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 nisABTC/PRKf€G 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 Tn5276 (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 posttranslational 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...
& Saris, 1996). nisl 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 nisl, 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 ΔnisB 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 SstI–AccI fragment containing the nisT gene and flanking regions was cloned into a SstI–AccI-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 SpeI fragment, resulting in the removal of the codons for amino acid residues 318–395. To construct the single-crossover integrative plasmid pNZ9134 (nisC), an internal 753 bp NdeI–NcoI (filled in with Klenow) fragment of nisC was cloned into a NdeI–NcoI-digested pUC19 vector, which had an additional erythromycin resistance marker from pE194 (Leenhouts et al., 1991). To construct the integrative plasmid pNZ9147, a 47 kbp SpeI–BclI fragment containing the nisI gene and flanking regions was cloned into a XbaI–BamHI-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 HpaI (partial digest)–AvaI (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 (Holm & 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, radio-labelling 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 al., 1995).
DNA sequencing was performed by the dideoxy-nucleotide 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 *L. lactis* production 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).

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-GATCCGAACCGAGTAC-3'</td>
<td>nisB</td>
</tr>
<tr>
<td>2</td>
<td>5'-GATGTTGAAAATCTCTTG-3'</td>
<td>nisB</td>
</tr>
<tr>
<td>3</td>
<td>5'-GGACGATGGAATTTGG-3'</td>
<td>nisT</td>
</tr>
<tr>
<td>4</td>
<td>5'-CATATGTTGAAGTTTGCAAC-3'</td>
<td>nisC</td>
</tr>
<tr>
<td>5</td>
<td>5'-GAGAGATATTATATTCTTGTG-3'</td>
<td>nisI</td>
</tr>
<tr>
<td>6</td>
<td>5'-ATGGTTGAGTGAAGATTTTCC-3'</td>
<td>nisI</td>
</tr>
<tr>
<td>7</td>
<td>5'-GTGTTTGATGGTCGGGACTGGACAT-3'</td>
<td>nisR</td>
</tr>
<tr>
<td>NIS121</td>
<td>5'-CTGATTAATTCTGAAACATGGT-3'</td>
<td>Promoter fragment of nisA and nisF</td>
</tr>
</tbody>
</table>

**Introduction of in-frame deletions in chromosomal nisin genes by gene replacement.** The plasmids pNZ9135 (ΔnisB), pNZ9143 (ΔnisT) and pNZ9147 (ΔnisI), all pUC19 derivatives that cannot replicate in *L. lactis*, were used to transform the nisin A producing *L. lactis* NZ9700. Erythromycin-resistant (Em') 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' 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' 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' 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' 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 ΔnisA 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%; +, 10-30%; -, <1%. Immunity levels: ++++, 100%; +++, 70-80%; ++, 30-40%; +, 10-30%; -, <5%. Transcription of nisFEG levels: ++++, 100%; +++, 50-70%; ++, 10-30%; +, 5-10%; -, <5%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular nisin production</th>
<th>Immunity</th>
<th>Transcription of nisFEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No induction</td>
<td>Pre-induction with nisin</td>
<td>No induction</td>
</tr>
<tr>
<td>MG1614</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NZ9700</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
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<tr>
<td>NZ9800 ΔnisA</td>
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<tr>
<td>NZ9735 ΔnisB</td>
<td>--</td>
<td>+</td>
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<tr>
<td>NZ9743 ΔnisT</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>NZ9734 ΔnisC</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>NZ9747 ΔnisI</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
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</table>

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 nisl 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 (ΔnisI) and strain NZ9734 (ΔnisC) (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 nisl 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 nisABCTCIPRFKFG, a Southern analysis was performed with EcoRI/EcoRV-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...
In-frame deletions in the nisin gene cluster

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, nisl, 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 nisl 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 nisl gene was detected in Western analysis using a Nisl-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 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 (ΔnisB, ΔnisT, ΔnisC and ΔnisI) 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 nisABTCTRKFEG. 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 nisl gene did not produce the Nisl protein but could still produce nisin. The lack of the Nisl 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 Nisl 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 nisRKFEG genes. From these results some conclusions resulted in an immunity level of approximately 20% of strain NZ9747 corresponds well to the nisin resistance determinant consisting of only the nisRKFEG genes. This has been suggested by previous results of 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-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.

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