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Characterization of the nisin gene cluster nisABTCIPR of Lactococcus lactis
Requirement of expression of the nisA and nisl 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., Beertuyzen, 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 nisl, downstream of the structural gene nisA. The nisT, nisC and nisl 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 nisl 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 nisl 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 (P-methy1)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, 1992; Koletta and Entian, 1989; Reis and Sahl, 1991) and nisin (nis; Buchman et al., 1988; Koletta et al., 1989; Reis and Sahl, 1991) and nisin (nis; Buchman et al., 1988; Koletta 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 operons, 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). 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; Chun and Hansen, 1992; Klein et al., 1992; Klein et al., 1993), pep5 (pep; Koletta et al., 1989; Reis and Sahl, 1991) and nisin (nis; Buchman et al., 1988; Koletta 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 operons, 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
modification does not occur in nisin, implying that a counter-
part 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 Sahil, 1991).
With respect to the self-protection of nisin-producing strains,
it has been noticed that some mutants of a nisin-A-producing
strain produce different levels of immunity, the highest level of immu-
nity corresponding to the highest level of nisin-A production
(Rauch et al., 1991). This suggests that the production of
nisA 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 nisl is involved in the
expression of nisin-A production, and strain MG1614. Lactococcal cells
were grown overnight without aeration at 30°C in a medium con-
taining 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 nova
DSM 1790 (obtained from the German Collection of Micro-
organisms) 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 Smal–
HindIII multiple cloning site of M13mp19 was cloned into
the Smal/HindIII-digested vector, to introduce a unique PstI
site (van Alen-Boerrigter et al., 1991). In L. lactis, the plasmids
pIL253 (Simon and Chopin, 1988) and pNZ9010 (contai-
nng nisA and pNZ9013 (containing nisZ; Kuipers et al.,
1992) were used. Antibiotics were used in the following con-
centrations: 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 reac-
tion (PCR) was performed using the conditions described be-
fore (Kuipers et al., 1991).

DNA sequencing was performed by use of the dyeoxy-
nucleotide chain-termination method (Sanger et al., 1977).
Oligonucleotides, used as primers in sequencing reactions
and for PCR, were synthesized using a Cyclone DNA synthe-
sizer (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 hy-
bridization with a 300-bp DNA fragment, containing the nisA
gene. The PstI fragment was isolated and ligated to PstI-
linearized dephosphorylated pNZ184 DNA. After transfer-
formation of E. coli MC1061, plasmid DNA of ampicillin-
resistant transformants was analyzed by restriction digestions
and hybridizations with the nisA gene probe. One recombi-
nant plasmid, pNZ9003, contained the 9.2-kbp PstI fragment
that included nisA and approximately 7-kbp DNA down-
stream 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 trans-
formed into L. lactis strains MG1614 and NZ9900.

A DNA fragment harboring the entire nisI gene was ob-
ained as a 1-kbp AccI (blunt ended by Klenow-enzyme treat-
ment)–PstI fragment from pNZ9000. This fragment was
cloned in the Smal/PstI-digested vector pIL253 in MG1614.
The new construct was named pNZ9031. The antisense orien-
tation 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 frag-
ment of pNZ9111 (van der Meer et al., 1993) in the Smal/
**PsI-digested vector pL253.** 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 BamH I (blunt ended by Klenow-enzyme treatment), together with a synthetic 15-bp NcoI–EcoRI-linker (5'-AATTCTTTCACTTCATC-3'). The resulting plasmid that contains the complete nisT gene was denoted pET8nisT. The nisC gene was cloned as a 2-kbp NdeI–HaeIII fragment (obtained with the primers 5'-GCGCATATGTTGAAAGTTTGCTAAC-3' and encoding the first 80 amino acids) of pNZ9000 and cloned in pETXc, which had been digested with NcoI and BamH I (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, was transformed in 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 protein, 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 fragment (blunt ended by Klenow-enzyme treatment)–SphI fragment from pUC19E, containing the erythromycin-resistance gene, was isolated and ligated and in the XbaI-digested (blunt ended by Klenow-enzyme treatment) and SphI-digested vector pNZ9005.1, resulting in the plasmid pNZ9006. This pUC19-derailed plasmid, which cannot replicate in L. lactis, was used to transform the nisin-A-producing L. lactis NZ7900. 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.

**Inactivation of chromosomal nisA by gene replacement**

To disrupt the nisA gene on the chromosome of nisin-A-producing L. lactis NZ7900, a 2.7-kbp BclI–EcoRV fragment, which contained nisA and approximately 1.2-kbp of its flanking regions, was isolated from pNZ7900 and cloned into pUC19, which had been digested with BamH I and EcoRI (blunt ended by Klenow-enzyme treatment). The resulting plasmid, pNZ9005, was linearized by partial digestion with SgrI, 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 protein, 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 fragment (blunt ended by Klenow-enzyme treatment)–SphI fragment from pUC19E, containing the erythromycin-resistance gene, was isolated and ligated and in the XbaI-digested (blunt ended by Klenow-enzyme treatment) and SphI-digested vector pNZ9005.1, resulting in the plasmid pNZ9006. This pUC19-derailed plasmid, which cannot replicate in L. lactis, was transformed into L. lactis NZ7900. 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 x 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-

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**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 L. lactis NIZO R5 (nisP and nisR, van der Meer et al., 1993).

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Growing cells were induced at A_600 0.5 by addition of IPTG and proteins were radiolabelled after addition of L-[35S]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.
repeats 6218-6223; Fig. 2. Sequences of sites used are as follows: AccI, 6383-6388; Ben, 4518-4523; NdeI, 4533-4538; NcoI, 5646-5651; NcoI, 4440-4445; PstI, 5160-5165. RNA was precipitated with ethanol and dissolved in buffer A, centrifuged at 5000 rpm 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 prepared as follows: 2 g Macaloid was suspended in 100 ml buffer A. For storage, RNA was kept in ethanol at -20°C and precipitated before each use. 4% Macaloid was used as a hybridization probe. For primer extension studies, was radiolabelled by nick translation with [α-32P]dATP and purified used as a hybridization probe. For primer extension studies, was radiolabelled by nick translation with [α-32P]dATP and purified used as a hybridization probe. For primer extension studies, was radiolabelled by nick translation with [α-32P]dATP and purified used as a hybridization probe. 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sequence 5’-GGATAGTATCCATCTCTGAAC-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 R Nasin (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-[3S]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; Hugenholtz 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 A₆₅₀ 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, pET8Anis or pNZ9124 (nisR) at A₆₅₀ 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, 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 PstI 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 nisBTCI shows that the nis genes are either partially overlapping (nisI 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 PstI fragment was subcloned into the lactococcal vector pIL253 and the resulting plasmid pNZ9000, encoding nisABTCI, 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 the 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 nisl genes in E. coli

To detect the gene products of nisT, nisC and nisl, these genes were cloned individually into the T7 expression vector pET8c. Plasmid pET8nisT 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). Upon 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 pET8nisC was introduced into JM109(DE3) and the radiolabelled proteins obtained after induction in the presence of L-[35S]methionine, were separated
using SDS/PAGE. A construct containing a truncated form of nisc (pET3Anisc) 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 nisl gene and a 5'-deletion form were cloned into vector pET8c, resulting in plasmids pET8nisl and pET8Anisl, 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 nisl-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 AnisA, 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 PstI-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 nis operon. The sequence of the AnisA 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 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 nisl 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 nisl gene in an antisense orientation with respect to the vector-located promoter (pNZ9032).

Nisin immunity of E. coli expressing nisl

E. coli JM109(DE3) cells, harboring pET8c-derived plasmids expressing either nisl, Anisl or nisz, 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 colonies were counted. The results of these experiments are shown in Table 2. With 2.5 μg/ml nisin A, the E. coli cells harboring nisl 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 nisl 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; Pₘₙ, lac promoter.

<table>
<thead>
<tr>
<th>L. lactis strain (plasmid)</th>
<th>Characteristics of MG1614 derivatives</th>
<th>Extracellular product</th>
<th>Immunity</th>
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<tr>
<td>MO1614</td>
<td>–</td>
<td>nisin A</td>
<td>&lt;0.5</td>
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<tr>
<td>NZ9700</td>
<td>Tn5276</td>
<td>nisin A</td>
<td>100</td>
</tr>
<tr>
<td>NZ9800</td>
<td>Tn5276 ΔnisA</td>
<td>nisin A</td>
<td>8–20</td>
</tr>
<tr>
<td>NZ9800 (pNZ9010)</td>
<td>Tn5276 ΔnisA; Pₘₙ nisA</td>
<td>nisin Z</td>
<td>&gt;80</td>
</tr>
<tr>
<td>NZ9800 (pNZ9013)</td>
<td>Tn5276 ΔnisA; Pₘₙ nisz</td>
<td>–</td>
<td>&gt;80</td>
</tr>
<tr>
<td>NZ9900</td>
<td>mutated Tn5276</td>
<td>nisin A</td>
<td>8–20</td>
</tr>
<tr>
<td>NZ9900 (pNZ9000)</td>
<td>mutated Tn5276; nisABTC1</td>
<td>–</td>
<td>1–4</td>
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<tr>
<td>MG1614 (pNZ9000)</td>
<td>nisABTC1</td>
<td>–</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>MG1614 (pNZ9111)</td>
<td>nisABTCIR</td>
<td>–</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>MG1614 (pIL253)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MG1614 (pNZ9030)</td>
<td>nisR</td>
<td>–</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>MG1614 (pNZ9031)</td>
<td>nisl</td>
<td>–</td>
<td>1–4</td>
</tr>
<tr>
<td>MG1614 (pNZ9032)</td>
<td>nisl antisense</td>
<td>–</td>
<td>&lt;0.5</td>
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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 \(2.7 \times 10^6\) to \(5.0 \times 10^4\) (pET8c), \(5 \times 10^4\) to \(8 \times 10^4\) (pET8nisI), \(1 \times 10^4\) to \(2 \times 10^4\) (pET8AnisI) and \(5 \times 10^4\) to \(12 \times 10^4\) (pNZ9124).

<table>
<thead>
<tr>
<th>Nisin</th>
<th>Colony-forming units of E. coli JM109(DE3) for pET8c</th>
<th>pET8nisI</th>
<th>pET8AnisI</th>
<th>pNZ9124(nisR)</th>
<th>µg/ml</th>
<th>%</th>
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<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>2.5</td>
<td>54</td>
<td>77</td>
<td>15</td>
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<tr>
<td>12.5</td>
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<tr>
<td>25</td>
<td>0.1</td>
<td>17</td>
<td>0.1</td>
<td>0.2</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

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 ΔnisA 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 is3 region (Fig. 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 ΔnisA gene (Fig. 6). The absence of the wild-type-size nisZ transcript is explained by the fact that in pNZ9013 the cloning of the nisA 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 ΔnisA transcripts.
The first four lanes indicate the sequencing ladder obtained with a
DNA fragment containing nisA and flanking regions, using oligonu-
cleotide 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 ΔnisA
(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 ex-
cluded, this finding raises some questions about the func-
tional role of the C-terminal residues at positions 852–993
of NisB. The deduced amino acid sequence of NisC, a pro-
tein that could be involved in modification reactions, is iden-
tical 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, nisl, is reported here, that encodes a pro-
tein which is involved in nisin immunity. Both in L. lactis
and in E. coli, nisl 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 ΔnisA 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 de-
scribed 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 Staphylo-
coccus 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-termi-
nus, 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 im-
munity protein NisI interacts in a highly specific way only
with the lantibiotic it protects against. However, the possi-
bility 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 lip-
protein 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 nisl is expressed by read through of the rep pro-
moter 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 nisl 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 nisl-deficient strain L. lactis NZ9800,
a reduced level of immunity is also observed. Although the
ΔnisA gene is not transcribed anymore, the downstream nisl
gene is probably still being transcribed, since a significant
level of immunity is observed. This would imply that down-
stream of nisl, 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 nisl promoter can
also occur, followed by a processing step to release the small
nisA transcript, as has previously been suggested (Buchman
e et al., 1988). Up to now, it has not been possible to clone the
nisl gene under the control of the strong lac promoter of L.
lactis. Possibly, overexpression of nisl 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 sti-
mulation 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 nisl, 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 trans-
locator NisT, the protease NisP or the NisB and NisC pro-
teins, all encoded by the nis-gene cluster, might affect their
function. It is also possible that the production of other bio-
synthetic 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
**Fig. 7. Signal peptides of lipoprotein precursors in bacteria.** Cleavage site for signal peptide (\(\text{L}\)): N-terminal cysteine of mature protein that is coupled to a lipid moiety (*\(\ast\)*). (1) This study, nisin immunity protein; (2) Vas et al. (1990), Haandriksen et al. (1991), murein lipoprotein; (3) Pedersen et al. (1991), periplasmic oligopeptide (binding)-transport protein; (4) Chang et al. (1982), \(\beta\)-lactamase; (5) McCaffery et al. (1981), \(\beta\)-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.

**REFERENCES**


**Gram-positive bacteria**

<table>
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<tr>
<th>Species</th>
<th>Peptide</th>
<th>Reference</th>
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<td>Lactococcus lactis</td>
<td>NisI</td>
<td>MRK YLTIVAGLTLGAS CYQKYP 1</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>NisM</td>
<td>MKKRNKVN LTVAGLTLGAS CYQKYP 2</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Upa</td>
<td>MKNWIVVTLTGLGA CGQKYP 3</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>Pfp</td>
<td>MRLYSTLKKW AAVVLPSCVLGAC QKQY 4</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Pfp</td>
<td>MKR LFYTVIALGA CSHINA 5</td>
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<td>Streptococcus pneumoniae</td>
<td>NisA</td>
<td>MKKRNWVATAGVLLAVAAWLVAC SSSSKS 6</td>
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**Gram-negative bacteria**

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<td>Klebsiella pneumoniae</td>
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<td>Escherichia coli</td>
<td>ColEl3</td>
<td>MKRN ITGIILLAVLISA CQHay 11</td>
</tr>
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**consensus characteristics**

- positively hydrophobic
- polar charged

**E. coli** cells showed a survival of approximately 0.1% when treated with 25 \(\mu\)g/ml nisin A for 30 min, prior to plating. However, up to a 170-fold increase in survival was found when 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 Gucht 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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