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Published in:
Microbiology

DOI:
10.1099/13500872-145-5-1227

Microbiology (1999), **145**, 1227–1233 Printed in Great Britain

Effects of gene disruptions in the nisin gene cluster of *Lactococcus lactis* on nisin production and producer immunity

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The lantibiotic nisin is produced by several strains of *Lactococcus lactis* subsp. *lactis*. The chromosomally located gene cluster *nisABTCIPRKFEG* is required for biosynthesis, development of immunity, and regulation of gene expression. In-frame deletions in the *nisB* and *nisT* genes, and disruption of *nisC* by plasmid integration, eliminated nisin production and resulted in a strongly reduced level of immunity of the strains. The transcription of two nisin operons was inactivated in these mutant strains, but could be restored by addition of small amounts of nisin to growing cultures. The immunity levels of the mutants were also raised by adding nisin to growing cultures, albeit not to wild-type level. A strain with an in-frame deletion in the *nisI* gene was still able to produce active nisin, but the production and immunity levels were markedly lower. By measuring immunity levels of the knock-out strains and determining mRNA levels, it is concluded that NisI has an important function for nisin immunity and must cooperate with *nisFEG*-encoded proteins to provide a high level of immunity. Maximal immunity could not be obtained in the mutant strains, probably because the wild-type transcription levels from *nisA* and *nisF* promoters are not reached when essential *nis* genes are disrupted. Using Southern hybridization with a consensus promoter probe, no other DNA sequences similar to the *nisA* and *nisF* promoters could be detected, indicating that these two elements are probably the only ones in the chromosome regulated by nisin and are thus the only ones involved in the regulation of producer immunity.

Keywords: *Lactococcus lactis*, nisin, producer immunity, in-frame deletions

INTRODUCTION

The antimicrobial peptide nisin belongs to the family of lantibiotics and is produced by several strains of *Lactococcus lactis* (Hurst, 1981). It is used as a natural preservative in the food industry because it inhibits the growth of food-spoilage bacteria (Delves-Broughton *et al*., 1996). Nisin is ribosomally synthesized as a precursor peptide that undergoes post-translational modifications, i.e. dehydration of serine and threonine residues and formation of five intramolecular thioether ring structures called (*β*-methyl)lanthionine residues (Gross & Morell, 1971). The eleven genes required for nisin synthesis are located in a gene cluster on the nisin-sucrose transposon Tn*5276* (Buchman *et al*., 1988; Kaletta & Entian, 1989; Steen *et al*., 1991; Rauch & de Vos, 1992; Engelke *et al*., 1992; van der Meer *et al*., 1993; Kuipers *et al*., 1993; Engelke *et al*., 1994; Immonen *et al*., 1995; de Vos *et al*., 1995a, b; Siegers & Entian, 1995). The organization of these genes is shown in Fig. 1. The *nisA* gene encodes the 57 aa precursor peptide; *nisB* and *nisC* probably encode membrane-associated proteins that are involved in the postranslational modification of nisin (Engelke *et al*., 1992; Kuipers *et al*., 1993; Siegers *et al*., 1996). *nisT* encodes a protein that shares significant homology with ATP-dependent translocator proteins, and recently it has been shown that NisT is involved in the translocation of the fully modified precursor nisin across the cytoplasmic membrane (Qiao

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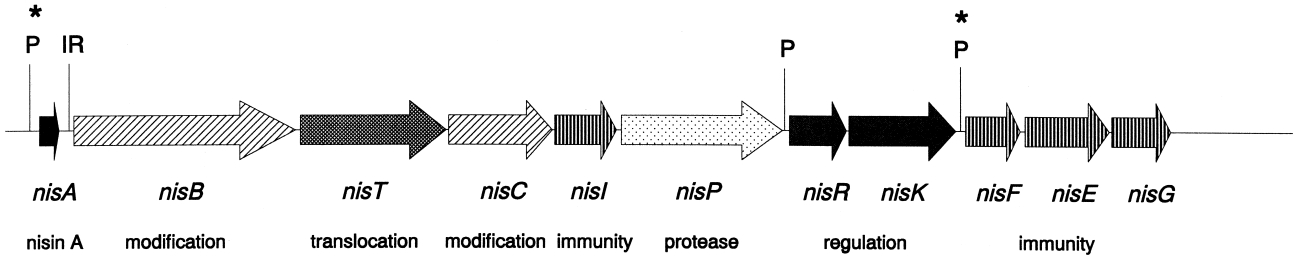

Fig. 1. Organization of nisin biosynthetic, regulatory and immunity genes. Regulated promoters are indicated by P* and constitutive promoters by P. An inverted repeat is indicated by IR.

& Saris, 1996). *nisI* encodes a lipoprotein that is involved in immunity (Kuipers *et al*., 1993; Qiao *et al*., 1995) and *nisFEG* encode putative transporter proteins that probably, together with *nisI*, accomplish full nisin immunity of the producer strain (Siegers & Entian, 1995; Dodd *et al*., 1996). *nisP* encodes an extracellular subtilisin-like protease involved in precursor processing (van der Meer *et al*., 1993;Qiao *et al*., 1996). *nisR* and *nisK* encode a response regulator (van der Meer *et al*., 1993) and a sensor of the histidine protein kinase family (de Vos *et al*., 1995a; Immonen *et al*., 1995; Siegers & Entian, 1995), respectively, that belong to the class of two-component regulatory systems (Stock *et al*., 1990). It has been shown that both genes are involved in the regulation of nisin biosynthesis (van der Meer *et al*., 1993; Engelke *et al*., 1994). Recently, it has been demonstrated that fully modified nisin is the extracellular input signal for the sensor NisK (Kuipers *et al*., 1995). Small amounts of fully modified nisin can activate the transcription of its own structural gene, the transcription of the downstream genes, and transcription of *nisFEG*, via this two-component regulatory system (Kuipers *et al*., 1995; de Ruyter *et al*., 1996; Ra *et al*., 1996).

In this paper we describe disruptions in several genes of the nisin gene cluster, of which all except one were made in-frame to avoid polar effects on downstream genes. The effects of these disruptions on the transcription of nisin genes, nisin production and producer immunity were analysed. Remarkably, several levels of immunity could be distinguished in the knock-out strains relative to the wild-type strain, depending on the production levels of NisI and NisFEG, but the wild-type level could never be reached.

METHODS

Bacterial strains, plasmids and media. *L. lactis* strain NZ9700 (Kuipers *et al*., 1993) is a nisin A producing transconjugant, which was obtained from a mating between *L. lactis* MG1614 (Gasson, 1983) and the nisin A producer *L. lactis* NIZO R5 (Rauch & de Vos, 1992). NZ9800 is a derivative of NZ9700, with a 4 bp deletion in the pronisin-encoding part of the *nisA* gene, which is unable to produce nisin (Kuipers *et al*., 1993). *L. lactis* strains were grown in M17 broth (Difco) supplemented with 0·5 % (w/v) glucose or sucrose at 30 °C without aeration. *Escherichia coli* strain MC1061 (Casadaban *et al*., 1980) was used as a host strain for cloning experiments; it was grown in Tryptone Yeast (TY) medium (Sambrook *et al*., 1989) at 37 °C. Antibiotics were used in the following concentrations: ampicillin, 50 μg ml^{-1}; erythromycin, 2·5 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}.

Construction of plasmids. The construction of the integrative plasmid pNZ9135, used for the disruption of the *nisB* gene by gene replacement, has been described previously (Kuipers *et al*., 1995). The gene replacement results in a Δ*nisB* gene, in which the codons for amino acid residues 474–535 were specifically deleted. To construct the integrative plasmid pNZ9143 for disruption of the *nisT* gene, a 3·9 kb *Sst*I–*Acc*I fragment containing the *nisT* gene and flanking regions was cloned into a *Sst*I/*Acc*I-digested pUC19 vector, which had an additional erythromycin resistance marker. The *nisT* gene was changed by introducing a 231 bp in-frame deletion in the middle of the gene; this was accomplished by removing an internal *Spe*I fragment, resulting in the removal of the codons for amino acid residues 318–395. To construct the single-crossover integrative plasmid pNZ9134 (Δ*nisC*), an internal 753 bp *Nde*I–*Nco*I (filled in with Klenow) fragment of *nisC* was cloned into a *Nde*I/*Sma*I-digested pUC19 vector, which had an additional erythromycin resistance marker from pE194 (Leenhouts *et al*., 1991). To construct the integrative plasmid pNZ9147, a 4·7 kbp *Spe*I–*Bcl*I fragment containing the *nisI* gene and flanking regions was cloned into a *Xba*I/*Bam*HI-digested pUC19 vector, which had an additional erythromycin resistance marker. The *nisI* gene was almost completely removed by introducing a 399 bp in-frame deletion, resulting in removal of the codons for amino acid residues 57–190 of NisI. This was accomplished by removing an internal *Hpa*I (partial digest)–*Ava*I (filled in with Klenow) fragment from the gene.

DNA, RNA and protein techniques. Plasmid and chromosomal DNA of *L. lactis* were isolated as described previously (Vos *et al*., 1989). *L. lactis* cells were transformed by electroporation (Holo & Nes, 1989). Plasmid isolations from *E. coli* cells, and transformations of *E. coli* strains, were carried out according to established procedures (Sambrook *et al*., 1989). Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Gibco-BRL Life Technologies, New England Biolabs, Pharmacia or Promega and used as recommended by the manufacturers. Cloning procedures, radiolabelling of DNA fragments, agarose-gel electrophoresis and Southern blot hybridizations were carried out according to established procedures (Sambrook *et al*., 1989). PCRs were performed using the conditions described before (Kuipers *et*

Table 1. Primers used in this study

Primer	Sequence	Location
1	5′-GAGTCCGAACCGAGTAC-3′	*nisB*
2	5′-GAGTTGAAATGTCTCGTG-3′	*nisB*
3	5′-GGAACATGGAACATTGGG-3′	*nisT*
4	5′-CATATGTTGAAAGTTTGCTAAC-3′	*nisC*
5	5′-GAGAAGATATTTAATACTTATTGTGG-3′	*nisI*
6	5′-ATGCTTCAGTAAGAGATTTTCCTC-3′	*nisI*
7	5′-GTTTTGATGCGTCGCAACTTCATAG-3′	*nisR*
NIS121	5′-CTGAITAIATTCTGAAIITTGTT-3′	Promoter fragment of *nisA* and *nisF*

al., 1991). DNA sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al*., 1977). Oligonucleotides, used as primers in sequencing reactions and for PCR, were purchased from Pharmacia. Primers used in this study are listed in Table 1. RNA isolation, Northern blotting and subsequent hybridization with radiolabelled probes were performed as described previously (Ra *et al*., 1995, 1996). The methods for nisin immunity assays for *L. lactis* (Kuipers *et al*., 1993) and nisin induction experiments (Kuipers *et al*., 1995) have also been described before. Pre-induction was performed by adding nisin A (0·01 μg ml^{-1}) to the freshly diluted culture. The nisin produced was quantified using an agar diffusion test with *Micrococcus luteus* as indicator. Dilution series of the samples were assayed in order to determine the minimal inhibitory concentration (MIC). This MIC value was used to compare percentage differences, which gives a more reliable estimation than values based on arithmetic measures of diffusion of nisin through agar. Western analyses were done with the KH1422 NisI-specific antiserum as described previously (Qiao *et al*., 1995).

Introduction of in-frame deletions in chromosomal nisin genes by gene replacement. The plasmids pNZ9135 (Δ*nisB*), pNZ9143 (Δ*nisT*) and pNZ9147 (Δ*nisI*), all pUC19 derivatives that cannot replicate in *L. lactis*, were used to transform the nisin A producing *L. lactis* NZ9700. Erythromycin-resistant (Em^r) colonies were obtained that were the result of the integration of the plasmid caused by a recombination event involving one of the flanking regions on the plasmid and the corresponding regions on the chromosome. After subculturing for 100–200 generations in the absence of erythromycin, Em^s colonies were obtained as a result of a second recombination event with the flanking region on the other side of the disrupted region relative to the first recombination event. The expected disruptions in the chromosomal genes were confirmed by PCR analysis, Southern analysis and sequence analysis.

RESULTS

In-frame deletions in *nisB*, *nisT* and *nisI* and single-crossover disruption of *nisC*

After electroporation of NZ9700 with pNZ9135 to disrupt the *nisB* gene, one of several Em^r colonies with the plasmid integrated in the correct location of the chromosome as judged by Southern analysis was grown without erythromycin. After plating, several Em^s colonies were obtained in which a second recombination event had occurred resulting in excision of the plasmid from the chromosome. Southern blot hybridization, PCR analysis with primers 1 and 2 (Table 1) and sequence analysis confirmed the expected in-frame disruption of the *nisB* gene on the chromosome in one of the colonies. The resulting strain was named NZ9735. Using a similar approach with other integrative plasmids, in-frame deletions were also obtained in *nisT* (NZ9743) and *nisI* (NZ9747). Attempts to obtain a double-crossover integration to disrupt the *nisC* gene on the chromosome of *L. lactis* NZ9700 were unsuccessful. After changing the strategy to obtain single-crossover integrants, Southern blot analysis of a picked Em^r colony revealed that four copies of pNZ9134 had integrated in the same spot of the *nisC* gene on the chromosome. The integrated plasmids could not be excised by culturing without erythromycin.

Effects of in-frame deletions and plasmid integration on nisin production and immunity

The effects of the various gene disruptions in the nisin gene cluster on nisin production were studied. Supernatants of overnight cultures were tested in a bioassay, and TCA-precipitated supernatants were analysed by SDS-PAGE for the production of nisin or nisin precursor (Table 2). Nisin production was blocked in all cases with one exception, i.e. strain NZ9747, carrying the in-frame deletion in the *nisI* gene, which was still able to produce nisin, although the production level was reduced to approximately 20–40 % of that of the wild-type NZ9700. This confirms that intact *nisB*, *nisC* and *nisT* genes are essential for the production of active nisin in the supernatant and shows for the first time that intact *nisI* is not essential.

A disruption in the *nisI* gene resulted in an immunity level that was still approximately 10–30 % of the maximum immunity level of the wild-type nisin-producing strain. Disruptions in *nisB*, *nisT* and *nisC* resulted in very low immunity levels, comparable to the immunity level of the Δ*nisA* strain NZ9800 (Kuipers *et al*., 1993), which is more than 100 times lower than that of the wild-type nisin producer NZ9700, but still much higher than that of strain MG1614, carrying no nisin

Table 2. Effects of gene disruptions on nisin production, immunity and transcription of *nisFEG*

The amount of nisin used for pre-induction of the cultures was 0·01 μg ml^{-1}. Nisin production levels: + + +, 100 %; + +, 20–40 %; −, no production. Immunity levels: + + + +, 100 %; + + + 30–60 %; + +, 10–30 %, +, 5–10 %, −, <1 %. *nisFEG* transcript levels: + + + +, 100 %; + + +, 50–70 %; + +, 10–30 %, −, <1 %.

Strain	Extracellular nisin production	Immunity		Transcription of *nisFEG*	
		No induction	Pre-induction with nisin	No induction	Pre-induction with nisin
MG1614	−	−	−	−	−
NZ9700	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
NZ9800 Δ*nisA*	−	−	+ +	−	+ +
NZ9735 Δ*nisB*	−	−	+ +	−	+ +
NZ9743 Δ*nisT*	−	−	+ +	−	+ +
NZ9734 Δ*nisC*	−	−	+	−	+ +
NZ9747 Δ*nisI*	+ +	+ +	+ +	+ + +	+ + +

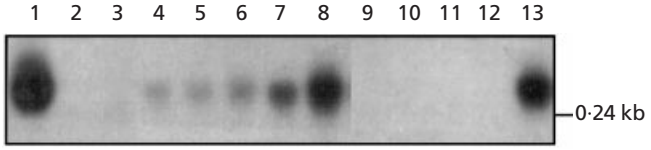

Fig. 2. Northern blot using total RNA from various *L. lactis* strains with the structural *nisA* gene as a probe. Lanes: 1, NZ9700; 2 and 3, MG1614; 4 and 9, NZ9800 (Δ*nisA*); 5 and 10, NZ9735 (Δ*nisB*); 6 and 11, 9743 (Δ*nisT*); 7 and 12, NZ9734 (Δ*nisC*); 8 and 13, NZ9747 (Δ*nisI*). The RNA in lanes 4–7 was isolated from cells that had been pre-induced with 0·01 μg nisin A ml^{-1}.

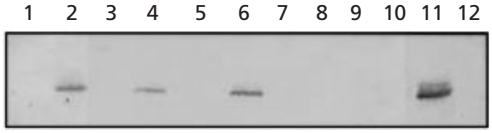

Fig. 3. Western blotting analysis of cells of *L. lactis* strains. A polyclonal NisI-specific antiserum was used to recognize the NisI protein. Lanes: 1 and 2, NZ9800 (Δ*nisA*); 3 and 4, NZ9735 (Δ*nisB*); 5 and 6, NZ9743 (Δ*nisT*); 7 and 8, NZ9734 (Δ*nisC*); 9 and 10, NZ9747 (Δ*nisI*); 11, NZ9700; 12, MG1614. The cells of the samples in lanes 2, 4, 6 and 8 were pre-induced with 0·01 μg nisin A ml^{-1}.

genes. However, when cells were pre-induced with 0·01 μg nisin A ml^{-1}, the immunity levels were elevated to approximately 20 % of the wild-type level (Table 2). Although no production of nisin was found in the extracellular medium after induction, it is possible that an intracellular, partly modified precursor nisin is produced by strains NZ9735 (Δ*nisB*), NZ9734 (Δ*nisC*) and NZ9743 (Δ*nisT*). However, we were not able to detect any accumulation of intracellular nisin precursor with antibodies against the nisin leader peptide. The presence of a partly modified nisin precursor secreted by nisin-induced cells of strains NZ9735 or NZ9734 could also not be detected.

Effects of gene disruptions and nisin induction on transcription of nisin immunity genes

Strains NZ9735, NZ9743, NZ9734 and NZ9747, with disruptions in the *nisB*, *nisT*, *nisC* and *nisI* genes, respectively, were checked for the presence of a *nisA* and a *nisFEG* transcript by Northern hybridizations. In all cases, except in NZ9747, *nisA* and *nisFEG* transcription was absent (Fig. 2, Table 2). Addition of nisin to the growing cultures restored the transcription of *nisA* and *nisFEG* in all knock-out strains (Fig. 2, Table 2). In accordance with this, Western blotting analysis with NisI-specific antiserum showed that without induction, NisI is not produced in the mutant strains, whereas induced cells produce NisI, except strain NZ9747 (Δ*nisI*) and strain NZ9734 (Δ*nisC*) (Fig. 3). In strain NZ9734 four plasmids had integrated into the *nisC* gene and this is likely to hinder transcriptional readthrough of the *nisC* to the *nisI* gene from the induced *nisA* promoter. Interestingly, this strain was also less immune than the other knock-out strains, even after induction.

Analysis of the number of promoters similar to the *nisA* and *nisF* promoters

In order to evaluate whether nisin induction could potentially initiate the transcription of genes other than *nisABTCIPRKFEG*, a Southern analysis was performed with *Eco*RI/*Eco*RV-digested chromosomal DNA of a nisin-producing strain using a degenerate oligonucleotide probe which hybridizes to the conserved regions of the two known nisin promoters. The expected size of the fragments with these promoters is 3 and

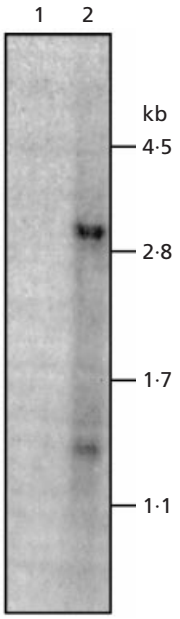

Fig. 4. Southern blot analysis of *L. lactis* strain MG1614, which does not contain the nisin operons (lane 1), and the nisin-producing strain NZ9700 (lane 2). A degenerate probe (NIS121) recognizing the homologous sequences of the *nisA* and *nisF* promoters was used.

1·3 kb. The Southern blot result (Fig. 4) did not visualize any other bands than the expected ones, indicating that a nisin producer has no additional promoters in the chromosome with high homology to the *nisA* or *nisF* promoters.

DISCUSSION

Gene disruptions with polar effects have previously been reported for the *nisA*, *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisF* and *nisE* genes (Kuipers *et al*., 1993; Siegers & Entian, 1995; Siegers *et al*., 1996; Dodd *et al*., 1996; Ra *et al*., 1996; Qiao *et al*., 1996). However, correlating a phenotype to a specific locus using a mutant strain that contains a polar mutation, especially if the mutation is in a large operon like the biosynthetic operon expressed from the *nisA* promoter, is not without risk. To avoid possible ambiguities, in-frame deletions were constructed in the *nisB*, *nisT* and *nisI* genes. Attempts to obtain an in-frame deletion in the *nisC* gene were unsuccessful and resulted in a strain with this gene disrupted by plasmid integration. Nisin production had ceased in the strains with the in-frame mutations in *nisB* and *nisT* and could not be restored by addition of subinhibitory amounts of nisin. Transcription of the genes downstream of the mutated *nisB* or *nisT* genes was deduced to take place, because after nisin induction the product of the downstream *nisI* gene was detected in Western analysis using a NisI-specific antiserum. The fact that all nisin genes were transcribed after nisin induction and still no nisin was produced by the NZ9735 and NZ9743 strains conclusively demonstrates that NisB and NisT are essential for the biosynthesis of nisin. The putative structure of NisT and the recent results of Siegers *et al*. (1996) and Qiao & Saris (1996) suggest that NisT is involved in transport of precursor nisin and NisB in dehydration of the nisin precursor.

Analysis of the NZ9734 strain with the disrupted *nisC* gene showed that the plasmid insertion in *nisC* resulted in polar effects as judged from the fact that that no NisI protein could be detected after nisin induction. Because no modified nisin precursor was secreted by strain NZ9734 upon nisin induction, as would be expected if NisC was not essential for the biosynthesis of nisin, it can be concluded that NisC is essential for nisin maturation.

Nisin induction of the knock-out strains (Δ*nisB*, Δ*nisT*, Δ*nisC* and Δ*nisI*) did not result in full restoration of transcription of the nisin operons. A lower transcription level of the nisin operons is likely to explain the lower immunity level of induced mutant strains compared to nisin-producing strains (Dodd *et al*., 1996). A wild-type level of transcription of the nisin operons might require gradually increasing nisin concentrations as in a nisin producer and such fine-tuning may not occur when nisin is externally added at a certain time point to mutant strains. The increase in immunity levels of the nisin mutant strains by nisin induction could also be a result of induction of nisin-inducible genes other than *nisABTCIPRKFEG*. If such genes did exist, their promoter would most likely share sequence similarity with the nisin-inducible promoters in front of *nisA* and *nisF* (de Ruyter *et al*., 1996; Ra *et al*., 1996). The Southern blot analysis of DNA of a nisin-producing strain using a degenerate probe recognizing the conserved sequences of the *nisA* and *nisF* promoters (de Ruyter *et al*., 1996; Ra *et al*., 1996) suggests that a nisin-producing strain does not contain additional nisin-inducible promoters. Therefore, the increase in the level of nisin immunity of the nisin mutant strains observed after nisin induction is likely to be the sole result of the increased transcription of the two known nisin-inducible operons, i.e. *nisABTCIP* and *nisFEG*.

The NZ9747 strain with a deletion in the *nisI* gene did not produce the NisI protein but could still produce nisin. The lack of the NisI immunity protein affected the growth of the strain. It did not grow to as high cell densities as the parental strain and the maximum amount of nisin produced was also lower: approximately 20% of the highest amount that could be produced by the wild-type strain. The cells of strain NZ9747 could tolerate the amount of nisin that they produced themselves, probably due to the expression of the *nisFEG* genes, but the sensitivity to externally added nisin was approximately five times higher than in the wild-type. The nisin immunity level of strain NZ9747 showed that without the NisI lipoprotein the NisF, NisE and NisG immunity proteins can protect the cells to approximately 20% of the wild-type level. Duan *et al*. (1996) described from *L. lactis* a plasmid-encoded nisin

resistance determinant consisting of only the *nisRKFEG* genes. The presence of this nisin resistance plasmid resulted in an immunity level of approximately 20% of a wild-type nisin producer. The nisin immunity level of strain NZ9747 corresponds well to the nisin resistance level of the nisin resistance plasmid containing the *nisRKFEG* genes. From these results some conclusions can be drawn concerning the question whether NisI cooperates with the NisFEG polypeptides or whether they represent separate immunity systems. Expressed to wild-type and higher levels without other *nis*-encoded genes NisI gives only 1–4% of the wild-type immunity level (Kuipers *et al*., 1993; Qiao *et al*., 1995). If NisI represents a non-cooperating immunity system, then the question arises why the immunity level of NZ9747 is only 20% of the wild-type level and is not in the range of >95 % as would be expected if NisI formed an independent immunity system. Therefore, NisI clearly cooperates with some of the transposon-encoded polypeptides, probably with the NisFEG polypeptides. The observed 80% reduction of nisin immunity in the NisI-deficient strain can be partly explained by assuming that the efficiency of the immunity proteins is also influenced by the presence of functional complexes with other *nis*-encoded membrane proteins, e.g. NisB, NisP or NisT. This has been suggested by previous results of Kuipers *et al*. (1993) showing that expression of *nisI* resulted in at most 1–4% of wild-type immunity, whereas when *nisI* was expressed together with the *nisABTC* genes the immunity level was higher, ranging between 8 and 20% of the wild-type level. Thus, full nisin immunity seems to require nisin production and fully induced *nisI* and *nisFEG* genes. This notion is further supported by recent studies showing that the production of antisense-*nisEG* or antisense-*nisG* RNA severely reduced the immunity levels in the *L. lactis* strain tested (Immonen & Saris, 1998).

The killing activity of nisin requires pore formation in the target cell. It has been speculated that NisI as a lipoprotein could destabilize this pore formation (Entian & de Vos 1996; Saris *et al*., 1996) or assist the putative transport function of NisFEG. Recently Qiao (1996) has shown by circular dichroism (CD) spectroscopy and biomolecular interaction analysis (BIA) that purified NisI does indeed have physical interactions with nisin. On the basis of sequence homology the *nisFEG*-encoded proteins belong to the family of ABC transporters (Siegers & Entian, 1995), which strongly suggests that nisin immunity is dependent on nisin translocation. Our present view of nisin immunity comprises cooperative interactions of NisI with the putative NisFEG complex, in which translocation of nisin from the membrane to the cell exterior by NisFEG activity is mediated or facilitated by NisI interactions with nisin.

ACKNOWLEDGEMENTS

We thank Roland Siezen, Michiel Kleerebezem and Cindy van Kraaij for critically reading the manuscript. This work was in part supported by contract BIOT-CT91-0265 of the BRIDGE programme of the European Union.

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Received 25 November 1998; revised 8 February 1999; accepted 12 February 1999.