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Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* Requirement of expression of the *nisA* and *nisI* genes for development of immunity

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The nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*, located on a 10-kbp DNA fragment of the nisin-sucrose transposon Tn5276, was characterized. This fragment was previously shown to direct nisin-A biosynthesis and to contain the *nisP* and *nisR* genes, encoding a nisin leader peptidase and a positive regulator, respectively [van der Meer, J. R., Polman, J., Beerthuyzen, M. M., Siezen, R. J., Kuipers, O. P. & de Vos, W. M. (1993) *J. Bacteriol.* 175, 2578–2588]. Further sequence analysis revealed the presence of four open-reading frames, *nisB*, *nisT*, *nisC* and *nisI*, downstream of the structural gene *nisA*. The *nisT*, *nisC* and *nisI* genes were subcloned and expressed individually in *Escherichia coli*, using the T7-RNA-polymerase system. This resulted in the production of radio-labelled proteins with sizes of 45 kDa (NisC) and 32 kDa (NisI). The *nisT* gene product was not detected, possibly because of protein instability. The deduced amino acid sequence of NisI contained a consensus lipoprotein signal sequence, suggesting that this protein is a lipid-modified extracellular membrane-anchored protein. Expression of *nisI* in *L. lactis* provided the cells with a significant level of protection against exogenously added nisin, indicating that NisI plays a role in the immunity mechanism. In EDTA-treated *E. coli* cells, expression of *nisI* conferred up to a 170-fold increase in immunity against nisin A compared to controls. Moreover, a lactococcal strain deficient in nisin-A production, designated NZ9800, was created by gene replacement of *nisA* by a truncated *nisA* gene and was 10-fold less resistant to nisin A than the wild-type strain. A wild-type immunity level to nisin and production of nisin was obtained in strain NZ9800 harboring complementing *nisA* and *nisZ* plasmids. Transcription analyses of several *L. lactis* strains indicated that an expression product of the *nisA* gene, together with NisR, is required for the activation of *nisA* transcription.

The antimicrobial peptide nisin (Gross and Morell, 1971; Hurst, 1981) belongs to the rapidly expanding family of lantibiotics (Schnell et al., 1988), a group of small peptides (less than 4 kDa), which contain (β -methyl)lanthionine residues. Nisin is produced by several strains of the Gram-positive bacterium *Lactococcus lactis* and is applied in the food industry as a natural preservative because it effectively inhibits the growth of food spoilage bacteria (Delves-Broughton, 1990). The biosynthesis of nisin and other lantibiotics is thought to take place by successive enzyme-catalyzed modifications of the ribosomally synthesized precursor peptide (Schnell et al., 1988). These specific modifications of precursor nisin include the following: dehydration of serine and threonine residues, resulting in dehydroalanine and dehydrobutyrine residues, respectively; the addition of free thiol groups of cysteine residues to the double bonds of dehydrated residues, resulting in *meso*-lanthionine and β -methyl-lanthionine residues; cleavage of the leader peptide from the mature precursor nisin after secretion into the medium (van

der Meer et al., 1993). Several genes have been characterized that encode proteins involved in biosynthetic processes of different lantibiotics, e.g. epidermin (*epi*; Schnell et al., 1992), subtilin (*spa*; Banerjee and Hansen, 1988; Chung and Hansen, 1992; Klein et al., 1992; Klein et al., 1993), pep5 (*pep*; Kaletta et al., 1989; Reis and Sahl, 1991) and nisin (*nis*; Buchman et al., 1988; Kaletta and Entian, 1989; Steen et al., 1991; Mulders et al., 1991; Engelke et al., 1992; van der Meer et al., 1993). The proteins encoded by the *nisB* and *nisC* genes (Engelke et al., 1992), and their counterparts in other lantibiotic-encoding gene clusters, might be involved in modification reactions of the prelantibiotic. The protein encoded by the *nisT* gene (Engelke et al., 1992) and its counterparts in the *epi* and *spa* gene clusters, have been implicated in the translocation process of lantibiotics, since their deduced sequences show a high degree of similarity to those of transport ATPases, exemplified by HlyB of *Escherichia coli* (Mackman et al., 1986). The gene product of *nisP* is involved in the extracellular processing of a fully matured precursor nisin (van der Meer et al., 1993). Finally, the *nisR* gene (van der Meer et al., 1993) and its homologues in the *spa* and *epi* operons, encode regulatory proteins involved in gene expression. In the epidermin biosynthetic operon the *epiD* gene encodes a flavoprotein, which is probably involved in the formation of *S*-[(*Z*)-2-aminovinyl]-D-cysteine in epidermin (Kupke et al., 1992). This post-translational

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Abbreviations. IPTG, isopropyl-thio- β -D-galactoside; PCR, polymerase chain reaction.

Note. The novel nucleotide sequence data published here have been deposited with Genbank and are available with accession number L16226.

modification does not occur in nisin, implying that a counterpart of *epiD* is not likely to occur in the nisin gene cluster.

Thus far, the only known protein encoded by a lantibiotic operon that is involved in immunity is produced by *pepI*, which confers immunity to *Staphylococcus epidermidis*, but only in the presence of the *pepA* gene (Reis and Sahl, 1991). With respect to the self-protection of nisin-producing strains, it has been noticed that some mutants of a nisin-A-producing strain, that produce low amounts of nisin or none at all, display different levels of immunity, the highest level of immunity corresponding to the highest level of nisin-A production (Rauch et al., 1991). This suggests that the production of nisin or its precursor, together with an unknown factor, is required for reaching a high level of producer immunity. As yet, no gene or gene product, apart from *nisA* itself, has been found that could be involved in immunity of a nisin-producing strain.

In a search for such a gene and for other biosynthetic genes, we have now cloned, sequenced and analyzed 10 kbp of the nisin-gene cluster that starts with *nisA*. This region, located on the nisin-sucrose transposon Tn5276 (Rauch and de Vos, 1992), was found to contain the genes *nisABTCIPR*. Analysis of these genes revealed that *nisI* is involved in the development of immunity against nisin. When the *nisA* gene was disrupted in a nisin-A-producing strain (strain NZ9800), a lower level of immunity to nisin was observed, which was elevated to wild-type levels again when complementing *nisA*-containing or *nisZ*-containing plasmids (Kuipers et al., 1992) were introduced. In strain NZ9800, no transcription of the truncated *nisA* gene was observed, suggesting the involvement of a *nisA* expression product in the transcription of *nisA*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

L. lactis strain NZ9700 is a transconjugant from a mating between the nisin-A-producing strain NIZO R5 (Pette and Kooy, 1952; Mulders et al., 1991) and the plasmid-free strain MG1614 (Gasson, 1983), and contains a single copy of the nisin-sucrose transposon Tn5276 at the same chromosomal site as in *L. lactis* T165.1 and T165.5 (Rauch and de Vos, 1992). *L. lactis* NZ9900 is a transconjugant from a mating between the non-nisin-producing strain NIZO R520 (Rauch et al., 1991), containing a mutant Tn5276 deficient in nisin-production, and strain MG1614. Lactococcal cells were grown overnight without aeration at 30°C in a medium containing 1% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 0.2% NaCl, pH 7.0, 0.002% MgSO₄ · 7H₂O and 1% KH₂PO₄ (SPYS medium; De Vuyst and Vandamme, 1992). *E. coli* strain MC1061 (Casadaban et al., 1980) was used as an intermediate host for cloning and was manipulated as described in Sambrook et al., 1989. Other *E. coli* strains included HMS174 (Studier and Moffat, 1986) and JM109(DE3) (Promega Corporation) that were used as recipient strains in cloning and expression experiments. *E. coli* strains were grown in TY broth at 37°C. *Micrococcus flavus* DSM 1790 (obtained from the German Collection of Microorganisms) was used to determine antimicrobial activity as described previously (Kuipers et al., 1992). The following plasmids were used in cloning experiments in *E. coli*: pUC19 and M13mp18/19 (Yanisch-Perron et al., 1985); pET8c, pET3b, pLysS and pLysE (Rosenberg et al., 1987); pUC19E, a pUC19 derivative described by Leenhouts et al. (1990),

containing the erythromycin-resistance gene of pE194 (Hori-nouchi and Weisblum, 1982); pNZ184, a derivative of pA-CYC184 (Chang and Cohen, 1978), in which the *SmaI*-*HindIII* multiple cloning site of M13mp19 was cloned into the *SmaI/HindIII*-digested vector, to introduce a unique *PstI* site (van Alen-Boerrieger et al., 1991). In *L. lactis*, the plasmids pIL253 (Simon and Chopin, 1988) and pNZ9010 (containing *nisA*) and pNZ9013 (containing *nisZ*; Kuipers et al., 1992) were used. Antibiotics were used in the following concentrations: ampicillin, 50 µg/ml; erythromycin, 5 µg/ml; chloramphenicol, 10 µg/ml for *E. coli* and 5 µg/ml for *L. lactis*. Induction of the T7 expression system in *E. coli* JM109(DE3) was performed by adding 1 mM isopropyl-thio-β-D-galactoside (IPTG) to the growth medium and by adding 0.2 mg/ml rifampicin (Studier and Moffat, 1986).

DNA manipulations and sequencing

Plasmid and chromosomal DNA of *L. lactis* were isolated as described previously (Vos et al., 1989). *E. coli* cells were transformed by use of the CaCl₂ procedure (Sambrook et al., 1989); *L. lactis* cells were transformed by electroporation (Holo and Nes, 1989; Vos et al., 1989). Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Gibco/BRL Life Technologies, New England Biolabs or Promega Corporation and used as recommended by the manufacturers. Cloning procedures, radiolabelling of DNA fragments, agarose-gel electrophoresis and Southern-blot hybridisations were according to established procedures (Sambrook et al., 1989). DNA fragments were purified by the Gene-Clean procedure (Bio 101). Polymerase chain reaction (PCR) was performed using the conditions described before (Kuipers et al., 1991).

DNA sequencing was performed by use of the dideoxynucleotide chain-termination method (Sanger et al., 1977). Oligonucleotides, used as primers in sequencing reactions and for PCR, were synthesized using a Cyclone DNA synthesizer (Biosearch).

Cloning of nisin operon genes

A 9.2-kbp *PstI* fragment, containing the *nisA* gene, was identified in total DNA of *L. lactis* strain NIZO R5 by hybridization with a 300-bp DNA fragment, containing the *nisA* gene. The *PstI* fragment was isolated and ligated to *PstI*-linearized dephosphorylated pNZ184 DNA. After transformation of *E. coli* MC1061, plasmid DNA of ampicillin-resistant transformants was analyzed by restriction digestions and hybridizations with the *nisA* gene probe. One recombinant plasmid, pNZ9003, contained the 9.2-kbp *PstI* fragment that included *nisA* and approximately 7-kbp DNA downstream of *nisA*. The same 9.2-kbp *PstI* fragment was also inserted in the *PstI* site of plasmid pIL253 (Simon and Chopin, 1988), yielding pNZ9000 (Fig. 1), which was transformed into *L. lactis* strains MG1614 and NZ9900.

A DNA fragment harboring the entire *nisI* gene was obtained as a 1-kbp *AccI* (blunt ended by Klenow-enzyme treatment)-*PstI* fragment from pNZ9000. This fragment was cloned in the *SmaI/PstI*-digested vector pIL253 in MG1614. The new construct was named pNZ9031. The antisense orientation of *nisI* was obtained by cloning the same fragment in the *XhoI/PstI*-digested pIL253; this construct was named pNZ9032. The *nisR* gene was cloned as a *ScaI-PstI* fragment of pNZ9111 (van der Meer et al., 1993) in the *SmaI*

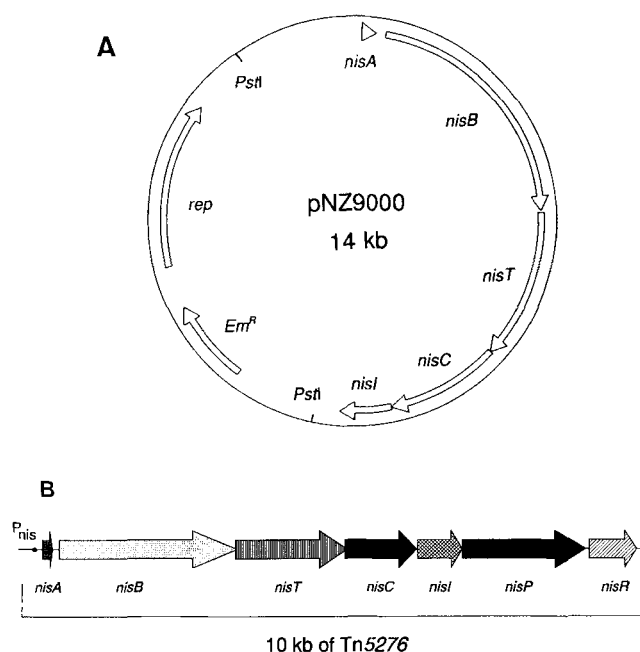


Fig. 1. Genetic organization of nisin biosynthetic genes. (A) Map of plasmid pNZ9000, harboring the 9.2-kbp *PstI* fragment of Tn5276. (B) Organization of the nisin biosynthetic gene cluster of Tn5276 of *L. lactis* NIZO R5 (*nisP* and *nisR*, van der Meer et al., 1993).

PstI-digested vector pIL253. The resulting construct was designated pNZ9030.

Construction of expression plasmids for *E. coli* and heterologous gene expression

A 2.8-kbp *EcoRI*–*NcoI* (blunt ended by Klenow-enzyme treatment) fragment from pNZ9000 (Figs 1 and 2), was cloned in pET8c, which had previously been digested with *NcoI* and *BamHI* (blunt ended by Klenow-enzyme treatment), together with a synthetic 15-bp *NcoI*–*EcoRI*-linker fragment consisting of 5'-CATGGATGAAGTGAAAG-3' and 5'-AATTCCTTTCCTTCATC-3'. The resulting plasmid that contains the complete *nisT* gene was denoted pET8*nisT*. The *nisC* gene was cloned as a 2-kbp *NdeI*–*HaeIII* fragment of pNZ9000 in pET3b digested with *NdeI* and *BamHI* (blunt ended by Klenow-enzyme treatment), together with a 240-bp PCR-amplified *NdeI* fragment (obtained with the primers 5'-GCCATATGAGGATAATGATGAATAAAAAAAT-3' and 5'-GCGCATATGTTGAAAGTTTGCTAAC-3' and encoding the first 80 amino acids of *nisC*). This plasmid was named pET3*nisC*. A truncated form of *nisC*, lacking the DNA encoding the first 80 amino acids, was cloned into pET3b, giving plasmid pET3*AnisC*. The *nisI* gene was obtained as a 1.2-kbp *NcoI*–*PstI* (blunt ended by T4 polymerase) fragment of pNZ9000 and cloned in pET8c, which had been digested with *NcoI* and *BamHI* (blunt ended by Klenow-enzyme treatment), resulting in plasmid pET8*nisI*. A deletion derivative of *nisI*, lacking the first 200 bp of *nisI*, was also cloned in pET8c. This construct was named pET8*AnisI*. The cloning of *nisR* in pET8c resulted in plasmid pNZ9124 (van der Meer et al., 1993).

Expression of nisin operon genes from the T7 promoter was analyzed in *E. coli* JM109(DE3) or, if necessary, in JM109(DE3) containing pLysS or pLysE. Exponentially

growing cells were induced at A_{620} 0.5 by addition of IPTG and proteins were radiolabelled after addition of L-[35 S]methionine (Amersham) and 0.2 mg/ml rifampicin for 2 h (Studier and Moffat, 1986; Studier et al., 1990). Radiolabelled proteins were prepared from the cell pellet of 1 ml induced cultures by suspending the pellet in sample buffer (Laemmli, 1970) and boiling them for 5 min. Proteins were analyzed by SDS/PAGE following established procedures (Sambrook et al., 1989) and visualized by autoradiography.

Inactivation of chromosomal *nisA* by gene replacement

To disrupt the *nisA* gene on the chromosome of nisin-A-producing *L. lactis* NZ9700, a 2.7-kbp *BclI*–*EcoRV* fragment, which contained *nisA* and approximately 1.2-kbp of its flanking regions, was isolated from pNZ9700 and cloned into pUC19, which had been digested with *BamHI* and *EcoRI* (blunt ended by Klenow-enzyme treatment). The resulting plasmid, pNZ9005, was linearized by partial digestion with *SstI*, treated with T4 polymerase to remove four extending nucleotides, ligated and used to transform *E. coli* MC1061. A 4-bp deletion was generated in the middle of the *nisA* gene, causing a frameshift and the occurrence of a stop codon at amino acid 16 of the encoded pronisin, excluding the possibility of the production of an active nisin species. This plasmid was named pNZ9005.1 and the mutated *nisA* gene was denoted *AnisA*. An approximately 1-kbp *EcoRI* (blunt ended by Klenow-enzyme treatment)–*SphI* fragment from pUC19E, containing the erythromycin-resistance gene, was isolated and ligated in the *XbaI*-digested (blunt ended by Klenow-enzyme treatment) and *SphI*-digested vector pNZ9005.1, resulting in the plasmid pNZ9006. This pUC19-derived plasmid, which cannot replicate in *L. lactis* (Leenhouts et al., 1990), was used to transform the nisin-A-producing *L. lactis* NZ9700. Erythromycin-resistant transformants were obtained, which contained a single chromosomal copy of plasmid pNZ9006. One transformant was allowed to grow for approximately 100 generations in the absence of erythromycin. After plating, colonies were screened for sensitivity to erythromycin and loss of the capacity to produce nisin. Several erythromycin-sensitive colonies, which did not produce nisin, were further characterized using PCR amplification of the *nisA* region and subsequent DNA sequencing of this region, and by hybridization of digested total DNA with a probe for the *nisA* gene. Following these experiments one strain, designated NZ9800, was selected that contained a single copy of the *AnisA* gene and an intact upstream and downstream region.

RNA isolation and Northern blotting

RNA was isolated according to the method of R. Raya (unpublished results). Briefly, *L. lactis* cells grown to A_{600} 0.6 were pelleted, suspended in buffer A (0.5 ml 10 mM Tris/HCl, pH 7.5, 1 mM EDTA) at 0°C. The suspension was transferred to a microfuge tube containing 0.6 g zirconium glass beads (Biospec Products), 0.17 g 4% Macaloid (Rheox), 0.5 ml phenol, pH 7.5, and 0.05 ml 10% SDS. The tubes were shaken at maximal setting in a homogeniser (Biospec products) at room temperature for 3 × 1 min at 1-min intervals, during which time the samples were allowed to cool to –20°C. The microtubes were subsequently centrifuged at 6000 g for 15 min. Four layers were visible and the upper one, containing RNA, was collected and extracted successively with phenol/chloroform (1:1, by vol.) and chlo-

TAAGTCATACAAAGCTATTTCATAAACTAATCTATTTCCTCAAAACAATGATTCAAAAATATTACATCATTTATACTAGTTTGTATGGAACAACCTTTTGGAGTTTTTAAAGAGAG	4440
V S L I Q A I S K I N T Y S Q T M I Q N I Y I I Y N T S L F M E Q L F E F L K R	331
AAAGTGTAGTTCCACAAAAATAGAACTACTGAAATATGCAATCAACATATAGGAAGCTTTAAAGATTAATTTATCATAATGTTTACCCTAATTCGAATGCCTTTGGCCTAAAGAAATA	4560
E S V V H K K I E D T E I C N H I G T V K V I N L S Y V Y P N S N A F A L K N	371
TCAATTTATCCTTTGAAAAAGGAGAATTAACCTGCTATTGTAGGAAAAATGGTTACGGGAAAAAGTACACTAGTAAAGATAATTCAGGATTATATCAACCACTATGGGAATAATCCAAT	4680
I N L S F E K G E L T A I V G K N G S G K S T L V K I I S G L Y Q P T M G I I Q	411
ACGACAAATGAGAAGTAGTTGATGCCTGAGGAGTTTATCAGAAAAACATATCGGTGCTGTTCCAAGATTTTGAAGTATGAGTTAACGATAAGAGAGAATATAGGATTGAGTGATT	4800
Y D K M R R S S L M P E E F Y Q K N I S T G Q D F V K Y E L T I R E N I G L S D	451
TGCTTCTCAATGGGAAGATGAGAAAAATTAAAGTACTAGATAATTTAGGACTCGATTTTTTGAACCTAATAATCAATATGTACTTGATACGCAGTTAGGAAATGGTTTCAAGAAG	4920
L S S Q W E D E K I I K V L D N L G L D F L K T N N Q Y V L D T Q L G N W F Q E	491
GGCATCAACTTTCAGGAGGTCAGTGGCAAAAAATGCAATAGCAGGACATCTTTPAAGAAAGCTTCAATTTATTTAGATGAACCAAGTGCCTCGACTCGATCCTGTAGCTGAAAAAG	5040
G H Q L S D G G Q W Q K I A L A R T F F K K A S I Y I L D E P S A A L D E P V A K E K	531
AAATATTGATTTTGTGTCTTTCCGAAAAATAATTTCAATTTTCATTTCTCATAGTTTGAATGCTGCGAGAAAAAGCAATAAAATCGTGGTTATGAAAGATGGACAGTTCGAAG	5160
E I F D Y F V A L S E N N I S I F I S H S L N A A R K A N K I V V M K D G Q V E	571
RBS	
ATGTTGGAAGTCATGATGTCCTTCGAGAAGATGTCAACTACTATCAAGAACTTTATTTATTCAGAGCAATATGAGGATAATGATGAATAAAAAAATATAAAAAAAGAAATGTTGAAAAAAT	5280
D V G S H D V L L R R C Q Y Y Q E L Y Y S E Q Y E D N D E *	600
<i>nisC</i> → M N K K N I K R N V E K I	13
ATTGCTCAATGGGATGAGAGAACTAGAAAAATAAGAAAACTTCGATTTCGGAGAGTGACTCTCTCAGGAGTTGCCCTGGTATAATTTTAAATGTTAGCGGAGTTAAAAATAAAGAT	5400
I A Q W L R E D R T R K N I A L A R T F F K K A L S T G L S T F L S T G L S T F L S T G L S T F L S T G L S T F L S T G L S T F L S T G L S T F L S T G L S T	53
AACTCAAAGATATATCAGAAAAAGATAGACAATTTATTTGAATATATTTAGTAACTTTCAACATATGGGGCTTTTAAACAGGATCACTTTATTCGGGAGCAGCTGGCATTGCATTAAGT	5520
N S K I Y Q K K I D N Y I E Y' I V S K L S T Y G L L T G S L Y S G A A G I A L S	93
ATCTACATTTACGAGAAATGACAAAAATAAAGAACTTCTTGTAGACCTAAATAGATATATCGAATATTTTCGTCAGAGAAAAATGAAGGATTTAATTTGAAAAAATTTACTCCT	5640
I L H L R E D D E K Y K N L L D S L N R Y I E Y F V R E K I E G F N L A E P L A G V	133
CCTGATTATGACGTGATGAAAGTTTATCTGGGACTTTCCTATCTATTTAATCAACAGCAGCAATATGATGATTGAAAAACTCATTTATCAATTTTATCAAATCTGCATAAA	5760
P D Y D V I E G L S G I L S Y L L L I N D E Q Y D D L K I L I I N F L S N L T K	173
GAAAACAAGGACTAATCTGCTTTCATCAATCGGAGAATCAGATCTCAATCAGAAAGTGAATGATCCACTAGGCTGTTTGAATATGGGATAGCACATGGACTTGTGAGTG	5880
E N N G L I S L Y I K S E N Q M S Q S E S E M T F L G C L N M G L A H G L A G V	213
GGCTGATCTTAGCTTATGCCACATAAAGGATATAGTAATGAAGCCTCGTTGTCAGCTTTGCAAAAAATTTATTTTATTTATGAAAAGTTTGAACITGAAAGGAAAAACAGTTTCTA	6000
G C I L A Y A H I K G Y S N E A S L S A L Q K I I F I Y E K F E L E R K K Q F L	253
TGGAAGAAGTGGACTGTAGCAGATGAATAAAAAGAGAAAGTAATAGGGAAGCAAGTTCATTTAGAGATGATGCTGCTATGGAGTCCAGGATTTAGTCTGCTACTTATACCGA	6120
W K D G L V A D E L K K E K V I R E A S T F I R D A W C Y G G P G I S L L Y L Y G	293
GGATTAGCACTGGATAATGACTATTTGTAGATAAAGCAGAAAAATATAGAGTCAAGTATGCAAAAGGAACTGGTATTTGATTCATATGATTTGCCATGGCTATTCGGTTAATA	6240
G L A L D N D Y F V D K A E K I L E S A M Q R K L G I D S Y M I C H G Y S G L I	333
GAAATTTGTTCTTTATTTAAGCGGCTATTAATACAAAAAAGTTTGGATTACATACATGGAAGAATTTAATGTTAATAGTGAAGCAATTTTGAAGAATACCGGAGATGAAAGTGGCACGGGT	6360
E I C S L F K R L L N T K K F D S Y M E E F N V N S E Q I L E E Y G D E S G T G	373
RBS	
TTCTTGAAGGAATAAGTGCTGTACTGGTATTATCGAAAATTGAATATCAATCAATTTTACTTTATGGAGACAAGCACTGTTACTTTTTGACGATTTTTTGAAGGAGGGAAGAGG	6480
F L E G I S G C I L V L S K F E Y S I N F T Y W R Q A L L L F D D F L K G G K R	413
AAATGAGAAGATTTAATACTTTATTTGGCCCTTAATAGGGATAACAGGTTTATCAGGGTGTATCAACAAGTCAATAAAAAGGTGAGGTTTGACGAAGGAAAGTTATACTAATTTTATTT	6600
K *	414
M R R Y L I L I V A L I G I T G L S G C Y Q T S H K K V R F D E G S Y T N F I	39
<i>nisI</i> →	
ATGATAATAAATCGTATTTTCGTAAGTAAAGGAGATTCCTCAGGAGAAGCTTAAACAATCCAAAAGTAAAATTTTAAAGCTGTGATTTGACATGAAAAGTGAAGAACTTTTATCAA	6720
Y D N K S Y F V T D K E I P Q E N V N N S K V K F Y K L L I V D M K S E K L L S	79
GTAGCAACAAAATAGTGTGACTTTGGTCTTAAATAATTTATGAGGCTTCTGACAAGTCTGATGTTGGGATTTAAACAGCAGATACTATAAGATACTCCCAAGTATAAGGGGG	6840
S N S N I Y E A S D K S L C M G I N D R Y Y K I L P E S D G K	119
CGGTCAAAGCTTTGAGATTACAAAATTTGATGTGACAAGCGATATTTCTGATGATAATTTGTTATTTGATAAAAAATGATTCAGCAAAAATGACTATATGGGAAATTTTACAGTATAT	6960
A V K A L R L Q N F D V T S D I S D D N F V I D K N D S R K I D Y M G N I Y S I	159
CGGACACCCTGATCTGATGAAGAATTTGGGAGAATATCAGGATGTTTACGTTGAAGTACGTTGTTGATTTAGTGGCAAAAGTATCCCGAGTCTGAATGGGGAGAATTGATA	7080
S D T T V L D G E Y Q D V L A E V R V G T F V D S V S G K S I P R S E W G R I D	199
AGGATGTTCAAATCCAAACAGAGTAGGACGGAATGGGATTTAGCGAAAATCCATCTTATAGAGAAAATCTCTTACTGAAGCATTTGCCGTTGAGATAAATGATGATTTAAGCTTG	7200
K D G S N S K Q S R T E W D Y G E I H S I R G K S L T E A F A F E I S L N D D F K L	239
RBS	
CAACGAAGGTAGGAAACTAGAGTGAAAAAATACTAGGTTTCCTTTTATCGTTTGTTCGTTGGGTTTATCAGCAACTGTGCATGGGGAGACAACAATTCACAACAGTTACTCTCAAAT	7320
A T K V G N * V K K I L G F L F I V C S L G L S A T V H G E T T N S Q Q L L S N 245/33	
<i>nisP</i> →	
AATATTAATACGGAATTAATTAATCATAATTTCTAATGCAATTTTATCTTCAACAGAGGGATCAACGACTGATTCGATTAATCTAGGGGAGCAGTCACTCGAG -->	7423
N I N T E L I N H N S N A I L S T E G S T T D S I N L G A Q S P A etc	77

Fig. 2. Sequences of *nisA*, *nisB*, *nisT*, *nisC* and *nisI* of Tn5276 of *L. lactis* NIZO R5. Putative ribosome-binding sites (RBS) and inverted repeats (→) are indicated, as is the transcription-initiation site of the *nisA* gene and its preceding canonical sequences. Positions of restriction sites used are as follows: *AccI*, 6383–6388; *BclII*, 2914–2919; *EcoRI*, 3461–3466; *EcoRV*, 1805–1810; *HaeIII*, 6509–6512; *NcoI*, 6218–6223; *NdeI*, 4518–4523; *PstI*, 7418–7423; *SstI*, 283–288, 1547–1552 and 2463–2468.

roform. RNA was precipitated with ethanol and dissolved in 100 µl buffer A. For storage, RNA was kept in ethanol at -20°C and precipitated before each use. 4% Macaloid was prepared as follows: 2 g Macaloid was suspended in 100 ml buffer A, boiled for 5 min and sonicated with a sterilized probe sonicator (Heat Systems) until a gel formed (approximately 1 min); the gel was allowed to cool, washed with buffer A, centrifuged at 5000 g and the supernatant was discarded, yielding approximately 50 ml gel.

For Northern-blot analysis, RNA was glyoxylated, fractionated using a 1.0% agarose gel, blotted and hybridized as described previously (van Rooijen and de Vos, 1990). A gel-purified *PstI*-*HindIII* restriction fragment of approximately 280 bp from pNZ9010, harboring the complete *nisA* gene, was radiolabelled by nick translation with [α -³²P]dATP and used as a hybridization probe. For primer extension studies, either oligonucleotide X3 with the sequence 5'-CCAAATCC-AAGTTAAAATC-3' (10 ng) or oligonucleotide 010 with the

sequence 5'-GGATAGTATCCATGTCTGAAC-3', was hybridized to 10 µg RNA in 14 µl containing 70 mM Tris/HCl, pH 8.3, 10 mM MgCl₂, 15 mM dithiothreitol and 40 U RNasin (Promega). This mixture was heated for 5 min at 65°C and allowed to cool to room temperature. Subsequently, 1.6 µl dNTP was added containing 1 mM dCTP, 1 mM dGTP and 1 mM dTTP and 100 µM dATP and the final volume was adjusted to 16 µl with 0.2 µl deoxyadenosine 5'-(α -[³⁵S]thio)triphosphate and 0.2 µl (20 U) avian myeloblastosis reverse transcriptase. After 30 min at 42°C, the reaction mixture was extracted successively with phenol/chloroform (1:1, by vol.) and with chloroform and precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in 3 µl water and 3 µl stop solution (Pharmacia). The samples were heated for 3 min at 98°C and analyzed using an acrylamide sequencing gel. Controls, consisting of RNA to which no primer was added, were treated similarly.

DNA and deduced-protein-sequence analyses

Computer analyses of DNA and amino acid sequence data were performed with the programs PC/GENE (Genofit). Amino acid sequence similarity searches in the EMBL/Genbank and Swissprot/PIR databases were performed using the FASTA and TFASTA programs in the GCG package, provided by CAOS-CAMM, while multiple sequence alignments were made using the PILEUP and CLUSTAL programs.

Determination of (precursor) nisin production

Production of nisin was analyzed using a bioassay with an agar-diffusion test (Tramer and Fowler, 1964; Hugenholz and de Veer, 1991; de Vos et al., 1993), or by SDS/PAGE or reverse-phase HPLC. For SDS/PAGE and HPLC, culture supernatants of *L. lactis* strains were precipitated by addition of 10% trichloroacetic acid. Pellets were dissolved in 50 mM sodium acetate, pH 6.0. Proteins were separated using tricine-SDS/PAGE gels and were detected by silver staining; alternatively proteins were analyzed by reverse-phase HPLC (Mulders et al., 1991). Molecular-mass markers from BRL were used as size markers for SDS/PAGE, whereas purified nisin A and nisin Z were used as references in reverse-phase HPLC.

Nisin-immunity assays for *L. lactis* and *E. coli*

To test the sensitivity of lactococcal cells of different strains to nisin, overnight cultures of these strains were diluted 20-fold in SPYS broth and divided into 10-ml tubes, which contained SPYS broth to which 0–25 µg/ml nisin A had been added. The tubes were incubated at 30°C and at regular intervals samples were removed and the A₆₀₀ of each sample was measured. For strains having immunity to nisin A higher than 5% of the wild-type immunity, growth curves were obtained and sensitivity was expressed as the highest amount of nisin A allowing the same extent of growth observed for the wild type. When no outgrowth occurred after two days in the presence of 0.125 µg/ml nisin A, strains were classified as having no detectable immunity.

The sensitivity of *E. coli* for nisin A was tested by induction of 5 ml *E. coli* JM109(DE3) cells, containing either pET8c, pET8nisI, pET8AnisI or pNZ9124 (*nisR*) at A₆₀₀ 0.6 by IPTG for 40 min. Cells were washed once with TY broth,

divided into four aliquots and subsequently incubated for 30 min in 1 ml 50 mM sodium acetate, pH 6.0, 20 mM EDTA containing 0, 2.5, 12.5 or 25 µg/ml nisin A. EDTA was added to disturb the integrity of the outer membrane, rendering the *E. coli* cells susceptible to nisin (Stevens et al., 1991). Undiluted cells and dilutions of 1:100 and 1:10000 were plated and after overnight incubation the number of surviving colony-forming bacteria in 0.1 ml was determined. The number of colonies obtained for bacteria not treated with nisin was taken as 100% for each strain, and the number of surviving cells in the presence of different amounts of nisin A was determined relative to this value. Independent experiments were performed three times.

RESULTS

Cloning and sequencing of nisin operon genes

Plasmid pNZ9003 is a pACYC184 derivative, containing a 9.2-kbp *Pst*I fragment of the nisin-sucrose transposon Tn5276 and was used in subcloning experiments and for determination of the DNA sequence. Restriction fragments of the insert were subcloned in both M13mp18 and M13mp19. Sequencing on single-stranded phage DNA in both orientations revealed the presence of four open-reading frames downstream of *nisA*, named *nisB*, *nisT*, *nisC* and *nisI*, respectively. Fig. 2 shows the entire nucleotide sequence and the deduced amino acid sequence of this region of Tn5276, including that of the previously reported gene *nisA* (Rauch and de Vos, 1992; Rauch, 1993). Together with the recently reported genes *nisP* and *nisR* (van der Meer et al., 1993), this region completes the nisin-gene cluster of Tn5276. The sequence organization of *nisBTCl* shows that the *nis* genes are either partially overlapping (*nisT* and *nisC*; *nisC* and *nisI*), or separated by only ten nucleotides (*nisB* and *nisT*) or one nucleotide (*nisI* and *nisP*), suggesting translational coupling. The 9.2-kbp *Pst*I fragment was subcloned into the lactococcal vector pIL253 and the resulting plasmid pNZ9000, encoding *nisABTCl*, was introduced into *L. lactis* MG1614 and its derivative NZ9900, carrying a mutated Tn5276 deficient in nisin biosynthesis. Plasmid pNZ9000 conferred nisin-A production to *L. lactis* NZ9900, probably caused by the presence of intact *nisP* and *nisR* genes in this strain. However, in MG1614 harboring pNZ9000 no expression of *nisA* was observed. This is most likely caused by the absence of the essential gene for transcription regulation, *nisR*, since we showed previously that a plasmid which also contained *nisR* (pNZ9111) resulted in the production and secretion of fully matured, but non-processed precursor nisin A by *L. lactis* MG1614 (van der Meer et al., 1993).

Heterologous expression of *nisT*, *nisC* and *nisI* genes in *E. coli*

To detect the gene products of *nisT*, *nisC* and *nisI*, these genes were cloned individually into the T7 expression vector pET8c. Plasmid pET8*nisT* was introduced into JM109(DE3), containing either pLysS or pLysE to reduce transcription levels of the cloned gene, because it could not be stably maintained in JM109(DE3). After induction with IPTG and selective radiolabelling with L-[³⁵S]methionine in the presence of rifampicin, no additional protein band could be observed after SDS/PAGE. Plasmid pET3*nisC* was introduced into JM109(DE3) and the radiolabelled proteins obtained after induction in the presence of L-[³⁵S]methionine, were separated

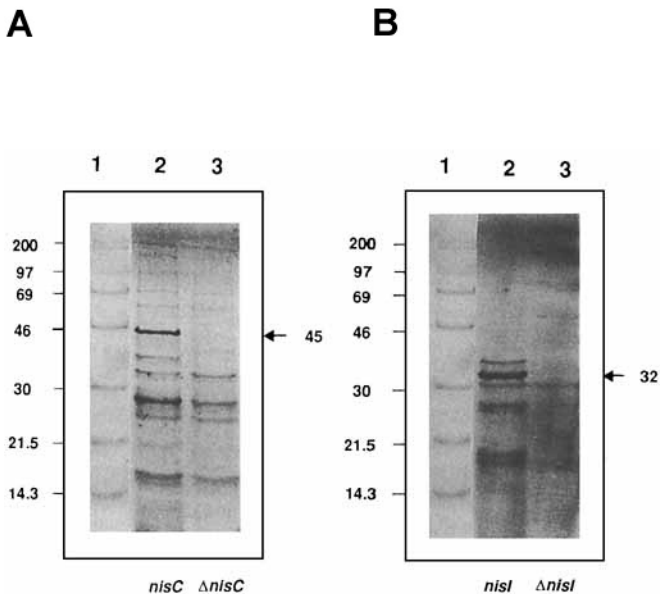


Fig. 3. Expression of *nisC* and *nisI* in *E. coli* JM109(DE3) after induction with IPTG. (A) Lane 1, molecular-mass marker (kDa); lane 2, pET3*nisC*; lane 3, pET3*ΔnisC*. (B) Lane 1, molecular-mass marker; lane 2, pET8*nisI*; lane 3, pET8*ΔnisI*. The molecular masses of NisC (45 kDa) and NisI (32 kDa) are indicated.

using SDS/PAGE. A construct containing a truncated form of *nisC* (pET3*ΔnisC*) was also made and introduced into JM109(DE3) as a negative control. The results obtained after autoradiography are shown in Fig. 3A. The estimated molecular mass of the *nisC* gene product was 45 kDa, which agrees well with the calculated molecular mass of 47.5 kDa. Both the intact *nisI* gene and a 5'-deletion form were cloned into vector pET8c, resulting in plasmids pET8*nisI* and pET8*ΔnisI*, respectively. These plasmids were introduced into JM109(DE3), and after induction and radiolabelling, the gene products were visualized by autoradiography as shown in Fig. 3B. The estimated molecular mass of the *nisI*-encoded protein is approximately 32 kDa, which is somewhat higher than the calculated molecular mass of 27.8 kDa.

Inactivation of chromosomal *nisA* by gene replacement

The procedure followed for gene replacement of *nisA* on Tn5276 by the truncated gene *ΔnisA*, that contains a 4-bp deletion at positions 284–287 (Fig. 2), is shown in Fig. 4. The resulting strain, designated *L. lactis* NZ9800, does not produce nisin A. Southern-blot hybridizations with *Pst*I-digested chromosomal DNA and *nisA* as a probe, showed exactly the same results as for the wild type, indicating the integrity of this part of the nisin operon. The sequence of the *ΔnisA* gene and approximately 200 bp of the flanking regions was confirmed by sequence analysis of a fragment obtained by PCR amplification using *nisA* flanking primers (Kuipers et al., 1992) and total DNA of *L. lactis* NZ9800 as a template. Expression plasmids containing either the *nisA* or the *nisZ* gene (Kuipers et al., 1992) were introduced into strain *L. lactis* NZ9800, leading to the production of nisin A or nisin Z in equal or higher amounts compared to the wild-type strain *L. lactis* NZ9700.

Immunity of *L. lactis* strains to nisin

Several strains that produced either nisin, precursor nisin or neither, were tested for their sensitivity to nisin A. The

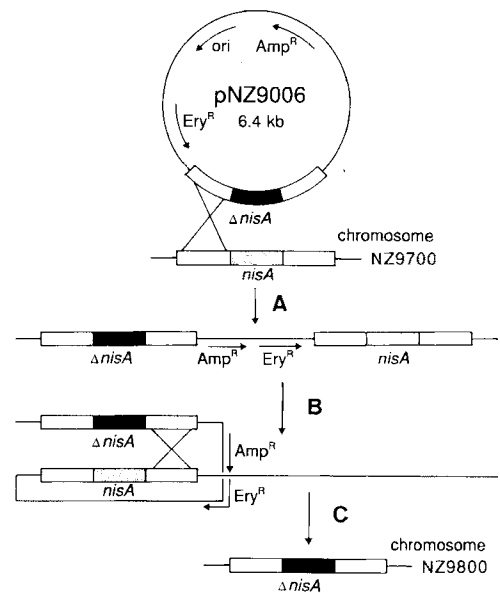


Fig. 4. Construction of *nisA*-deficient *L. lactis* NZ9800 by chromosomal integration. First recombination event (A); second recombination event (B); resulting strain NZ9800 containing *ΔnisA* (C).

outgrowth of these strains in the presence of different amounts of nisin A was monitored. All strains were derivatives of the plasmid-free strain *L. lactis* MG1614. The level of immunity of the wild-type nisin-A producer NZ9700 was 25 µg/ml nisin A, which was used as a reference for the other strains. Table 1 shows the immunity levels as determined for various strains. These results show that several levels of self-protection against nisin can be distinguished. A maximal level of immunity is only reached when mature nisin is produced. When a strain is used which instead produces precursor nisin, such as MG1614 harboring pNZ9111, or which is deficient in only the *nisA* gene, such as NZ9800, the immunity level decreases by almost one order of magnitude. Immunity can be restored to the wild-type level by introduction of a *nisA* or *nisZ* expression plasmid in strain NZ9800. When *nisI* was expressed in MG1614 by read through from the *rep* gene of pIL253, as in pNZ9031, a low but significant level of immunity to nisin is obtained. *L. lactis* MG1614 harboring pNZ9000 encoding *nisABTCI* showed a similar low level of immunity. No such immunity was obtained with several negative-control constructs, like those that gave expression of *nisR* (pNZ9030), or contained the *nisI* gene in an antisense orientation with respect to the vector-located promoter (pNZ9032).

Nisin immunity of *E. coli* expressing *nisI*

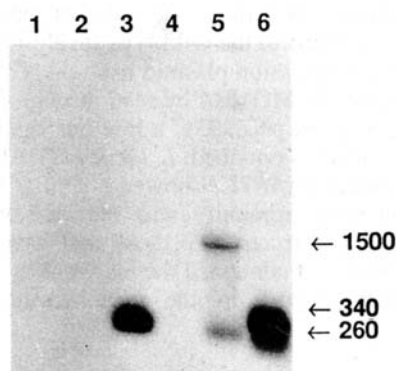
E. coli JM109(DE3) cells, harboring pET8c-derived plasmids expressing either *nisI*, *ΔnisI* or *nisR*, were induced with IPTG, and subsequently incubated at pH 6.0 in the presence of 20 mM EDTA and varying amounts of nisin A. After washing, plating and overnight incubation the surviving colony-forming bacteria were counted. The results of these experiments are shown in Table 2. With 2.5 µg/ml nisin A, the *E. coli* cells harboring *nisI* showed a slightly higher level of immunity. With 12.5 µg/ml and 25 µg/ml nisin A, a 40–170-fold increase in the number of surviving bacteria compared to controls was observed, indicating that expression of *nisI* is also able to confer protection against nisin to *E. coli*.

Table 1. Immunity levels of several *L. lactis* strains. Immunity is expressed relative to wild-type immunity. —, none; P_{lac}, *lac* promoter.

<i>L. lactis</i> strain (plasmid)	Characteristics of MG1614 derivatives	Extracellular product	Immunity
			%
MG1614	—	—	<0.5
NZ9700	Tn5276	nisin A	100
NZ9800	Tn5276 <i>Δ</i> <i>nisA</i>	—	8–20
NZ9800 (pNZ9010)	Tn5276 <i>Δ</i> <i>nisA</i> ; P _{lac} <i>nisA</i>	nisin A	>80
NZ9800 (pNZ9013)	Tn5276 <i>Δ</i> <i>nisA</i> ; P _{lac} <i>nisZ</i>	nisin Z	>80
NZ9900	mutated Tn5276	—	8–20
NZ9900 (pNZ9000)	mutated Tn5276; <i>nisABTCI</i>	nisin A	>80
MG1614 (pNZ9000)	<i>nisABTCI</i>	—	1–4
MG1614 (pNZ9111)	<i>nisABTCIR</i>	precursor nisin A	8–20
MG1614 (pIL253)	—	—	<0.5
MG1614 (pNZ9030)	<i>nisR</i>	—	<0.5
MG1614 (pNZ9031)	<i>nisI</i>	—	1–4
MG1614 (pNZ9032)	<i>nisI</i> antisense	—	<0.5

Table 2. Survival of *E. coli* cells expressing either *nisI* or other genes after treatment with EDTA and nisin A. All experiments were performed three times. The standard errors are less than 20% for each value. Numbers of surviving bacteria (100%) ranged from 2.7×10^6 – 5.0×10^6 (pET8c), 5×10^4 – 8×10^4 (pET8*nisI*), 1×10^5 – 2×10^5 (pET8*Δ**nisI*) and 5×10^4 – 12×10^4 (pNZ9124).

Nisin μg/ml	Colony-forming units of <i>E. coli</i> JM109(DE3) for			
	pET8c	pET8 <i>nisI</i>	pET8 <i>Δ</i> <i>nisI</i>	pNZ9124(<i>nisR</i>)
	%			
0	100	100	100	100
2.5	54	77	15	14
12.5	0.7	45	0.5	1.2
25	0.1	17	0.1	0.2

**Fig. 5. Autoradiography of Northern blots using total RNA from various lactococcal strains with a *nisA* gene fragment as a probe.** Lane 1, MG1614; lane 2, MG1614 + pNZ9000; lane 3, NZ9700; lane 4, NZ9800; lane 5, NZ9800 + pNZ9000; lane 6, NZ9800 + pNZ9013. Molecular mass is indicated in kDa.

Transcription analysis of the *nisA* gene in *L. lactis* strains

Northern blotting with RNA obtained from different lactococcal strains and hybridization with a *nisA* gene probe, showed remarkable differences in transcription of *nisA* (Fig. 5). In the wild-type strain NZ9700, a clear *nisA* transcript of approximately 260 bases is present that corresponds

well with the one described previously (Buchman et al., 1988), which is absent in the Tn5276-free and plasmid-free strain MG1614. Not even a faint hybridization signal was observed with the RNA of strain NZ9800, although a 4-bp deletion in the middle of *nisA* was the only difference between NZ9800 and NZ9700. However, when either plasmid pNZ9010 (harboring *nisA*) or pNZ9013 (harboring *nisZ*) is introduced, two small transcripts of approximately 260 nucleotides and 340 nucleotides are visible. It was shown that the former transcript originates by transcription from the *nisA* promoter located in front of the *Δ**nisA* gene and the latter originates from the plasmid-encoded wild-type gene under control of the *lac* promoter, which is located 0.1 kbp upstream of the cloned *nisA* gene. Two transcripts hybridizing with a *nisA* gene probe were also found in NZ9800 harboring pNZ9000; one of approximately 260 nucleotides and one of approximately 1500 nucleotides. Assuming that the transcription terminator downstream of the *nisA* gene (position 394, Fig. 2) is used, the 1500-nucleotide transcript should initiate far upstream from the *nisA* gene in the iso-IS904 element IS1068 (Rauch and de Vos, 1992; Rauch, 1993). Primer-extension experiments using primer 010, which is complementary to a region downstream of *nisA*, showed that the transcription start of *nisA* was exactly at position 132 (Figs 2 and 6). In NZ9800 which harbors plasmid pNZ9013, primer extension using the same primer showed that the product was four nucleotides smaller than the wild-type product, giving evidence for transcription of the *Δ**nisA* gene (Fig. 6). The absence of the wild-type-size *nisZ* transcript is explained by the fact that in pNZ9013 the cloning of the *lac* promoter had deleted the –35 region of the *nis* promoter. Instead, a larger transcript, resulting from transcription directed by the *lac* promoter was detected (Fig. 6).

DISCUSSION

The sequences of the genes downstream of *nisA* in *L. lactis* NIZO R5, i.e. *nisB*, *nisT* and *nisC*, are almost identical to those reported for *L. lactis* 6F3 (Engelke et al., 1992). The deduced sequence of the 993-amino-acid NisB from *L. lactis* NIZO R5 appears to be identical to that reported for NisB from *L. lactis* 6F3, except for residue 19, which is serine in this study and cysteine in the sequence of *L. lactis* 6F3 (Engelke et al., 1992). Although residue 19 is also serine in *L.*

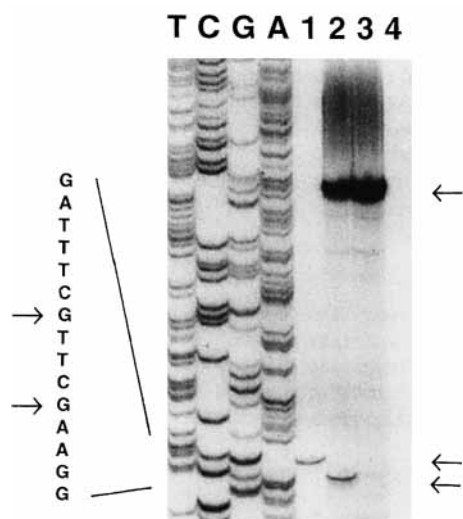


Fig. 6. Primer-extension mapping of *nisA* and *AnisA* transcripts. The first four lanes indicate the sequencing ladder obtained with a DNA fragment containing *nisA* and flanking regions, using oligonucleotide 010 as a primer. Lane 1, primer-extended product of NZ9700; lane 2, primer-extended product of NZ9800 harboring pNZ9013; lane 3, primer extension of MG1614 harboring pNZ9013; lane 4, primer extension of NZ9800. The *nisA* (upper) and *AnisA* (lower) primer-extended products and the transcript derived from the *lac* promoter, are indicated by arrows.

lactis 11454, this sequence differs considerably from that of 6F3 and NIZO R5, because it already terminates at amino acid 851 (Steen et al., 1991). If a sequencing error is excluded, this finding raises some questions about the functional role of the C-terminal residues at positions 852–993 of NisB. The deduced amino acid sequence of NisC, a protein that could be involved in modification reactions, is identical in *L. lactis* strains 6F3 and NIZO R5. For the putative translocator NisT, however, a difference between the encoded protein from *L. lactis* NIZO R5 and *L. lactis* 6F3 is found at amino acid 14, being asparagine in the former and tyrosine in the latter case. This minor difference is not likely to cause any changes in the biological properties of this protein. The possible function and putative membrane location of this protein have been discussed before (Engelke et al., 1992).

A novel gene, *nisI*, is reported here, that encodes a protein which is involved in nisin immunity. Both in *L. lactis* and in *E. coli*, *nisI* gene expression provides the cells with a significant level of protection against nisin (Tables 1 and 2). However, full immunity is only reached when mature nisin is produced by the lactococcal cells (Table 1). This is most clearly demonstrated by the results obtained with strain *L. lactis* NZ9800, which contains the truncated *AnisA* gene, but an intact copy of all other nisin operon genes. How nisin, the leader peptide, or a precursor of nisin could fulfill this role of increasing immunity is not clear yet. Similar results have been obtained with the only other immunity protein described thus far for a lantibiotic, i.e. PepI, which is encoded by the *pep5* operon (Reis and Sahl, 1991). In that study, it was shown that *pepI* only conferred immunity on *Staphylococcus epidermidis* when the *pepA* structural gene was also present. We did not detect any sequence similarity between the deduced amino acid sequence of PepI (69 residues) and NisI (245 residues; predicted mature length 226 residues) except for the fact that both contain a hydrophobic N-terminus, which might serve as a membrane anchor. No similarity

of NisI with other protein sequences in the data bases was found, which is not surprising since we assume that the immunity protein NisI interacts in a highly specific way only with the lantibiotic it protects against. However, the possibility exists that a protein similar to NisI could be encoded by the *spa* gene cluster involved in the biosynthesis of the related lantibiotic subtilin, which awaits further investigation.

The localization of NisI is likely to be at the outside of the cytoplasmic membrane. This is suggested by the presence of a typical lipoprotein signal sequence (von Heijne, 1989; Hayashi and Wu, 1990), consisting of 19 amino acids. A sequence comparison between several typical bacterial lipoprotein signal sequences and the NisI signal sequence is shown in Fig. 7. It is predicted that after translocation and processing, the NisI protein becomes a peripheral membrane protein, attached to the membrane by a lipid-modified N-terminal cysteine. No other membrane-spanning fragments are predicted in the mature sequence of NisI.

When *nisI* is expressed by read through of the *rep* promoter of pIL253 in *L. lactis*, a low but significant level of protection against nisin A is found (Table 1). When the *nisR* gene is expressed (pNZ9111), in addition to the *nisA*, *nisB*, *nisT*, *nisC* and *nisI* genes, giving rise to precursor nisin-A production (van der Meer et al., 1993), the level of immunity increases approximately fivefold. However, in this case the immunity level is still one order of magnitude lower than the wild-type level, which is reached when either nisin A or nisin Z is produced. In the *nisA*-deficient strain *L. lactis* NZ9800, a reduced level of immunity is also observed. Although the *AnisA* gene is not transcribed anymore, the downstream *nisI* gene is probably still being transcribed, since a significant level of immunity is observed. This would imply that downstream of *nisA*, another promoter(s) could be present, as has previously been suggested (Steen et al., 1991; Engelke et al., 1992). However, this does not exclude the possibility that in NZ9700 read through from the putative *nisA* promoter can also occur, followed by a processing step to release the small *nisA* transcript, as has previously been suggested (Buchman et al., 1988). Up to now, it has not been possible to clone the *nisI* gene under the control of the strong *lac* promoter of *L. lactis*. Possibly, overexpression of *nisI* alone has a negative effect on the viability of the *L. lactis* cells.

Several explanations for the role of nisin production itself in raising the level of immunity are possible. A general stimulation of expression of the gene cluster downstream of *nisA* seems the most plausible reason for this phenomenon. Furthermore, the presence of NisR (with or without the presence of nisin) might also affect the expression of genes downstream of *nisA* including *nisI*, thereby raising the level of immunity. An interaction between nisin, the nisin leader peptide, or precursor nisin with NisI could also enhance its protective action. Although the involvement of NisI in immunity is clearly demonstrated, it cannot be excluded that this is not the only function of this protein. Interactions of NisI with other membrane-bound proteins such as the translocator NisT, the protease NisP or the NisB and NisC proteins, all encoded by the *nis*-gene cluster, might affect their function. It is also possible that the production of other biosynthetic gene products can enhance the protective action of the NisI protein, in an as yet undefined way.

Although it is well known that Gram-negative bacteria are highly resistant to the action of nisin, it has been reported that it is possible to increase their susceptibility for nisin by destabilizing the protective outer membrane, e.g. by using EDTA (Stevens et al., 1991). We found that EDTA-treated

Gram-positive bacteria						
<i>Lactococcus lactis</i>	NisI	MRR	YLILIVALIGITGLSG	CYQTSH	1	
<i>Lactococcus lactis</i>	PrtM	MKKKMRLLK	VLLASTATALLLLSG	CQSNQT	2	
<i>Bacillus subtilis</i>	OppA	MKKR	WSIVTLMILFIVLVA	CGFGGT	3	
<i>Bacillus licheniformis</i>	PenP	MKLWFSTLKLKK	AAAVLLFSCVALAG	CANNQT	4	
<i>Staphylococcus aureus</i>	PenP	MKK	LIFLIVIALVLSA	CNSNHA	5	
<i>Streptococcus pneumoniae</i>	AmiA	MKKNR	VFATAGLVLLAALVLA	CSSSKS	6	
↓*						
Gram-negative bacteria						
<i>Klebsiella pneumoniae</i>	PulS	MR	NFILFPMMAVLLSG	CQQRNP	7	
<i>Klebsiella pneumoniae</i>	PulA	MLR	YTCNALFLGSLILLSG	CDNSSS	8	
<i>Escherichia coli</i>	TraT	MKMKK	LMMVALMSSTLALSG	CGAMST	9	
<i>Escherichia coli</i>	Lpp	MKATK	LVLGAVILGSTLLAG	CSSNAK	10	
<i>Escherichia coli</i>	ColE3	MKK	ITGIILLLLAVIILSA	CQANYI	11	
consensus characteristics		positively	hydrophobic	polar		
		charged				

Fig. 7. Signal peptides of lipoprotein precursors in bacteria. Cleavage site for signal peptidase (↓); N-terminal cysteine of mature protein that is coupled to a lipid moiety (*). (1) This study, nisin immunity protein; (2) Vos et al. (1989), Haandrikman et al. (1991), maturation protein for proteinase; (3) Perego et al. (1991), periplasmic oligopeptide (binding)-transport protein; (4) Chang et al. (1982), β -lactamase; (5) McLaughlin et al. (1981), β -lactamase; (6) Alloing et al. (1990), oligopeptide (binding)-transport protein; (7) D'Enfert and Pugsley (1989), pullulanase-secretion protein; (8) Chapon and Raibaud (1985), pullulanase; (9) Ogata et al. (1982), complement resistance; (10) Nakamura and Inouye (1979), murein lipoprotein; (11) Watson et al. (1984), colicin E3 lysis protein.

E. coli cells showed a survival of approximately 0.1% when treated with 25 μ g/ml nisin A for 30 min, prior to plating. However, up to a 170-fold increase in survival was found with EDTA-treated cells of *E. coli* expressing the *nisI* gene, which was not observed in the controls (Table 2). This suggests that at least some of the synthesized NisI is located at the outer side of the cytoplasmic membrane in *E. coli*, where it is expected to be functional in preventing the pore-forming activity of nisin.

L. lactis NZ9800 differs from NZ9700, harboring the wild-type Tn5276, only in a 4-bp deletion (nucleotide positions 284–287, Fig. 2) in the *nisA* gene. This small deletion, which was intended only to prevent the production of an active nisin species, abolished the transcription of the *AnisA* gene. Two transcripts were detected in strain NZ9800 harboring plasmids expressing either *nisA* or *nisZ* under control of the *lac* promoter (Kuipers et al., 1992). Based on their sizes of approximately 260 nucleotides and 340 nucleotides, we conclude that the largest one originates from transcription initiating at the *lac* promoter that is located 89 bp upstream of the plasmid-located *nisA* or *nisZ* gene, whereas the smaller one represents the transcript initiating at the *nisA* promoter of the Tn5276-located *AnisA* gene. This conclusion is also supported by the results of primer-extension experiments (Fig. 6), which confirm the expected sizes of the transcripts. Engelke et al. (1992) proposed that the conserved hexanucleotides of this promoter are located at positions 38–43 and 59–64. We noticed, however, that these canonical promoter sequences can also be found at positions 94–99 and 120–125 (Fig. 2), and conform to those proposed for *L. lactis* (De Vos, 1987; van de Guchte et al., 1992). This promoter would be placed at a better position relative to the transcription-start site of *nisA* at position 132, than the previously proposed location of the *nisA* promoter (Engelke et al., 1992). Plasmids pNZ9010 and pNZ9013 conferred full production of nisin on this strain, which can only be explained by assuming that nisin or a nisin precursor in some stage of modification is required for transcription of its own gene from the putative promoter. The intriguing phenomenon of the involvement of the *nisA* translation product, either

modified or unmodified, in the transcription of its own gene and possibly also of downstream located genes, is the subject of future investigations.

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