PR2	5'-AACTGGCAAATCATGG	right hand region of *attB1*; 3'-end 86 nt to the right of the TTTTTG hexanucleotide
IPL1	5'-GGTTGTTTGTTTCGGAAG	Tn*5276* left end; 5'-end located 5434 nt to the right of the TTTTTG hexanucleotide and 57 nt to the left of the nearest *Eco*RI site (Kuipers *et al.*, 1993)
SPR1	5'-AATCATGAGGAAGATTAGGG	Tn*5276* right end; nt 158-177 in Rauch and De Vos (1992a)

described previously (Rauch and De Vos, 1992a). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (MilliGen Biosearch Division, San Rafael, Calif.).

For the amplification of the left end right junctions of Tn*5276* inserted into *attB1* of *L. lactis* MG1614-derived transconjugants, primer pairs PL1 and PL2 (annealing temperature 43°C; 270 bp product) and PR1 and PR2 (annealing temperature 41°C; 240 bp product) were used, respectively. For amplification of the joints of Tn*5276* circles, primers PL1 and PR1 (annealing temperature 45°C; 250 bp product) were used.

Inverse or circular PCR for the amplification of the left junction of Tn*5276* in *L. lactis* T165.4S2 was carried out using a protocol based on the conditions described by Ochman *et al.* (1990). The protocol was as follows. Approximately 1 µg of total DNA was digested with *Eco*RI and after phenol extraction it was ethanol precipitated. After resuspension in 10 mM Tris.HCl, 1 mM EDTA (pH8.0), approximately 10 ng of *Eco*RI-linearized total DNA was circularized through overnight ligation at 15°C in 50 µl of T4 ligation buffer (Gibco/BRL Life Technologies, Gaithersburg, Md.) using 1 Weiss unit of T4 DNA ligase (Gibco/BRL). From the ligation mixture, 2 µl was used in a PCR amplification reaction using IPL1 and PL1 as primers (annealing temperature 47°C). The size of the resulting amplification product (approximately 300 bp) was as expected from the size (5.8 kb) of the *Eco*RI fragment containing the left junction of Tn*5276* (Rauch and De Vos, 1992a) and the position of the Tn*5276* *Eco*RI site closest to the left end (5.6 kb from the TTTTTG hexanucleotide; Kuipers *et al.*, 1993).

For the amplification of the right junction of Tn*5276* from total DNA of *L. lactis* T165.4S2, a modification of the PCR mediated by a single primer described by Parks *et al.* (1991) was used. Total DNAs from *L. lactis* MG1614 and *L. lactis* NIZO R5 served as negative controls. The procedure was as follows. Oligonucleotide SPR1 was used as a primer in standard reaction mixtures composed as described above. The PCR consisted of two stages. The first (low stringency) stage consisted of 20 cycles, which included a denaturing step at 93°C for 1 min, a primer annealing step at 30°C for 1.5 min, and two extension steps at 56°C (1 min) and 72°C (2.5 min). The second (high stringency) stage was identical to the first stage, except that the temperature of the annealing step was raised to 47°C. Under these conditions *L. lactis* NIZO R5 DNA failed to produce amplification products, while *L. lactis* MG1614 DNA produced a significant amount of an amplification product of approximately 700 bp, next to low amounts of smaller products. The

DNA of *L. lactis* T165.4S2, next to these products, gave rise to an amplification product of approximately 500 bp.

DNA manipulations, agarose gel electrophoresis, DNA transfer, and hybridization. DNA manipulations and agarose gel electrophoresis were carried out as described previously (Rauch and De Vos, 1992a). DNA was transferred from agarose gels to GeneScreen Plus nylon membranes (Du Pont, NEN Research Products, Wilmington, Del.) also as described previously (Rauch and De Vos, 1992b). Membranes were hybridized to an oligonucleotide probe specific for the left end of Tn*5276* (PL1; Table 1) at a hybridization temperature of 54°C. Hybridization and washing conditions were as recommended by the membrane manufacturer.

DNA sequencing. Subclones in M13mp19 were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio). Double stranded DNAs (pNZ780 dérivatives and amplification products) were sequenced by the method of Hsiao (1991). When sequencing amplification products (isolated from agarose gels), 0.5% Non-idet P40 (Gibco/BRL) was added to all reaction mixtures and the amount of primer was raised to 10 pmol. Oligonucleotide primers specific for the regions to be sequenced were chosen from the list in Table 1.

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CHAPTER 8

DISTRIBUTION AND EVOLUTION OF NISIN-SUCROSE ELEMENTS IN *LACTOCOCCUS LACTIS*

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A preliminary account of part of the work described in this chapter has been presented previously in "Nisin and Novel Lantibiotics" (G. Jung and H.-G. Sahl, Eds.) ESCOM, Leiden, The Netherlands, pp. 243-249

Abstract The distribution, architecture, and conjugal capacity of nisin-sucrose elements in wild-type *Lactococcus lactis* strains were studied. Element architecture was analyzed with the aid of hybridizations to different probes derived from the nisin-sucrose transposon Tn*5276* of *L. lactis* NIZO R5, including the *nisA* gene, the left and right ends, and IS*1068* (formerly designated iso-IS*904*) located between the left end and the *nisA* gene. Three classes of nisin-sucrose elements could be distinguished in the thirteen strains investigated. Classes I and II consist of conjugative transposons containing a *nisA* gene and a *nisZ* gene, respectively. Representative conjugative transposons of these classes include Tn*5276* (class I) from *L. lactis* NIZO R5 and Tn*5278* (class II) from *L. lactis* ILC11. The class II transposon found in *L. lactis* NCK400 and probably all class II elements are devoid of IS*1068*-like elements, which eliminates the involvement of an iso-IS*1068* element in conjugative transposition. Members of class III contain a *nisZ* gene, are non-conjugative and do not contain sequences similar to the left end of Tn*5276* at the appropriate position. The class III element from *L. lactis* NIZO 22186 was found to contain an iso-IS*1068* element, termed IS*1069*, at a position corresponding to that of IS*1068* in Tn*5276*, but in an inversed orientation. The results suggest that an iso-IS*1068*-mediated rearrangement is responsible for the dislocation of the transposons left end in this strain. A model for the evolution of nisin-sucrose elements is proposed and the practical implications for transferring nisin A or nisin Z production and immunity are discussed.

INTRODUCTION

Interest in the structure, function, and application of the lantibiotic nisin, produced by a number of *Lactococcus lactis* strains, has led to the identification of conjugative transposons carrying the genetic information for nisin biosynthesis, sucrose fermentation, and other traits (for a review see 29). The recently described transposons Tn*5276* from strain NIZO R5 (27), Tn*5301* from strain NCFB 894 (15), and Tn*5307* from strain ATCC 11454 (2, 11, 33) are all approximately 70 kb in size and show a similar organization. Upstream from the nisin structural gene, *nisA*, the transposons contain an identically orientated IS element, designated IS*904* in Tn*5301* (4) and IS*1068* (formerly called IS*904* or iso-IS*904*) in Tn*5276* (26, 27). The nucleotide sequence of IS*1068* differs from that of IS*904* in eight positions and as a consequence has an increased coding capacity (25). The nucleotide sequences of the ends of Tn*5276* and Tn*5301* are identical and both contain a single copy of the IS element that is separated from the left end by approximately 250 bp (15, 27). In contrast to earlier suggestions (2, 4, 33), it is unlikely that this IS element is involved in

conjugative transposition since (i) the sites in which Tn*5276* and Tn*5301* were found to insert show no homology to the IS element, and (ii) exision of the ends of Tn*5276* was found to depend on the activity of *int* and *xis* genes that are located at the right end of Tn*5276* (28).

Recently it was found that there are two natural variants of nisin, nisin A and nisin Z encoded by the *nisA* and *nisZ* gene, respectively (3, 22). Both variants contain dehydrated amino acids and lanthionine rings (13, 22), but they differ in a single amino acid (Asn instead of His in nisin Z at position 27). Due to this substitution, nisin Z shows larger inhibition zones in agar diffusion assays than nisin A (3). It was found that the *nisA* and *nisZ* genes are distributed equally among 23 naturally occurring, sucrose-fermenting *L. lactis* strains (3). All conjugative transposons studied up to now code for the production of nisin A. Since conjugal transfer of the ability to produce nisin is of considerable significance in industrial strain improvements (1, 8, 12), it was of interest to determine whether the production of nisin Z could also be conjugally transferred simultaneously with the capacity to ferment sucrose. Therefore, we compared the conjugative capacity of nisin Z- and nisin A-producing strains and examined the organization of their nisin-sucrose elements. We found that the *L. lactis* nisin-sucrose elements are heterogeneous and can be grouped into three classes based on their architecture. A new iso-IS*1068* element, designated IS*1069*, was characterized in *L. lactis* NIZO 22186, which produces nisin Z (22). There appeared to be a strong correlation between the integrity of the extreme ends of the investigated elements and the ability to conjugally transfer sucrose fermentation and the production of nisin.

MATERIALS AND METHODS

Bacterial strains and culture and conjugation conditions. *Escherichia coli* TG1 (10) was used as a host for M13-derived vectors. The *L. lactis* strains used are listed in Table 1. Conjugal matings and the identification of transconjugants were carried out as described previously (27).

DNA manipulations and hybridizations. Bacteriophage and plasmid DNAs were isolated from *E. coli* essentially by using established protocols (30). Isolation of total *L. lactis* DNA was carried out as described previously (27). DNA was digested with restriction enzymes (Gibco/BRL Life Technologies, Gaithersburg, Md.) as recommended by the manufacturer and separated by agarose gel electrophoresis as described (30). DNA fragments were recovered from agarose gels with a USBioclean kit (U.S. Biochemical Corp., Cleveland, Ohio) or transferred to GeneScreen Plus nylon

membranes (Du Pont, NEN Research products, Wilmington, Del.) with the use of a VacuGene XL unit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and using the alkaline blotting protocol supplied by the manufacturer. Hybridization, washing, and deprobing conditions were as recommended by the membrane manufacturer.

As a probe for sequences homologous to the left end of Tn*5276* (Tn*5276*-L probe), an oligonucleotide with the sequence 5'-CCAAAGCAAAACTATACAGC (nt 107-126 in the sequence of the Tn*5276* left end [27]) was used at a hybridization temperature of 54°C. As probes for IS*1068*-like sequences oligonucleotides with the sequences 5'-GAGCTGAGGCTACTCA (IS-I probe; nt 1165-1180 in Chapter 3, Fig. 2) and 5'-GGCATGAACCTCAAGC (IS-II probe; complementary to nt 224-239 in Chapter 3, Fig. 2) were used at a hybridization temperature of

TABLE 1. *L. lactis* strains used in this study and classification of their nisin-sucrose elements

Strain	Source (reference)[a]	Type of nisin gene[b]	Conjugative capacity[c]	Class[d]	Lane no. in Fig. 1
NIZO R5	NIZO (6)	*nisA*	+	I	1
INRA 2	INRA (3)	*nisA*	+	I	3
INRA 3	INRA (3)	*nisA*	-	I	4
INRA 5	INRA (3)	*nisA*	+	I	5
NZI	UP (3)	*nisA*	+	I	6
ILC13	ILC (3)	*nisA*	+	I	8
ILC11	ILC (3)	*nisZ*	+	II	7
ILCpSL5	ILC (3)	*nisZ*	+	II	11
NCK400	NCSU (14)	*nisZ*	+	II	13
NIZO 22186	NIZO (22)	*nisZ*	-	III	2
ILC19	ILC (3)	*nisZ*	-	III	9
ILC126	ILC (3)	*nisZ*	-	III	10
ILCpSL20	ILC (3)	*nisZ*	-	III	12
MG1614[e]	(7)	none	-	-	-

[a] INRA, G. Limsowtin, Institut National de Recherche Agronomique, Jouy en Josas, France; UP, M. Nuti, University of Padua, Padua, Italy; ILC, G. Giraffa, Instituto Sperimentale Lattiero-Caesiario, Lodi-Milan, Italy; NCSU, T. R. Klaenhammer, North Carolina State University, Raleigh, USA.

[b] see ref. 3.

[c] +, conjugative; -, non-conjugative: result of 3-10 independent conjugation experiments.

[d] for definitions of classes see text.

[e] plasmid-free, streptomycin- and rifampin-resistant strain used as recipient in conjugation experiments.

48°C. As a probe for the *nisA* or *nisZ* genes, an oligonucleotide with the sequence 5'-ATGGGTTGTAATATGAAAAC (*nis* probe; 27) was used at a hybridization temperature of 45°C. Two different probes homologous to sequences at the right end of Tn*5276* were used. One was an oligonucleotide with the sequence 5'-GGTTAGTCTCTTGGTTAG (Tn*5276*-R probe; complementary to nt 118-135 in the nucleotide sequence of the Tn*5276* right end; 27) that was used at a hybridization temperature of 50°C. The other was the 2.3-kb *Hin*dIII-*Eco*RI fragment from pNZ774 (27) located at the right end of Tn*5276* that was hybridized at 65°C. The latter probe was labeled by nick-translation using [α-^{32}P]ATP whereas all oligonucleotides were end-labeled with [γ-^{32}P]ATP as described (30).

DNA sequencing. The nucleotide sequences of both strands of the iso-IS*1068* element (IS*1069*) upstream from the *nisZ* gene of *L. lactis* NIZO 22186 and its surrounding regions were determined following the dideoxy-chain termination method (31) adapted for Sequenase version 2.0 (U.S. Biochemical Corp.) with overlapping restriction fragments cloned into M13mp18 and M13mp19 (36) and using either the M13 universal primer or synthesized primers. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (MilliGen Biosearch Division, San Rafael, Calif.). The sequence data were assembled and analyzed with the PC/Gene program version 5.01 (Genofit, Geneva, Switzerland).

Polymerase Chain Reaction (PCR). PCR experiments were performed in reaction mixtures composed as described previously (19) containing approximately 100 ng of *L. lactis* total DNA as a template. The oligonucleotide primers used were nis10 (5'-GGATAGTATCCATGTCTGAAC; complementary to bases 201-221 in the nucleotide sequence of the *nisZ* gene of *L. lactis* NIZO 22186 [22]) in combination with either IS-I or IS-II. PCR was performed in 30 cycles, each cycle consisting of a denaturing step at 93°C for 1 min, a primer annealing step at 45°C for 1.5 min and an extension step at 72°C for 2.5 min, using a Biomed Thermocycler 60 (ICN Biomedicals, Inc., Amsterdam, The Netherlands).

Nisin bioassays and differentiation. Supernatants of *L. lactis* strains were analyzed for the production of nisin by the agar diffusion assay with *Micrococcus luteus* as indicator (3). Differentiation between the production of nisin A and nisin Z was obtained by analyzing supernatant samples concentrated by hydrophobic interaction chromatography using reversed-phase HPLC as described (3, 22).

RESULTS AND DISCUSSION

Conjugative transfer of sucrose fermentation and the production of nisin Z and nisin A. Nisin Z was initially found to be produced by *L. lactis* strain NIZO 22186, which also ferments sucrose (22). Numerous attempts to conjugally transfer the capacity to ferment sucrose and nisin Z production from strain NIZO 22186 to *L. lactis* MG1614 or other lactococcal strains failed (data not shown). In contrast, transfer of Tn*5276* from *L. lactis* NIZO R5 specifying nisin A production was easily achieved under the same conditions (27). Therefore, other available nisin Z producers were screened for their capacity to conjugally transfer the ability to ferment sucrose to strain MG1614 (Table 1). Three out of seven *L. lactis* strains containing the *nisZ* gene were found to transfer the capacity to ferment sucrose and nisin production, with similar frequencies as Tn*5276* was transferred from strain NIZO R5 (10^{-8} to 10^{-6} colony forming units (CFU) per CFU of donor). A transconjugant obtained from the mating of strains ILC11 and MG1614 was studied in more detail and as expected produced nisin Z (data not shown). This and other studies (see below) indicated that also nisin Z production was encoded by a conjugative transposon, which also carried sucrose genes. The transposon present in *L. lactis* ILC 11 was designated Tn*5278* (registered with the Plasmid Reference Centre [20]).

To determine whether conjugally inactive nisin-sucrose elements are only found among *L. lactis* strains carrying the *nisZ* gene, five nisin A producers other than strain NIZO R5, which carries Tn*5276*, were also tested for the capacity to transfer sucrose proficiency and nisin production (Table 1). Except for strain INRA 3, all nisin A producers contained nisin-sucrose elements that could be readily transferred in conjugal matings.

A likely explanation for these results is that nisin-sucrose elements similar to Tn*5276* or Tn*5278* were present in all strains, but that in some cases the transposon had lost its conjugative capacity.

Heterogeneity between nisin-sucrose elements in *L. lactis*. To characterize the Tn*5276*- or Tn*5278*-like elements in the 13 studied strains at the DNA level, we hybridized *Hin*dIII digests of total DNAs to a probe for the *nisA* or *nisZ* gene (*nis* probe). The results (Fig. 1A) show that all strains contain at least one copy of a *nis* gene. From previous studies (26, 27) and the complete nucleotide sequence analysis of the 10-kb *nis* operon of Tn*5276* (18, 35) we know that the *nisA* gene is located on a 4.2-kb *Hin*dIII fragment that is flanked by parts of IS*1068* (26, 27) and the *nisT* gene (18). In nisin A producers, this part of the *nis* operon is very well conserved at the

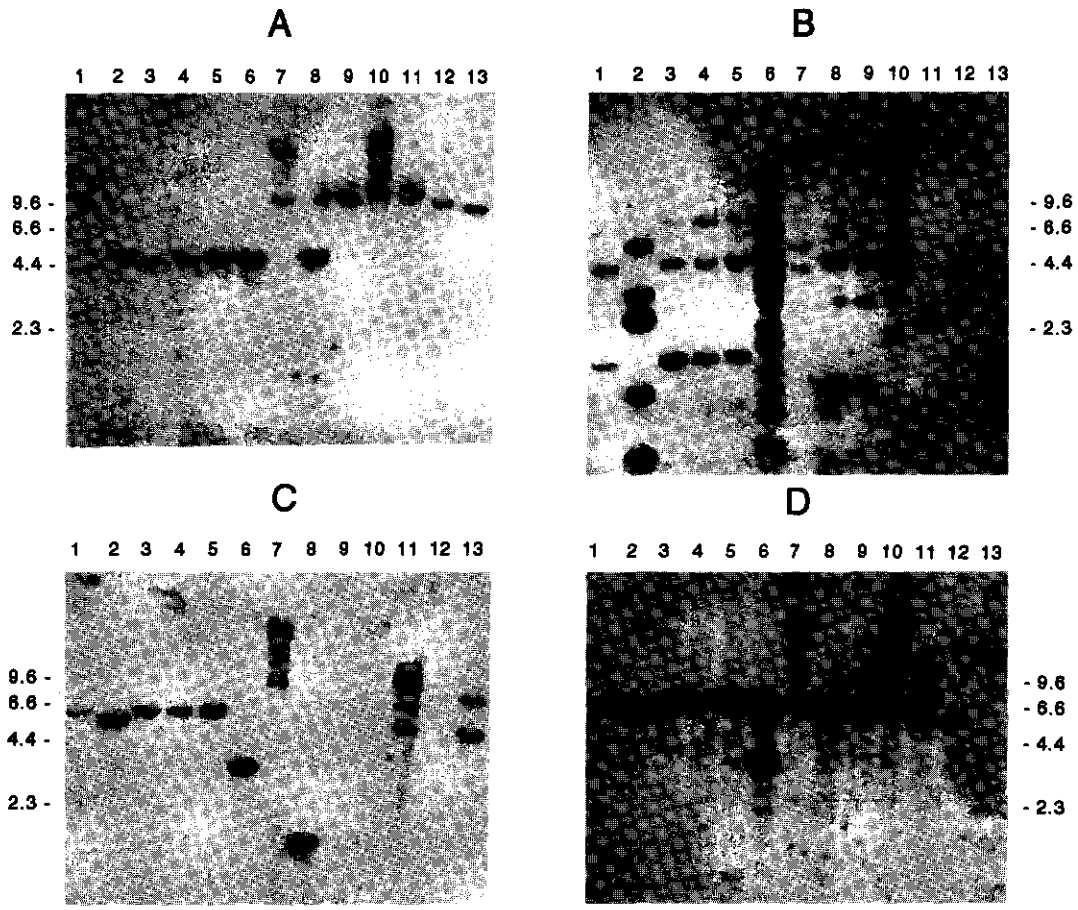
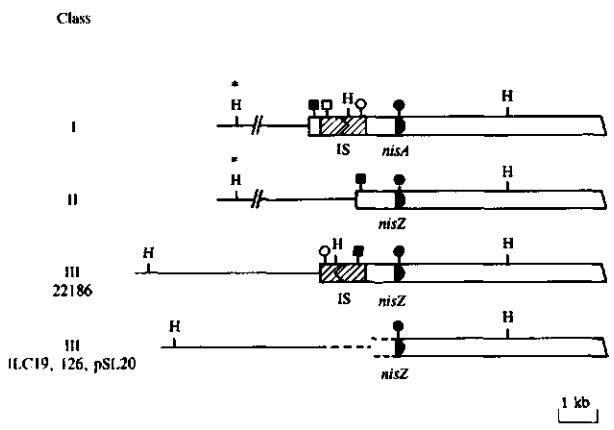


FIG. 1. Hybridizations of total DNAs from wild-type, nisin-producing *L. lactis* strains to Tn*5276*-specific probes. Total DNAs were digested with *Hin*dIII and separated by agarose gel electrophoresis. A Southern blot was hybridized to the *nis* probe (A), IS-I probe (B), Tn*5276*-L probe (C), and Tn*5276*-R probe (D). The lanes contain DNA from the following strains: 1, NIZO R5; 2, NIZO 22186; 3, INRA 2; 4, INRA 3; 5, INRA 5; 6, NZI; 7, ILC11; 8, ILC13; 9, ILC19; 10, ILC126; 11, ILCpSL5; 12, ILCpSL20; 13, NCK400. Lanes 7 and 10 contain small quantities of undigested DNA.

sequence level, as was found by comparing the Tn*5276 nis* genes and their upstream region with those of Tn*5301* (15), Tn*5307* (32), and *L. lactis* strain 6F3 (5). This explains why all nisin A producing strains contained an approximately 4.2-kb *Hin*dIII fragment that hybridized with the *nis* probe. However, the nisin Z producers contained *Hin*dIII fragments of either 4.7 kb (NIZO 22186) or approximately 9.6 kb (other strains) that hybridized to the *nis* probe (Fig. 1A), indicating that the organization of the region upstream from the *nisZ* gene is different from that preceding the *nisA* gene.

Distribution of IS*1068*-like elements and characterization of IS*1069* preceding the *nisZ* gene in *L. lactis* NIZO 22186. To study the organization upstream from the *nisZ* gene in the nisin Z producers, we first determined whether they contained an IS*1068*-like element by hybridization of their DNAs with the IS-I probe that is specific for iso-IS*1068* elements and is located on the 4.2-kb *Hin*dIII fragment also containing the *nisA* gene in Tn*5276* and Tn*5301* (4, 26, 27; Fig. 2). Similar experiments were done with the nisin A producers. The results (Fig. 1B) showed that IS*1068*-like elements are abundant in most strains and that they have possible use as a way to differentiate strains by IS typing as previously suggested (9). Only the nisin A producers appeared to contain an iso-IS*1068* element upstream from the *nisA* gene, since the same 4.2-kb *Hin*dIII fragment hybridized to the *nis* probe and the IS-I probe. However, to detect inverted IS*1068*-like elements upstream from the *nis* genes, the same digested DNAs were also hybridized to the IS*1068*-specific probe IS-II, which is complementary to sequences in between the *Hin*dIII site in IS*1068* or IS*904* and the left end of Tn*5276* or Tn*5301* (4, 26, 27; Fig. 1). The results (summarized in Fig. 2) showed that such an inverted IS*1068*-like element was present only upstream from the *nisZ* gene of strain NIZO 22186. To substantiate this further, the nucleotide sequence of the region



Class	hybridizing *Hin*dIII fragments (kb)[1]			
	Tn*5276*L	IS-II	IS-I	*nis*
I	x	x	4.2	4.2
II	x	-	-	x
III 22186	-	4.7	5.0	4.7
III ILC19, 126, pSI.20	-	-	-	8.9

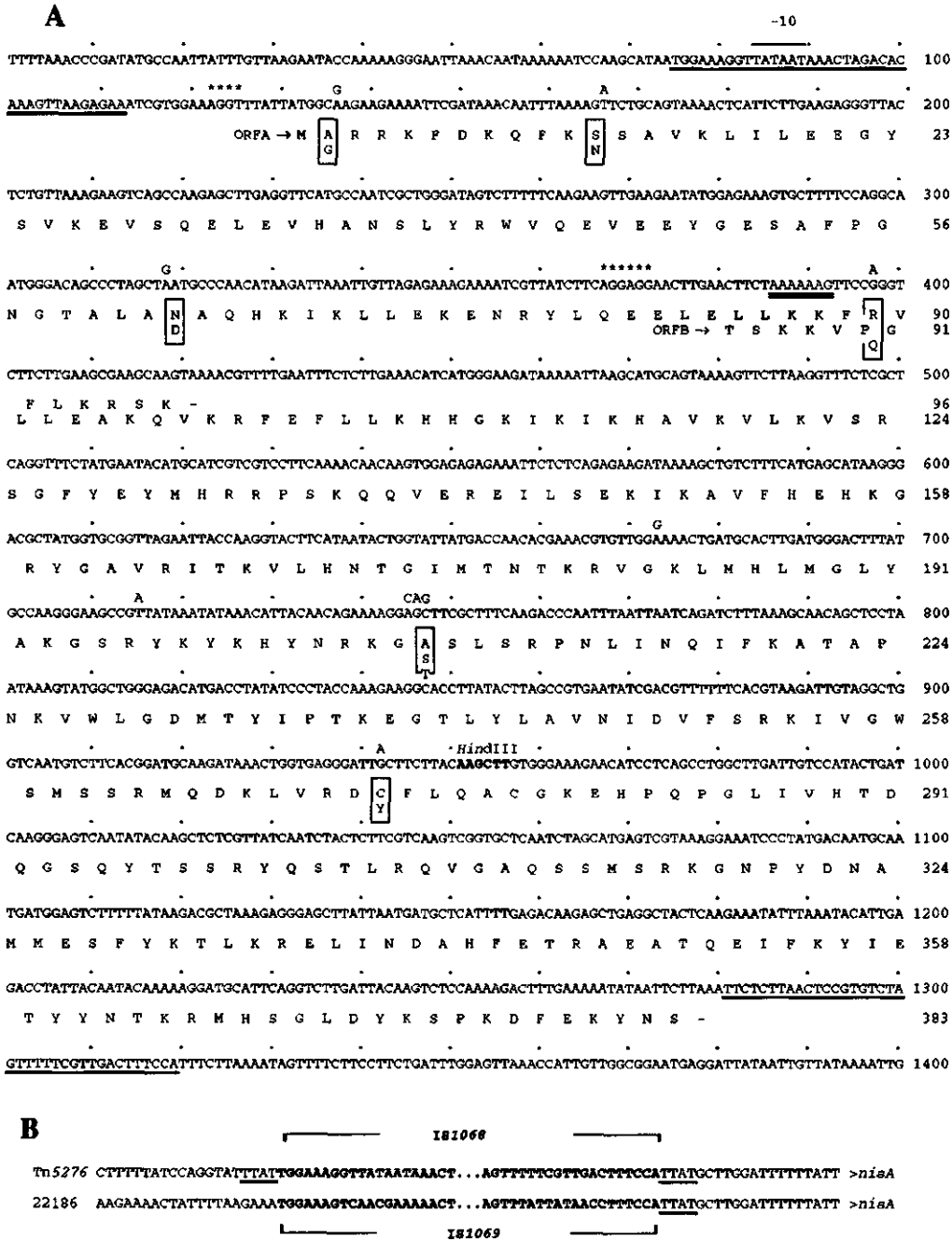
[1] -, no hybridization or hybridization to *Hin*dIII fragment(s) elsewhere in the genome; x, can vary from strain to strain (dependent on location marked (*) *Hin*dIII site) but is identical within strains

FIG. 2. Architecture of left end regions of nisin-sucrose elements and location of the probes used. Lengths of hybridizing fragments are from Fig. 1. H: *Hin*dIII site.

around the *Hin*dIII site upstream from the *nisZ* gene of *L. lactis* NIZO 22186 was determined (Fig. 3). Indeed, this region contained an IS-like element that was very similar to IS*1068* from Tn*5276*. We have designated this element IS*1069* (registered with the Plasmid Reference Centre [Lederberg, 1987]). As expected, the orientation of IS*1069* was opposite to that of IS*1068*. More importantly,the nucleotide sequence of the region left of IS*1069* was completely different from that of the corresponding region in NIZO R5 and only half of the TTAT target repeat that flanks IS*904* in Tn*5301* (4) and IS*1068* in Tn*5276* (26) was found to border IS*1069* (Fig. 3B). Since this region constitutes the left end of Tn*5276*, we propose that IS-mediated rearrangements have dislocated or deleted the left end of the nisin-sucrose element of *L. lactis* NIZO 22186, that as a consequence has become defective since conjugative excision, the first step of its conjugal transposition, would be impaired.

The here characterized IS*1069*, located upstream from the *nisZ* gene in *L. lactis* NIZO 22186, is the fifth in a group of *L. lactis* IS-like elements that show high (94-99%) nucleotide sequence identity and belong to the IS*3* group of insertion sequences (4, 16, 26). However, the small differences on the nucleotide level result in important differences in the coding capacities of these elements. IS*1068* and IS*1069* from Tn*5276* and *L. lactis* NIZO 22186, respectively, show an organization of ORFs that is similar to that found in most members of the IS*3* group: a small ORFA potentially translationally coupled to a larger ORFB, together covering almost the complete IS element (Fig. 3A; 24). The ORFAB fusion protein probably is the active transposase and production of the transposase as a fusion protein possibly plays a role in the regulation of IS transposition activity (23). In IS*904* from Tn*5301* several deletions with respect to IS*1068* and IS*1069* result in the lack of coding capacity for the first 440 bp of the element and only one ORF is present (4). This suggests that IS*904* is no longer transpositionally active. IS*1076*L and IS*1076*R from *L. lactis* Z270 (16) possess one large ORF covering the region that encompasses ORFA and ORFB in IS*1068*. Regulation of the transposition activity of the latter IS elements apparently is not effected through production of the transposase as a fusion protein.

Relation between the architecture of nisin-sucrose elements and their conjugative capacity. The results concerning the organization of the nisin-sucrose element in NIZO 22186 suggest that in other non-conjugative strains rearrangements in the region upstream from the nisin structural gene or elsewhere have rendered the nisin-sucrose elements non-conjugative. To test this possibility, we studied the architecture of nisin-sucrose elements using probes specific for the left


A
-10
ORFA → M A/G R R K F D K Q F K S/N S A V K L I L E E G Y 23
S V K E V S Q E L E V H A N S L Y R W V Q E V E E Y G E S A F P G 56
N G T A L A N/D A Q H K I K L L E K E N R Y L Q E E L E L L K K F R/Q V 90
ORFB → T S K K V P G 91
F L K R S K - 96
L L E A K Q V K R F E F L L K H H G K I K I K H A V K V L K V S R 124
S G F Y E Y M H R R P S K Q Q V E R E I L S E K I K A V F H E H K G 158
R Y G A V R I T K V L H N T G I M T N T K R V G K L M H L M G L Y 191
A K G S R Y K Y K H Y N R K G A/S S L S R P N L I N Q I F K A T A P 224
N K V W L G D M T Y I P T K E G T L Y L A V N I D V F S R K I V G W 258
S M S S R M Q D K L V R D C/Y F L Q A C G K E H P Q P G L I V H T D 291
Q G S Q Y T S S R Y Q S T L R Q V G A Q S S M S R K G N P Y D N A 324
M M E S F Y K T L K R E L I N D A H F E T R A E A T Q E I F K Y I E 358
T Y Y N T K R M H S G L D Y K S P K D F E K Y N S - 383
HindIII
B
IS1068
Tn5276 CTTTTTATCCAGGTATTTATTGGAAAGGTTATAATAAACT...AGTTTTTGGTTGACTTTCCATTATGCTTGGATTTTTTATT >nisA
22186 AAGAAAACTATTTTAAGAAATGGAAAGTCAACGAAAAACT...AGTTTATTATAACCTTTCCATTATGCTTGGATTTTTTATT >nisA
IS1069

and right ends of Tn*5276* (Figs. 1, 2C, and 2D) in the eight conjugative and the five non-conjugative nisin producers. The results (summarized in Table 1 and Fig. 2) showed that three classes of nisin-sucrose elements could be distinguished, designated class I, II, and III. *Class I* (or the Tn*5276*-like class) includes elements that contain a *nisA* gene and a left end region (including an iso-IS*1068* element) that is similar or identical to that of Tn*5276* of strain NIZO R5. All elements in this class are conjugative, except that of *L. lactis* INRA 3. The *class II* elements (or the Tn*5278*-like elements) contain a *nisZ* gene and do not contain a sequence that hybridizes to the IS*1068* probes immediately upstream from this gene. However, elements of this class do contain a region that is similar or identical to the left end of Tn*5276* upstream from the *nisZ* gene and all of them are conjugative. The class II element-containing strain NCK400 is completely devoid of IS*1068*-like sequences. This shows that iso-IS*1068* elements are not involved in the conjugative transposition of nisin-sucrose elements. The elements in *class III* all contain a *nisZ* gene but this is not preceded in its close vicinity by a sequence hybridizing to the Tn*5276*-left end probe. This is compatible with the finding that all class III elements are non-conjugative. There are two groups of class III elements (Fig. 2). Strains ILC19, ILC126, and ILCpSL20 belong to one class and completely lack sequences with homology to the left end of Tn*5276* (Fig. 1C). These strains also contain no IS*1068*-like element immediately preceding the *nisZ* gene as was found in the hybridization studies (Fig. 1B) and using PCR amplification. In the latter experiments, amplification was sought using IS-I or IS-II as a left primer and a primer located downstream from the nisin structural gene (nis10) as a right primer. With DNA from strains NIZO R5 and NIZO 22186, where the distance between the IS element and the *nis* gene is approximately 750 bp, amplification products of the expected sizes were obtained (NIZO R5: 1.2-kb product with IS-I and nis10; NIZO

FIG. 3. Nucleotide sequence of IS*1069* and its surrounding region. **A:** Nucleotide sequence of IS*1069*. The amino acid sequences deduced from the ORFs of significant length present in the sequence are given below the nucleotide sequence. Potential ribosome binding sites are denoted by stars over the bases that are complementary to the 3' end of *L. lactis* 16S rRNA (21). The *Hin*dIII site is in bold face. A putative -10 TATAAT box is underlined, as are the 32-bp imperfect inverted repeats of the IS element, and the potential frameshift promoting motif (AAAAAAG; 24). The amino acid numbering corresponds to ORFAB, generated by a frameshift similar to that found for IS*911* (23). Nucleotides in the DNA sequence and amino acids in the deduced ORFs that are different in IS*1068* are given over the nucleotide sequence and below the amino acid sequences, respectively (amino acid differences are also boxed). **B:** Nucleotide sequences of the regions flanking IS*1068* in Tn*5276* and IS*1069* in *L. lactis* NIZO 22186. The IS element sequence is in bold face. The 4-bp target repeat flanking IS*1068* is underlined.

22186: 1.2-kb product with IS-II and nis10), while DNA from strains ILC19, ILCpSL20, and ILC126 did not yield any products with either primer combination (data not shown). Since the limit of the PCR amplification reaction in our hands is approximately 2.5 kb, these results indicate that there is no iso-IS*1068* element present in approximately 2 kb of the region upstream from the *nisZ* gene in the DNA ofstrains ILC19, ILCpSL20, and ILC126. Strain NIZO 22186 is the only representative of the other group of class III elements (Fig. 2). It contains sequences that hybridize to the left end of Tn*5276* but these are not located at the appropiate position upstream from IS*1069* and the *nisZ* gene. This supports the assumption presented above that an iso-IS*1068* mediated rearrangement has occurred in strain NIZO 22186. This rearrangement might be an inversion of the region between two inversely oriented IS elements or deletion of the region between two directly repeated IS elements through homologous recombination. Alternatively, IS*1069* might have inserted as part of a composite transposon.

All strains were found to contain a region (or regions) that is (or are) similar or identical to the right end of Tn*5276*. Thus, all non-conjugative elements, with the exception of INRA 3, differ from the conjugative elements by lacking a sequence that is similar to the Tn*5276* left end at the appropriate location. As proposed for strain NIZO 22186, the absence of the left end presumably prevents excision and thereby conjugal transfer. Apparently, other causes underly the inability of strain INRA 3 to conjugally transfer sucrose proficiency.

Evolution of the nisin-sucrose elements in *L. lactis*. Class II nisin-sucrose elements are conjugative transposons encoding nisin Z production. Since they do not contain iso-IS*1068* elements upstream from their *nisZ* genes (Figure 1), it is likely that these class II elements are closest to the original nisin-sucrose element. The G+C content of IS*1068* (37.7%; 25) is close to the *L. lactis* G+C content of approximately 38% (17, 34), while the G+C contents of the region surrounding the IS element and of other regions of Tn*5276* average 30% (25). This suggests that IS*1068* was not originally part of the transposon and that the transposons origin lies outside *Lactococcus*. The nisin Z producer *L. lactis* NCK400, which was isolated from vegetables (14) and is completely devoid of iso-IS*1068* elements, could be a primary lactococcal source of an original nisin-sucrose element, which then would belong to class II. The class I element would then have been generated from a class II element by insertion of an iso-IS*1068* element, resulting in elements such as Tn*5276*. Since all class I elements contain a *nisA* gene, early in the generation of the class I element also a nucleotide substitution resulting in nisin A production must have occurred.

Elements of class I and II were able to spread themselves into different strains. Class III elements appear to be descendants of class II elements that were fixed in their positions by rearrangements that dislocated or deleted the left end of the transposon. Thus, class III elements are no longer transposons.

Unknown genetic elements related to Tn*5276* might be present in the genomes of several *L. lactis* strains. In the DNA of some strains more than one band hybridized to the Tn*5276*-L and -R probes (Fig. 1C,D). Strain ILCpSL5 (Fig. 1, lanes 11) apparently contains two copies of its class II nisin-sucrose element. Two *Hin*dIII fragments hybridized to the Tn*5276*-L probe (Fig. 1C) and, because there is no IS*1068*-like element between the left end and the *nisZ* gene in class II elements, these fragments also hybridized to the *nis* probe (Fig. 1A). The hybridization to the Tn*5276*-R probe showed two strong signals probably corresponding to the right ends of the two class II elements. In addition to these strong hybridization signals, two additional signals of lower intensity could be seen in the hybridizations with the left and right end probes. A possible explanation for this could be the presence of two genetic elements with ends that are similar to those of Tn*5276*.

In several other strains additional weak or even strong hybridizations with the Tn*5276*-L probe and/or the Tn*5276*-R probe were observed (Fig. 1C,D). IS-mediated duplications could possibly be responsible for the occurrence of additional hybridizations. Alternatively, the origin of strong additional hybridizations could be the presence of genetic elements that contain regions similar to the left or right end of Tn*5276*. Preliminary experiments with *L. lactis* MG1614 indicate that a region is present in this strain that is very similar to the right end of Tn*5276*, including the genes responsible for excision (25). For a further knowledge of gene transfer and evolution in *L. lactis* it would be of interest to identify this and other cryptic elements that show similarity to parts of Tn*5276*.

In conclusion, among a variety of non-conjugative nisin-sucrose elements, also nisin Z conjugative transposons have been found such as Tn*5278* in strain ILC11. This allows the construction of industrial strains that produce nisin Z and may have advantages in some applications (3).

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CHAPTER 9

SUMMARY AND CONCLUDING REMARKS

The bacteriocin nisin, produced by several *Lactococcus lactis* strains, is a promising alternative for the use of sodium nitrate against spoilage by *Clostridium butyricum* in Gouda cheese manufacture. The production of nisin by the starter culture would be an attractive means of supplying the bacteriocin during the cheese making process. Since wild-type nisin-producers are not suitable for use as starter cultures, transfer of nisin production to industrial strains would be the obvious solution. Most wild-type nisin-producers are able to transfer the ability to produce the bacteriocin to other *L. lactis* strains in a conjugation-like process. In this process, also the ability to take up and ferment sucrose is transferred. The aim of the study described in this thesis was to identify and characterize the conjugative element carrying the genetic information for nisin production and for sucrose proficiency.

Chapter 2 presents an overview of the current knowledge of nisin genetics and of the results obtained thus far in nisin protein engineering. Some attention is also paid to other aspects with which nisin research is concerned, namely nisin application, biosynthesis, immunity and mode of action.

In **Chapter 3**, the isolation and the characterization of the structural gene for the nisin A precursor (*nisA*) of *L. lactis* strain NIZO R5 are described. Since the cloning and characterization of the *L. lactis* NIZO R5 *nisA* gene, the analysis of the *nis* region has advanced significantly and now the nucleotide sequence, transcriptional organization, and regulation of the complete nisin gene cluster of this strain are known (7, 18). The *nisA* gene of *L. lactis* NIZO R5 was found to be preceded by an IS-like element that has been designated IS*1068* and belongs to the IS*3* family of insertion sequences.

Chapter 4 deals with the characterization of the sucrose region of the nisin-sucrose element. *L. lactis* imports sucrose by means of a phosphoenolpyruvate-dependent phosphotransferase system. The product of this translocation is sucrose 6-phosphate, which is subsequently cleaved by a specific sucrose-6-phosphate hydrolase to glucose 6-phosphate and fructose. Fructose is then converted to fructose 6-phosphate by an ATP-dependent fructokinase. All these activities have been shown to be induced during growth on sucrose (15, 16). We isolated a large part of the *L. lactis* NIZO R5 sucrose operon and determined the nucleotide sequence of the *sacA* gene, encoding sucrose-6-phosphate hydrolase, and its flanking regions. The sucrose operon was found to contain two divergent transcriptional units. Transcription initiated at two sucrose-inducible promoters with a back-to-back organization. One transcriptional unit includes the *sacB* gene. This gene most proba-

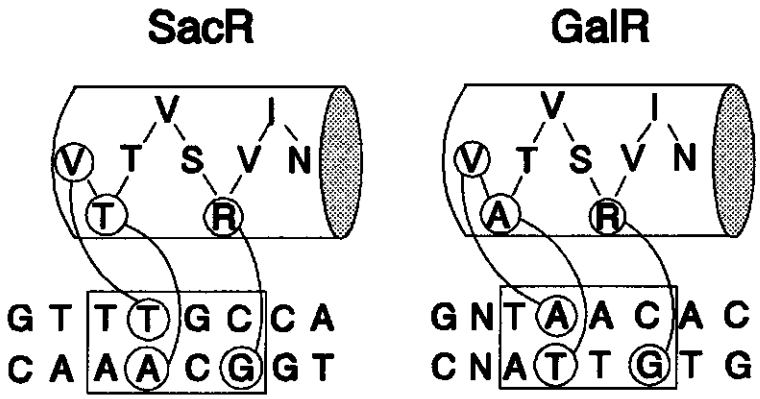

FIG. 1. Interaction of the *Escherichia coli* GalR helix-turn-helix motif with the *gal* operator half site (after reference 8) and the proposed interaction between *L. lactis* SacR and its putative operator.

bly encodes the sucrose-specific Enzyme II of the PTS, which is involved in uptake of sucrose with concomitant phosphorylation. The size of this transcriptional unit (3.2 kb) would also allow the presence of the gene encoding the sucrose-inducible fructokinase. The other transcriptional unit includes the genes *sacA* and *sacR*. The protein encoded by the *sacR* gene is likely to be involved in the regulation of *sac* operon expression, since its deduced N-terminus is homologous to helix-turn-helix DNA-binding domains found in several regulatory proteins and the sucrose operon promoter region contains a sequence that is similar to the consensus operator postulated for this group of regulatory proteins (Fig. 1).

The question remains what the nature of the regulation of the sucrose operon is and how it is effected at the molecular level. The simultaneous, sucrose-induced transcription of the *sacA* and *sacR* genes suggests an activator function for the *sacR* gene product. A comparison of the position of the putative operator with respect to both the *sacA* and the *sacB* gene to the positions of known and putative operators in *Escherichia coli* (3) also points in that direction. In order to learn more about the regulation of the sucrose operon, studies on the interaction between SacR and the putative operator will be necessary, next to nucleotide sequence analysis of the complete operon. Northern blot analysis of RNA from cells grown on a combination of glucose and sucrose showed that the operon was not induced under those conditions, suggesting a possible role for catabolite repression in regulation of the sucrose operon (9).

Chapter 5 describes the use of gene probes for the nisin and sucrose regions isolated from the nisin conjugative element of *L. lactis* NIZO R5, as reported in Chapters 3 and 4, to identify this element and to characterize it in more detail. The element was found to be a chromosomally located conjugative transposon, designated Tn*5276*. The transposon was found to insert at various locations in the chromosome of *L. lactis* MG1614, but showed a preference for orientation-specific insertion into a single target site (now designated *attB1*). A physical map of the 70-kb Tn*5276* was constructed and the single copy of IS*1068* present on the transposon, the nisin gene cluster, and the sucrose operon were located. The ends of Tn*5276* were found to be flanked by a direct hexanucleotide repeat in both the donor *L. lactis* NIZO R5 and in site 1 of a MG1614-derived transconjugant, but the Tn*5276* ends do not contain an inverted repeat, as is the case for most other transposons.

The identification and molecular analysis of genes involved in excision (and probably insertion) of Tn*5276* are described in **Chapter 6**. The right end of Tn*5276* was found to contain two genes, designated *xis* and *int*, that are involved in excision. In *E. coli*, the *int* gene product alone can promote excision of the Tn*5276* ends, but its activity is strongly promoted by the *xis* gene product. The *int* gene product (Int) shows high (up to 50%) similarity to various integrases, including that of the Tn*916*-related conjugative transposons and that of bacteriophage λ. The *xis* gene product, like almost all known excisionase (Xis) proteins, is a small, basic protein. Next to a number of direct or inverted repeats that might act as Xis or Int binding sites, several subsequences can be found in the ends of Tn*5276* that conform to the proposed consensus binding sites of two *E. coli* proteins that have been shown to be involved in bacteriophage λ site-specific recombination, i.e. Integration Host Factor (IHF; 4) and Factor for Inversion Stimulation (FIS; 5). Site-directed mutagenesis of the Tn*5276* ends followed by studying their effect in the *E. coli* Tn*5276* excision system in conjunction with studies using *E. coli* strains deficient in the production of IHF and FIS, could be instrumental in identifying the binding sites of Xis and Int and in studying the role of DNA bending in Tn*5276* recombination.

In **Chapter 7** the transposition (or rather site-specific recombination) mechanism of Tn*5276* is analyzed in more detail. It was shown that the circular form of Tn*5276* is present in a low percentage of the total cell population of Tn*5276*-containing *L. lactis* strains. In analogy to the related Tn*916* (12), this circular form is expected to be the transposition intermediate. On the basis of the analysis of different Tn*5276* recombination products, the regions involved in site-specific

recombination were identified. Tn*5276* appears to hold an intermediary position between the conjugative transposons Tn*916* and Tn*1545* on one hand and the temperate bacteriophages on the other hand: Tn*5276* can insert into several sites, which, like for the other conjugative transposons, show only limited sequence similarity to the inserting transposon. However, the regions in which the strand exchange reaction takes place (the overlap regions) are of identical or almost identical sequence. Sequence identity between the overlap regions is a feature of site-specific recombination of the temperate bacteriophages, while in Tn*916* and Tn*1545* site-specific recombination the recombining sites are generally not identical.

It appears that the gram-positive conjugal transposons are related to the mobile DNAs of temperate bacteriophages (and integrative plasmids) rather than to 'classical' transposons, which often generate target site duplications in their insertion. Several questions concerning the conjugal transposition of Tn*5276* remain. How is the transposition intermediate transferred from one cell to another, how is this transfer initiated, and is there an interplay between conjugation and site-specific recombination? The intercellular transfer of conjugative transposons in general is an unexplored territory. In this respect, the possible presence of descendants of several Tn*5276* transfer events in one transconjugant colony deserves attention. For the related conjugative transposon Tn*916* it has been shown that a contiguous region of approximately 9 kb is involved in conjugal transfer of the element, of which an approximately 4-kb segment (including the *xis* and *int* genes) is involved in transposition (2, 13). It is therefore possible that further nucleotide sequence analysis of the right end of Tn*5276* will reveal the presence of genes involved in conjugal transfer and, possibly, the regulation of both site-specific recombination and conjugal transfer.

Finally, **Chapter 8** is concerned with the distribution and evolution of nisin-sucrose elements. In an analysis of six nisin A- and seven nisin Z-producing wild-type strains isolated in the Netherlands (two strains), France (three strains), Italy (seven strains), and the Unites States of America (one strain), three classes of nisin-sucrose elements could be found: nisin A transposons (class I; usually conjugative; Tn*5276*-like; six members; Fig. 2), nisin Z transposons (class II; conjugative; three members) and nisin Z degenerate elements (class III; non-conjugative; four members). The experiments indicated that the class I elements have originated from the class II elements and thus that the *nisZ* gene is the ancestor of the *nisA* gene. The degenerate elements appeared to have been rendered transpositionally inactive by the loss of their left ends; they are no longer transposons. In the case of strain NIZO 22186, an IS-mediated rearrangement was probably

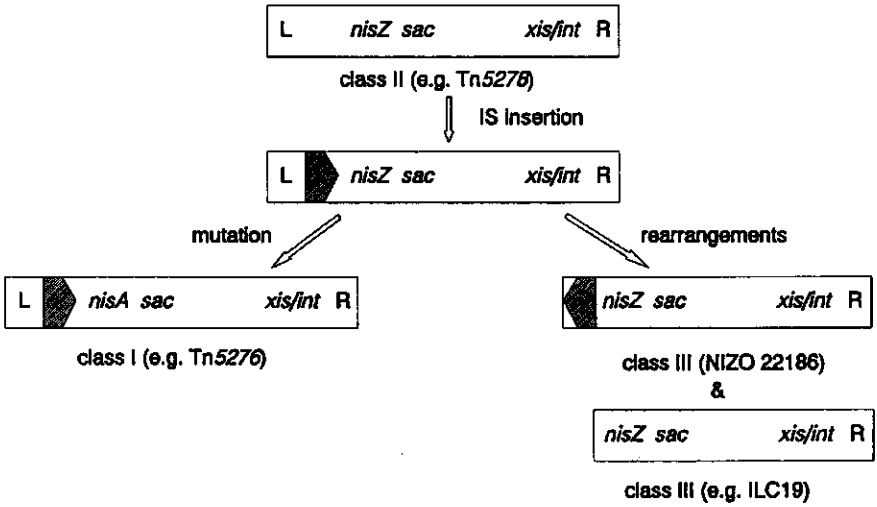

FIG. 2. Model for the evolution of the nisin-sucrose elements in *L. lactis*. Abbreviations: L and R, left and right ends; *nisA*, *nisZ*, *sac*, *xis/int*, schematic positions of nisin A or Z operon, sucrose operon, and transposition genes, respectively; hatched arrows, iso-IS*1068* elements and their orientations.

responsible for the loss of the elements left end. A tentative model for the evolution of the nisin-sucrose elements in *L. lactis* is given in Fig. 2. The hybridization results also suggested that elements related to the nisin-sucrose transposons might be present in the *Lactococcus* genome.

Where do the nisin-sucrose transposons (Fig. 3) come from and how have they evolved? The analyses described in Chapter 8 offer some clues. The G+C contents of all functional regions of

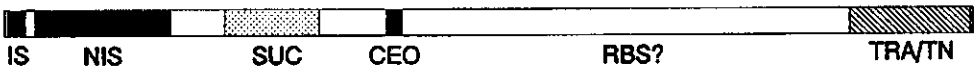

FIG. 3. Possible genetic organization of the nisin-sucrose transposon Tn*5276*. Abbreviations: IS, IS*1068*; *NIS*, nisin A gene cluster; *SUC*, sucrose operon; *CEO*: N^5-(L-1-carboxy-ethyl)-L-ornithine (N^5-CEO) synthetase gene; *RBS*: reduced bacteriophage sensitivity gene(s); *TRA/TN*: genes involved in conjugal transfer and transposition. The sizes of *NIS* and *SUC* have been derived from nucleotide sequencing results (7, 18) and Northern blot analysis (Chapter 4), respectively. The position of *CEO* is from Thompson *et al.* (16), but its possible presence on Tn*5276* has not yet been examined. The position of *RBS* is unknown. The size of *TRA/TN* has been presumed to be similar to the size of the corresponding region in Tn*916* (13). According to this map, the function of approximately 30 kb of the transposon remains unknown.

Tn*5276* analyzed at the nucleotide sequence level thus far range from 28.7 to 32.4% (9), while the G+C content of the *L. lactis* genome is 38% (6, 17). This suggests that the origin of the nisin-sucrose transposons lies outside of the genus *Lactococcus*. Since the search for novel bacteriocins is a very active field, the possibility exists that nisin or its ancestor will be rediscovered in its original source.

It has been found that plants provide a habitat for *L. lactis* subsp. *lactis*, but not for the other important lactococci (11). The plant environment is considered to be the most likely natural habitat from which *Lactococcus* has entered its present most important habitat (milk). Two observations point toward the possibility that the nisin-sucrose transposon has been acquired by *Lactococcus* in its original plant habitat. Firstly, all known wild-type nisin producers are from the subspecies *lactis*. Secondly, two of the traits encoded by the nisin-sucrose transposons appear to be more useful in a plant environment than in milk, i.e. sucrose proficiency and the production of N^5-(L-1-carboxyethyl)-L-ornithine (N^5-CEO) reported to be encoded by the nisin-sucrose transposon Tn*5306* from *L. lactis* K1 (15). Sucrose is the most common disaccharide found in plants (1) and, as pointed out by Thompson (14), two regioisomers of N^5-CEO are members of the octopine family of opines. These opines are found in crown gall tumor tissue formed in the symbiosis of *Agrobacterium tumefaciens* with its plant host, where one of their functions is that of chemical mediators between the bacterium and the plant cell (14). The role of N^5-CEO in the interaction between *L. lactis* and plants remains elusive at this moment. The other properties known to be encoded by the nisin-sucrose transposons, i.e. nisin production and reduced bacteriophage sensitivity, appear to present advantages in both the plant and the milk habitat and could be responsible for the preservation and continued transfer of the nisin-sucrose transposons in *L. lactis* in milk.

As mentioned in Chapter 6, the joining together of DNA regions from different sources would be an attractive model for the evolution of Tn*5276* and related transposons, since they carry the genes for a number of very different traits. Especially interesting in this respect is the presence on Tn*5276* of the genetic information for reduced sensitivity to isometric phages (9). This reduced sensitivity could be speculated to be a remnant of a superinfection immunity mechanism. The genes responsible for this mechanism, together with the genes encoding the Tn*5276* excision and insertion functions, could then have a bacteriophage origin.

The studies described in this thesis have led to the identification of the nisin-sucrose transposons and provided a detailed description of Tn*5276* from *L. lactis* NIZO R5. In addition, it became apparent that several classes of nisin-sucrose transposons exist. During the course of this work, also mutants of the prototype Tn*5276* were isolated that were impaired in nisin production, but were still immune to significant levels of nisin (10). The transposon of one of these strains (NIZO R520) was conjugally transferred to a *L. lactis* subsp. *cremoris* strain (9). The transconjugants obtained were sucrose proficient and were immune to the same level of nisin as the donor strain. The possibility to generate strains that produce nisin A or nisin Z and/or are immune to nisin, now allows the development of new nisin-producing, industrial strains *via* conjugation and the prediction of their characteristics.

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HOOFDSTUK 10

SAMENVATTING

DNA IN BEWEGING

Natuurlijke overdracht van nisine produktie: een onbekend genetisch element. Het DNA van bacteriën is voortdurend in beweging. Binnen de cel vinden er bijvoorbeeld uitwisselingen van genetisch materiaal plaats tussen het chromosoom (bij melkzuurbacteriën is dat ongeveer 2 miljoen baseparen groot) en in de cel aanwezige plasmiden - kleinere, cirkelvormige DNA-moleculen. Er kan echter ook uitwisseling van genetisch materiaal tussen bacteriecellen onderling plaatsvinden. Het natuurlijke proces waarbij erfelijk materiaal, en daarmee erfelijke eigenschappen, via verschillende soorten genetische elementen wordt overgedragen van de ene bacterie (de donor) naar de andere (de recipiënt) noemen we conjugatie (=verbinding). Een eigenschap die op deze manier van de ene melkzuurbacterie op de andere kan worden overgedragen is de produktie van het antimicrobiële eiwit nisine. Dit proefschrift beschrijft de ontdekking en nadere karakterisering van het genetische element van de melkzuurbacterie *Lactococcus lactis* subsp. *lactis* dat voor die overdracht verantwoordelijk is.

We wilden graag meer weten over dit genetische element, omdat er voor de overdracht van nisine-produktie een zeer interessante praktische toepassing te bedenken is. Nisine is namelijk een goed bruikbaar natuurlijk conserveermiddel dat reeds in een aantal voedselprodukten, o.a. smeerkaas, wordt toegepast. Een veel voorkomende bacteriële besmetting in dit soort produkten is de boterzuurbacterie *Clostridum tyrobutyricum*. Ook bij de bereiding van Goudse kaas is besmetting met boterzuurbacteriën, die als sporen via het veevoer in de melk terecht komen, een groot probleem. De uitgroei van deze sporen (met als gevolg een opgeblazen, stinkende kaas) wordt momenteel voorkomen door tijdens de kaasbereiding nitraat toe te voegen. Produktie van nisine door het bij de kaasbereiding gebruikte zuursel, d.i. het mengsel van melkzuurbacteriën dat zorgt voor verzuring van de melk, zou de toevoeging van nitraat overbodig maken. Een aantal, ook in melk voorkomende, *Lactococcus lactis* subsp. *lactis* stammen produceert nisine om zich te verweren tegen andere bacteriën in de concurrentiestrijd om beperkte voedselbronnen in hun leefmilieu. Deze van nature voorkomende stammen zijn echter vaak om verschillende redenen ongeschikt om er goede Goudse kaas mee te bereiden. Overdracht via conjugatie van nisine-produktie naar melkzuurbacteriën die wel goede Goudse kaas kunnen maken, zou daarvoor een oplossing kunnen zijn.

Isolatie van gedeeltes van het genetisch element. Toen de zoektocht naar het genetische element dat verantwoordelijk is voor de overdracht van nisine-produktie begon, wisten we er niet veel over. De aminozuurvolgorde, de zgn. primaire structuur, van nisine was bekend (Fig. 1). Nisine bestaat uit 34 aminozuren. Naast algemeen bekende aminozuren bevat nisine ook een aantal bijzondere aminozuren, die in de natuur niet zo vaak voorkomen (Fig. 1). Er waren sterke aanwijzingen dat de biosynthese van nisine plaats vindt doordat in een voorloper, die dan opgebouwd zou zijn uit de algemeen bekende aminozuren, een aantal aminozuren omgebouwd zou worden tot de bijzondere aminozuren die in nisine gevonden worden. Die modificatie van een voorloper zou dan waarschijnlijk uitgevoerd worden door speciale, nog onbekende enzymen.

Wat we ook wisten, was dat bij de natuurlijke overdracht van nisine-produktie van de ene bacterie naar de andere ook de mogelijkheid om saccharose (suiker) als energiebron te gebruiken werd overgedragen. Alle bacteriën die nisine produceren, zijn ook in staat om op saccharose te groeien als hen dat aangeboden wordt. Deze eigenschap kan zeer gemakkelijk op agarplaten worden aangetoond en is dan ook het kenmerk waarop in eerste instantie de nakomelingen van de conjugatieve overdracht worden geselecteerd.

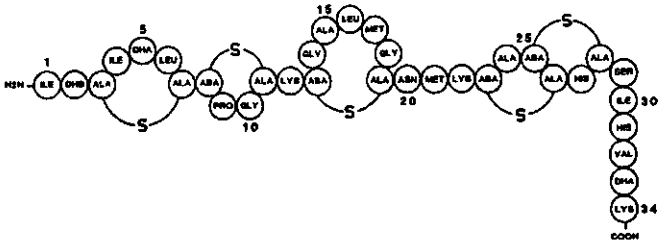

FIG. 1. Het nisine molecuul. De bolletjes stellen aminozuren voor. De bijzondere aminozuren zijn DHA (dehydroalanine) en DHB (dehydrobutyrine) en de residuen die zgn. thio-etherringen vormen (ALA-S-ALA: lanthionine; ABA-S-ALA: ß-methyllanthionine).

Het genetische element waarnaar we op zoek waren, bevatte dus de erfelijke informatie - de genen - voor de produktie van nisine en de opname en verwerking van saccharose. Bij deze eigenschappen zijn diverse eiwitten betrokken en er zijn dus ook meerdere genen die voor deze eigenschappen coderen (zie bijvoorbeeld voor de opname en verwerking van saccharose Fig. 2). Door nu deze genen op te sporen in het DNA van een nisine-producerende bacterie, zouden we dus op het spoor komen van het genetische element waarop ze zich bevinden. Gebruik makend van verschillende technieken hebben we een aantal van deze genen dan ook geïsoleerd uit het totale

DNA (plasmides en chromosoom) van de uit melk geïsoleerde nisine-producent *L. lactis* subsp. *lactis* stam NIZO R5.

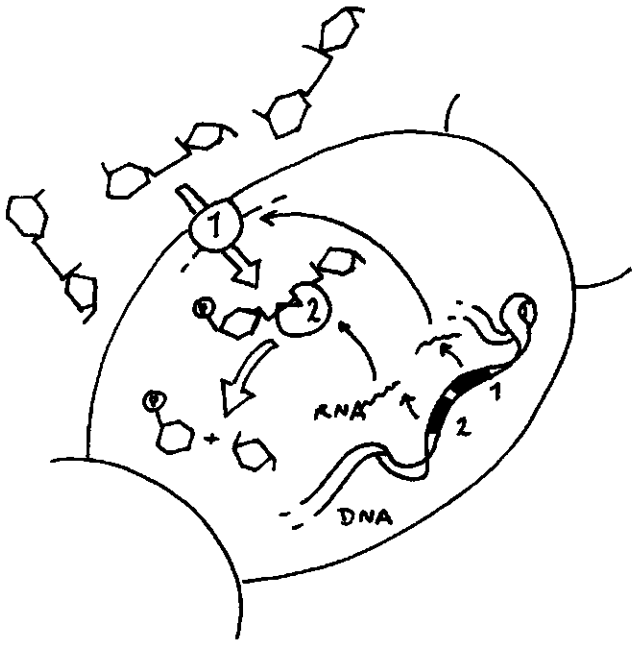

FIG. 2. De verwerking van saccharose door *L. lactis*. Minstens twee specifieke eiwitten zijn betrokken bij de verwerking van saccharose. Eiwit 1 zorgt dat de suiker de cel binnenkomt en eiwit 2 splitst de suiker. De ontstane moleculen worden opgenomen in de (algemene) stofwisseling. Bij het aanschakelen van de genen 1 en 2 is waarschijnlijk nog een derde eiwit betrokken.

Het genetische element is een groot transposon: Tn*5276*. Nu we een aantal van de genen in handen hadden die betrokken waren bij de produktie van nisine en de verwerking van saccharose, konden we deze gebruiken om het genetische element waarop ze gelegen zijn te identificeren. Er zijn twee soorten genetische elementen bekend die zichzelf via conjugatie van de ene cel naar de andere kunnen verplaatsen: conjugatieve plasmiden en conjugatieve transposons. Plasmiden zijn al kort genoemd, maar transposon is een nieuw begrip. Transposons zijn mobiele stukken DNA, die zich kunnen verplaatsen van de ene plek in het DNA van een organisme naar een andere plek. Bij normale transposons liggen die plekken in het DNA van één cel, maar bij conjugatieve transposons liggen die plekken in twee verschillende cellen (Fig. 3).

Door nu met behulp van de genen die we in handen hadden het spoor van het conjugatieve element te volgen van de donor-cel naar de recipiënt-cel, konden we vaststellen dat het genetische

element dat nisine-produktie overdraagt een groot (ongeveer 70.000 baseparen) conjugatief transposon is. Er waren al conjugatieve transposons bekend die betrokken bleken bij de verkrijging van antibioticum-resistenties door ziekte-verwekkende bacteriën. Het nisine transposon is echter een "onschuldig" transposon: het draagt geen antibioticum-resistentie over - een eigenschap die voor ziekte-verwekkende bacteriën gunstig is - maar nisine-produktie - een eigenschap die melkzuurbacteriën voordeel oplevert in hun strijd met andere melkzuurbacteriën om beperkte voedselbronnen. Transposons worden altijd aangeduid met Tn gevolgd door een centraal geregistreerd nummer. Het nisine transposon heeft de aanduiding Tn*5276* gekregen. We hebben de ligging van de genen die verantwoordelijk zijn voor nisine-produktie en saccharose-verwerking in kaart gebracht (zie Fig. 3 van het vorige hoofstuk). Tn*5276* bleek zichzelf op diverse plaatsen in het chromosoom van de gebruikte recipiënt te kunnen invoegen (inserteren), maar het had wel een voorkeur voor één bepaalde plaats.

Het mechanisme van transpositie van Tn*5276*. In de beweging van een conjugatief transposon van een plek in de ene cel (de donor) naar een plek in de andere (de recipiënt), zijn verschillende stappen te onderscheiden (Fig. 3):

1. Het transposon snijdt zichzelf uit het DNA van de donor (excisie).
2. Het uitgesneden transposon gaat naar de recipiënt (conjugatie).
3. Het transposon knipt het DNA van de recipiënt open en plakt zichzelf erin (insertie).

Over de tweede stap in het proces, de conjugatie, weten we nog helemaal niets, behalve dat er nauw celcontact tussen de donor en de recipiënt voor nodig is en dat er op de een of andere manier een verbinding tussen de twee cellen wordt gevormd. Over de manier waarop de eerste en de derde stap van het proces worden uitgevoerd (het mechanisme van transpositie) zijn we wel een aantal zaken te weten gekomen. Het mechanisme van transpositie van Tn*5276* blijkt te lijken op het mechanisme waarvan de zogenaamde gematigde bacteriofagen zich bedienen om in en uit het chromosoom van een bacterie gaan. Bacteriofagen zijn de virussen van de bacteriën. Gematigde bacteriofagen zijn in staat hun DNA in het chromosoom van een bacterie-gastheer te inserteren. Dat DNA kan daar vervolgens generaties lang onopgemerkt blijven (daarom heten deze bacteriofagen ook gematigd), maar dan plotseling onder invloed van omgevingsfactoren (stress) weer uit het chromosoom van de gastheer tevoorschijn komen (exciseren). Het DNA van de bacteriofaag wordt dan vervolgens vermenigvuldigd en ingepakt in eiwitmantels, waardoor er complete bacteriofagen ontstaan. Die nieuwe bacteriofagen breken vervolgens de gastheer-bacterie open, komen vrij in de

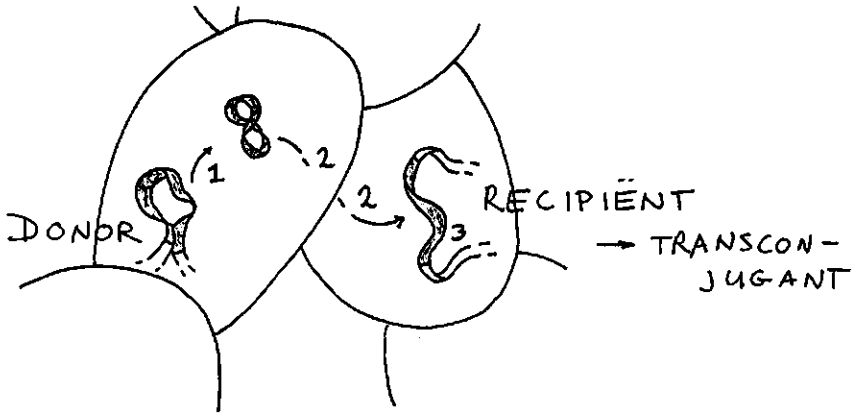

FIG. 3. Overdracht van een conjugatief transposon van donor naar recipiënt.

omgeving en kunnen weer nieuwe gastheren infecteren die zich nietsvermoedend in de omgeving ophouden. Zoals gezegd, bedient Tn*5276* zich van een vergelijkbaar mechanisme om in en uit de chromosomen van melkzuurbacteriën te springen. De tussenvorm (het intermediair) van Tn*5276*, dus de vorm die waarschijnlijk van de ene cel naar de andere cel beweegt, blijkt een cirkel te zijn, net als het (vrije) DNA van de gematigde bacteriofagen.

Verschillende elementen in verschillende nisine producenten. Niet alle melkzuurbacteriën die nisine produceren, kunnen die eigenschap ook overdragen op andere melkzuurbacteriën. Dit bleek toen er bij het NIZO een *Lactococcus lactis* stam (NIZO 22186) ontdekt werd die een ander nisine maakte dan tot dan toe bekend was. Dit nieuwe nisine (nisine Z) verschilt in één aminozuur van het bekende nisine (nisine A; Fig. 1). Omdat nisine Z mogelijk betere eigenschappen had dan nisine A bij het gebruik ervan in de kaasbereiding, was men uiteraard erin geïnteresseerd of de produktie van nisine Z ook op natuurlijke wijze overdraagbaar was. Dit bleek niet het geval voor stam NIZO 22186. Toen we dit verder uitzochten, bleek dat alle *Lactococcus lactis* stammen die nisine produceren Tn*5276*-achtige elementen bezitten, maar dat in sommige gevallen de structuur van die elementen zodanig veranderd is dat ze zichzelf niet meer uit het DNA kunnen snijden. Gelukkig werden er ook stammen gevonden die nisine Z produceren en de produktie ervan ook kunnen overdragen. Bij nader onderzoek van Tn*5276*-achtige elementen hebben we een aanwijzing gevonden dat deze elementen oorspronkelijk niet uit *Lactococcus lactis* afkomstig zijn. Bovendien

komen er mogelijk in *Lactococcus lactis* genetische elementen voor die iets weg hebben van Tn*5276*, maar niets met nisine-produktie of de verwerking van saccharose te maken hebben.

Tot besluit. We zijn heel wat meer te weten gekomen over het genetische element dat zorgt voor de natuurlijke overdracht van nisine-produktie. Deze kennis zal gebruikt kunnen worden bij de pogingen om nisine-produktie over te dragen naar stammen die gebruikt worden in de kaasbereiding. Een aantal interessante vragen blijft echter nog onbeantwoord. Hoe vindt de overdracht van de transposon-cirkel van de ene cel naar de andere plaats? Zijn er inderdaad genetische elementen die op Tn*5276* lijken en, zo ja, wat zijn dat voor elementen; zijn het bijvoorbeeld gematigde bacteriofagen? Wat was de voorvader van Tn*5276* en waar kwam die voorvader eigenlijk vandaan?

Het genetisch materiaal van organismen is continu aan veranderingen onderhevig. Het volgt daarbij niet alleen de langzame maar gestage weg van de evolutie door willekeurige mutaties, maar helpt zelf een handje mee, bijvoorbeeld met conjugatieve transposons - DNA in beweging.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren als Peter Jacobus Gerardus Rauch op 10 augustus 1963 te Vlodrop. Na het behalen van het diploma Gymnasium-ß aan het Bisschoppelijk College Schöndeln te Roermond in 1981, werd in hetzelfde jaar begonnen met de studie Moleculaire Wetenschappen aan de (toen nog) Landbouwhogeschool te Wageningen. De doctoraalfase omvatte als hoofdvakken Erfelijkheidsleer (Moleculaire Genetica) en Moleculaire Biologie (Plantecelbiologie) en als bijvak Moleculaire Fysica. De bij het hoofdvak Erfelijkheidsleer behorende stage werd vervuld bij de werkgroep Moleculaire Genetica van het Nederlands Instituut voor Zuivelonderzoek (NIZO). Het doctoraal examen werd in september 1987 behaald. In januari 1988 trad hij in tijdelijke dienst bij NIZO, waar in de werkgroep Moleculaire Genetica het in dit proefschrift beschreven onderzoek werd verricht. Sinds augustus 1992 is hij in tijdelijke dienst verbonden aan de vakgroep Microbiologie van de faculteit Scheikunde van de Universiteit van Amsterdam. Daar houdt hij zich binnen de werkgroep Moleculaire Microbiologie onder meer bezig met de natuurlijke transformatie van *Acinetobacter calcoaceticus*. Hij is getrouwd met Catelijne en is de trotse vader van Marijke en Lonneke.