Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in *Lactococcus lactis* N8

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Nisin is a small post-translationally modified lanthionine-containing peptide (lantibiotic) produced by certain *Lactococcus lactis* strains which has a high antimicrobial activity against several pathogenic Gram-positive bacteria. Northern blots and RT/PCR analyses of the nisin-producing strain N8 revealed that the *nisZBTCIPRKFE* gene cluster, responsible for nisin biosynthesis, immunity and regulation, consists of two operons, *nisZBTCIPRK* and *nisFE*. The promoter of the *nisFE* operon was mapped. The –35 to –1 region upstream of the transcription start of the *nisFE* promoter showed 73% identity with the corresponding region upstream of the *nisA* and *nisZ* gene. In contrast to earlier reports, nisin was found to be secreted during the early stages of growth as well as later in the growth cycle. The secreted nisin was adsorbed on the surface of the cells and was released to the medium during mid-exponential growth, when the pH in the medium fell below 5.5. In *nisZ* antisense and *nisT* deletion mutant strains constructed in this study the transcription of the nisin operons, nisin production and immunity were lost. Provision of external nisin restored the transcription of both operons in the mutant strains, showing that the operons are coordinately regulated by mature nisin. Nisin induction of the mutant strains also resulted in an increased amount of the NisI protein and an increase in the level of immunity. Induction using higher concentrations of nisin yielded a higher level of immunity. These results showed that the nisin promoters are under positive control in an autoregulatory manner and that antimicrobial peptides can also function as signal molecules.

Keywords: nisin regulon, transcriptional regulation, signal transduction, *Lactococcus lactis*

INTRODUCTION

Nisin, subtilin and epidermin are peptide antibiotics called lantibiotics. These polycyclic peptides are post-translationally modified and contain the unusual amino acids dehydroalanine, lanthionine and β -methyllanthionine. They are produced by various Gram-positive bacteria and are active against several Gram-positive spoilage bacteria and food pathogens such as *Listeria*, *Clostridium* and *Bacillus* species (Buchman *et al.*, 1988; Banerjee & Hansen,

1988; Schnell *et al.*, 1988). The bactericidal effect of these peptides is based on depolarization of bacterial cytoplasmic membranes by pore formation (Gao *et al.*, 1991). Nisin produced by *Lactococcus lactis* is the most important lantibiotic, and is widely used in the food industry (Delves-Broughton, 1990). Two naturally occurring nisin variants have been described, nisin A and nisin Z, which differ in a single amino acid residue at position 27 (Buchman *et al.*, 1988; Graeffe *et al.*, 1991; Mulders *et al.*, 1991). Recently the *nis(A/Z)BTCIPRKFE* gene cluster, comprising a 15 kb DNA fragment responsible for nisin production, regulation and immunity has been cloned and sequenced (Dodd *et al.*, 1990; Steen *et al.*,

Abbreviation: RT, reverse transcriptase.

Fig. 1. (a) Genetic organization of the nisin operons of *L. lactis*. (b) Schematic representation of the construction of *L. lactis* LAC46 by plasmid integration. E, *EcoRI*; H, *HindIII*.

1991; Engelke *et al.*, 1992, 1994; Kuipers *et al.*, 1993; van der Meer *et al.*, 1993; Immonen *et al.*, 1995; Siegers & Entian, 1995).

NisB and NisC are believed to be necessary for chemical modification, which involves dehydration and lanthionine formation. NisT is an ABC transport protein potentially responsible for the transport of prenisin across the cytoplasmic membrane. The lipoprotein NisI seems to play a role in nisin immunity (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Qiao *et al.*, 1995), while NisP is a protease (van der Meer *et al.*, 1993) which cleaves the leader of the modified nisin precursor peptide to active mature nisin. NisR and NisK are believed to form a two-component regulatory system necessary for nisin synthesis (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Immonen *et al.*, 1995). The *nisFEG* genes encode a putative ABC transport system, which has recently been suggested also to be involved in nisin self-protection (Siegers & Entian 1995).

Transcriptional studies have shown that the half-life of the structural nisin gene is 7–10 min, and its promoter has been mapped (Buchman *et al.*, 1988; Engelke *et al.*, 1992;

Kuipers *et al.*, 1993). Northern blot analyses with probes against *nisA* and *nisB* indicated two different monocistronic transcripts, a *nisA* 0.3 kb transcript and a *nisB* smear of 3 kb (Engelke *et al.*, 1992). By using RT/PCR we have shown that a *nisZB* transcript exists (Ra & Saris, 1995) and that RNA processing results in the two observed transcripts. Based on DNA sequence data, five other potential promoters have been suggested in front of the *nisB*, *nisT*, *nisC*, *nisR* and *nisF* genes, and two potential transcriptional terminators downstream of *nisB* and *nisK* (Engelke *et al.*, 1992; Dodd & Gasson, 1994; Siegers & Entian, 1995; Immonen *et al.*, 1995). There have been conflicting reports about the expression of the nisin gene and the detection of nisin activity. Northern analysis against the structural *nisA* gene with RNA isolated during all growth stages showed that the gene was expressed to about the same extent at all stages tested (Buchman *et al.*, 1988; de Vuyst & Vandamme, 1992). Whereas de Vuyst & Vandamme (1992) observed a stringent correlation between *nisA* expression and nisin activity, other workers have detected a delay of several hours between the onset of detectable nisin activity and the transcription of the structural gene (Hurst & Peterson, 1971; Buchman *et al.*, 1988). No transcriptional studies of the other genes of the nisin gene cluster have yet been described. Thus the transcriptional pattern of the nisin gene cluster and its regulatory factors has been poorly characterized.

In this paper we show that the *nisZBTCIPRKFEG* gene cluster is transcribed as two operons, *nisZBTCIPRK* and *nisFEG*, from two homologous promoters. We demonstrate that both operons are induced by external nisin and thus form one regulon. Our data show that mature nisin is produced at all growth stages, but is adsorbed onto the cell surface during early stages of growth, until the pH of the growth medium drops below 5.5.

METHODS

Bacterial strains and media. The nisin Z producing *Lactococcus lactis* subsp. *lactis* strain N8 (Graeffe *et al.*, 1991), the nisin A producing strain ATCC 11454 (American Type Culture Collection) and the nisin-negative strain MG1614 (Gasson, 1983) were grown in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose and 0.5% sucrose instead of lactose (M17GS medium) at 30 °C without shaking. The nisin-sensitive indicator strain *Micrococcus luteus* AL NCIMB 8166 (National Collection of Industrial and Marine Bacteria) and *Escherichia coli* DH5 α (Hanahan, 1983), the host for the plasmid vectors, were cultured at 37 °C in LB broth, with shaking. Growth was measured as OD₆₀₀, using a Shimadzu model UV-120-02 spectrophotometer.

Plasmid and mutant constructions. The superlinker from pSL1180 was cloned as an *EcoRI*–*HindIII* fragment into the Bluescript plasmid (Stratagene), yielding plasmid pLEB44. The multilinker was cloned from pLEB44 as a *PstI* fragment in the shuttle vector pVS68 (Valio, Helsinki, Finland), resulting in pLEB122. The lactococcal promoter P45 (Sibakov *et al.*, 1991) was cloned from pKTH1799 as a *ClaI*–*HindIII* fragment in pLEB23 and from there as an *BamHI*–*BglII* fragment in pLEB122, yielding the expression vector pLEB124. The structural gene of nisin (*nisZ*) was cloned in pLEB124 as a *BamHI* fragment from pKTH1984 in sense and antisense

Table 1. Probes for Northern analysis, primer combinations for RT/PCR and primers used for extension analysis

(a) DNA fragments used for generating probes for Northern analysis

Probe for	Enzyme(s) used to generate probe	Cleavage sites*	EMBL accession no.
<i>nisZ</i>	<i>SspI/SacI</i>	125–352	Z18947
<i>nisB</i>	<i>HindII</i>	2117–2957	Z18947
<i>nisT</i>	<i>EcoRI/HindIII</i>	3527–5046	Z18947
<i>nisI</i>	<i>HindIII</i>	6912–7259	Z18947
<i>nisP</i>	<i>AlwNI/HpaI</i>	84–1320	Z22725
<i>nisR</i>	<i>ScaI/EcoRI</i>	76–255	Z22813
<i>nisK</i>	<i>SaII/PstI</i> †	801–305	Z22813 and U17255†
<i>nisE</i>	<i>AclI/HpaV</i>	1119–1433	U17255

(b) Primer combination used for RT/PCR

RNA stretch amplified	Upstream primer	Downstream primer
<i>nis</i> 'BT'	GTCTCCCTGAACCTAGCACAGAG	GAAATGCTTCTTTTCAGAAATGATAAC
<i>nis</i> 'TCI'	AGTTTCTCACTTTTCATGTC	GAACCTTATTATTTCAGAGC
<i>nis</i> 'IP'	TCTAAGAGATACCACTTCCTTAGC	GTATCCCGAGGTCTGAATGGGGGAG
<i>nis</i> 'PRK'	TGCCATATCCGTCGACGC	AGGTCAGGATGATGCTATTAAC

(c) Primers used for extension analysis

Gene	Primer
<i>nisF</i>	CCAGTATCTGCAGAAATTAAACC
<i>nisR</i>	GAAATCAGTAATATCCAAGGGAAGTG

orientation, resulting in plasmids pLEB167 and pLEB168, respectively. These plasmids were electroporated into *L. lactis* ATCC 11454, yielding strains LAC15 and LAC16. Analysis of the plasmid content of the strains verified the presence of the electroporated plasmids. The *nisT* mutation was made by integration of an *E. coli* plasmid having a Gram-positive selection marker (*erm*; Axelsson *et al.*, 1988) and an internal *EcoRI*–*HindIII* fragment (1524 bp) of the *nisT* gene of *L. lactis* N8 (Immonen *et al.*, 1995) into the *nisT* gene of *L. lactis* N8 (Fig. 1). The correct integration was verified by Southern analysis and the transformant was named LAC46. The 3' deletion of the *nisT* gene resulted in a gene capable of encoding a truncated NisT protein with a deletion of 86 amino acids from the C-terminus. All plasmids were isolated by the method of Carter & Milton (1993). Otherwise established protocols were used (Maniatis *et al.*, 1982).

RNA techniques and Northern hybridizations. RNA isolations and RT/PCR were done according to Ra & Saris (1995). Thirty micrograms of RNA and 3 µg RNA ladder (GIBCO BRL; cat. no. 15620-16) were fractionated on 0.6% agarose gels containing formaldehyde. After electrophoresis the gels were soaked in water for 20 min and then for 1 h in transfer buffer (8 mM Na₂HPO₄, 17 mM NaH₂PO₄). RNA was transferred to Hybond N membrane with Bio-Rad's Trans-Blot cell overnight at 250 mA in a cold-room. After transfer, membranes were briefly rinsed in transfer buffer and then baked in a vacuum oven at 80 °C for 2 h. Membranes were prehybridized for 4 h at 57 °C (5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 200 µg denatured HS DNA ml⁻¹). After addition of the probe, hybridization was performed in the same buffer overnight at

57 °C. Membranes were washed for 10 min with 2 × SSC/0.1% SDS at 57 °C, for 20 min with 1 × SSC/0.1% SDS at 57 °C, for 20 min with 0.5 × SSC/0.1% SDS at 57 °C and for 20 min with this solution at 65 °C, and finally with 0.1 × SSC/0.1% SDS at 65 °C. Washing of membranes for reprobing was done with 10 mM TE/0.1% SDS (pH 8) at 90 °C for at least 2 h. Membranes were monitored by exposing them to Kodak X-Omat films to ensure that old probe had been fully removed before they were reprobbed. Membranes were exposed to Kodak X-Omat 100 films at –70 °C overnight before developing.

Labelling of probes. Labelling of 50 ng of probe was performed with the Multiprime labelling system (Amersham) according to the manufacturer's instructions, in the presence of Amersham's [³²P]dCTP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹). Free nucleotides were separated from the labelled DNA with Sephadex G-50 Nick columns (Pharmacia). Table 1 shows the DNA fragments used for generating the probes.

Primer extension analysis. Primer extension experiments were done mainly according to Myöhänen & Wahlfors (1993), with the following exceptions: 10 µg or 20 µg RNA with 10 pmol of each primer was incubated for 5 min at 65 °C in similar reverse transcription conditions as above. The tubes were transferred to 42 °C and 25 U of M-MuLV reverse transcriptase (RT) was added to the final reaction mixture (20 µl). The dNTP mix contained normal dGTP instead of deaza-dGTP. After 45 min the volume was adjusted to 200 µl and the reaction stopped with an equal volume of phenol/chloroform (1:1, v/v). Following organic extraction and precipitation the pellets were dissolved in 4 µl water containing 5 µg RNase A µl⁻¹.

Nisin-immunity assay. The sensitivity of lactococcal cells to nisin was tested by adding 0–1000 IU nisin ml⁻¹ to overnight cultures diluted 1:50 in M17GS. Cultures were incubated overnight at 30 °C and the growth was monitored by measuring OD₆₀₀. Cells immune to nisin could grow in the presence of 1000 IU nisin ml⁻¹ whereas sensitive cells stopped growing in nisin concentrations above 5 IU ml⁻¹. To determine the proportion of nisin-immune cells at a certain timepoint in fermentation experiments, the cells were washed in M17GS and incubated in M17GS containing nisin (0–1000 IU ml⁻¹) for 30 min at 30 °C followed by determination of the viable count.

Nisin bioassay. Three samples from each chosen timepoint of the growth culture were taken. The pH of the first sample was adjusted to 4.8 with HCl, to release nisin adsorbed onto the cells, followed by removal of the cells by centrifugation. The supernatant was heated at 56 °C for 15 min to inactivate the remaining cells. From this sample (nisin from the growth medium and the cells) and dilutions thereof, 3 µl was spotted on a plate of L-agar with 3 ml soft L-agar containing 10⁵ *M. luteus* cells ml⁻¹ on the surface (agar spot test). The second sample was directly centrifuged to separate the cells from the supernatant. The supernatant (nisin from the growth supernatant) was heat treated and used in the agar spot test as described above. The cells were resuspended in M17GS pH 4.8 to release nisin, and centrifuged. This supernatant (nisin from the cells) was also heat treated and applied to the surface of soft agar containing the indicator bacteria. The nisin bioassay plates with spots of the samples and dilutions of nisin (Sigma) were incubated at 37 °C overnight. The diameter of the inhibition haloes in the uniform lawn of the indicator bacteria was measured. The values obtained and the minimal inhibitory concentrations of the samples were used to estimate the amount of nisin in the samples.

Nisin induction of lactococcal strains. Stationary-phase cells of LAC16 and LAC46 were incubated with 0.1 IU nisin ml⁻¹ in 5 ml M17GS medium at 30 °C for > 10 h. Cells used in the Western analysis were also induced in higher nisin concentrations (50 IU ml⁻¹).

Protein purification and analysis. Immunodetection was done using the Protoblot kit (Promega) with a polyclonal rabbit antiserum recognizing the NisI protein. The NisI protein used for the immunization was isolated from *E. coli* cells producing a glutathione-S-transferase–NisI protein fusion using a glutathione-Sepharose column. The NisI protein was cleaved from the fusion protein with thrombin and purified by HPLC–MonoQ.

RESULTS

The transcriptional product of the *nisZ* promoter

Northern analyses with *nisZ*, *nisB*, *nisT*, *nisI*, *nisP*, *nisR* and *nisK* specific probes showed hybridization signals measuring from 0.24 kb to 11.0 kb. Only the *nisZ* probe gave a hybridization signal corresponding to the expected size of the gene; the other probes revealed smeared hybridization signals, similar to those that Engelke *et al.* (1992) obtained with a *nisB* probe. The lack of detectable distinct bands was most likely due to the generally very short half-lives (0.5–2.0 min) of bacterial mRNAs (King & Schlessinger, 1987), and the technical challenge of obtaining high-quality lactococcal RNA fast enough without any degradation. Because even a single attack

Fig. 2. RT/PCR analysis of the biosynthetic nisin operon. Lanes: 1, *Bcl*I digest of the amplified *nis'*PRK' region (1115 bp); 2, the *nis'*PRK' region; 3, a *Hind*III digest of the amplified *nis'*IP' region (758 bp); 4, the *nis'*IP' region; 5, *Sty*I digest of the amplified *nis'*TCI' region (1504 bp); 6, the *nis'*TCI' region; 7, *Sph*I digest of the *nis'*BT' region (569 bp); 8, the *nis'*BT' region; 9, *Sac*I digest of the amplified *nis'*ZB' region (628 bp); 10, the *nis'*ZB' region. The outer lanes contain size standards. The RT/PCR was done in more than triplicate experiments, with similar results each time. The sizes of the digestion products obtained correlated with the expected sizes.

anywhere on the 11 kb polycistronic transcript by an endonucleatic RNase alters the size of the hybridization signal, causing a smear, another approach was chosen. A series of RT/PCR reactions was run with primers annealing to the 3'-end of a gene and to the 5'-end of the adjacent upstream gene, with the following concept: if the genes are expressed on the same RNA, the RNA stretch between these two genes will be amplified; if the genes are only expressed on different mRNA species, no amplification will be detected (Ra & Saris, 1995). To assure the true identity of the amplified RT/PCR products, they were cut with restriction enzymes to give DNA fragments of expected sizes (Fig. 2). All amplification results were positive, and combined with Northern analyses confirmed that one long *nisZBTCIPRK* mRNA was transcribed.

O. P. Kuipers (unpublished results in de Vos & Simmons, 1994) has mapped a putative *nisR* promoter. Despite efforts with several different primers and with RNA isolated from a nisin Z producing strain, from a nisin A producing strain at various growth stages, and from the mutant strain LAC46, we could not identify the same 5'-end mRNA (results not shown). Instead, one strong signal 31 nt downstream of the identified transcriptional initiation site was always obtained. This site lies 10 nt downstream of the ribosome-binding site of *nisR* and could represent a functional RNA inactivation cleavage site (King & Schlessinger, 1987). Another very weak signal was also detected 20 nt upstream of the reported transcription initiation site, but no typical promoter sequences could be found in this region. When RNA from strain LAC16, which has no detectable *nisZ* promoter activity, was analysed with a *nisK* probe, no hybridization signals could be detected.

Fig. 3. Northern blot analysis of nisin-induced and noninduced lactococcal strains using a *nisZ* (a), *nisB* (b), *nisK* (c) and *nisE* (d) probe. (a) Lane 1, nisin-induced LAC46; lane 2, LAC46; lane 3, LAC16; lane 4, nisin-induced LAC16; lane 5, N8; lane 6, MG1614. (b–d) Lane 1, MG1614; lane 2, N8; lane 3, nisin-induced LAC16; lane 4, LAC16; lane 5, LAC46. The result in (a) was from one separate filter; the results in (b–d) were from another filter that was reprobed. In lane 5 in (c) and (d) the film was exposed for 1 d longer than the other lanes. In all panels RNA from the same isolations was used. The experiments were done in triplicate with freshly isolated RNA. Only minor variation in the results was obtained.

Fig. 4. Western blot analysis of nisin-induced and noninduced lactococcal strains using a NisI-specific antibody. Lane 1, N8; lane 2, MG1614; lane 3, LAC46; lane 4, nisin-induced LAC46. The experiment was done in triplicate, giving similar results each time.

The *nisFEG* operon and the mapping of its promoter

Northern blot analysis of the nisin producer *L. lactis* N8 using a *nisE* specific probe revealed a band of 2.3 kb (Fig. 3d). This size agrees with the expected size of a *nisFEG* transcript. Primer extension analysis revealed only one

strong signal (results not shown), which mapped the transcriptional starting site to the T nucleotide –27 bp upstream of the ATG initiation codon of the *nisF* gene. Upstream of the transcriptional start a characteristic Gram-positive –35 (CTGATT) and –10 (TGTTTATA) region was identified. Compared to the *nisA* promoter (Kuipers *et al.*, 1993) the –35 region differed by only one nucleotide, but the –10 region was noticeably different. However, if the proposed –10 region of the *nisA* gene is moved 4 nt upstream it would be separated by 1 nt from a preceding TG, which approximately half of the characterized lactococcal promoters possess (de Vos & Simmons, 1994). In addition, with this adjustment the –35 and –10 region of the *nisF* and *nisA* promoters show 80% identity. The total identity of the –1 to –35 region upstream of the transcription start of the *nisF* and *nisA* was 73%.

Inhibition of nisin production results in loss of transcription of the nisin operons

Nisin production was inhibited by a plasmid insertion into *nisT* (LAC46) or by introducing pLEB168, con-

Table 2. Nisin immunity of *L. lactis* strains

Immunity (expressed as a percentage of wild-type immunity to 1000 IU nisin ml^{–1}) was determined by the plate diffusion assay and from growth in liquid media containing varying amounts of nisin. ND, Not determined. The results presented are mean values from five experiments.

Strain	Description	Immunity to nisin (%)		
		No nisin induction	Nisin induction	
			0.01 IU ml ^{–1}	0.1 IU ml ^{–1}
MG1614	No nisin operons	≥0.5	≥0.5	≥0.5
N8	Nisin producer	100	ND	ND
LAC15	<i>nisZB'</i> sense	≥60	ND	ND
LAC16	<i>nisZB'</i> antisense	5	40	50
LAC46	<i>nisT</i> mutant	2.5	≥10	20

Fig. 5. Analysis of nisin production and immunity of the *L. lactis* strain N8. (a) Growth of the bacteria (OD₆₀₀, ▲) and pH of the medium (○). (b) Amount of nisin produced during growth: □, nisin from the surface of the cells; ○, nisin from the growth supernatant; ■, nisin from the cells and the growth supernatant. (c) Proportion of nisin-immune cells at different stages of growth: △, after 1 h; ●, after 3 h; ▽, after 4.5 h; ▼, after 6 h.

taining the pLEB124 expression vector with the *nisZ* gene and the 10 first codons of the *nisB* gene in the antisense orientation. In addition to the loss of nisin production the resistance to nisin dropped to less than 5% of wild-type immunity. Northern analysis of total RNA from strain LAC46 and the antisense-RNA mutant strain LAC16, using probes which hybridize to *nisZ*, *nisB*, *nisK* and *nisE* sequences showed that transcription of the two nisin operons was lost (Fig. 3). This showed that the two nisin promoters are strictly and coordinately regulated.

Nisin induction of the nisin operons

The loss of transcription from both nisin operons in strains LAC16 and LAC46 suggested that these operons are positively regulated by nisin or some of its precursor

forms. Addition of nisin to the growth cultures of strain LAC16 resulted in an activation of the transcription of the nisin operons (Fig. 3). Nisin-induced transcription also resulted in an increased level of the NisI protein (Fig. 4). The level of nisin immunity of the mutant strains was increased by addition of nisin in amounts that do not inhibit even the most sensitive indicator strain. Higher nisin concentrations induced the cells to a higher level of nisin immunity (Table 2).

Production of nisin

Cells of the nisin Z producer *L. lactis* N8 were grown in batch culture using a complex medium. In order to avoid transfer of nisin from the previous fermentation, the cells used as inoculum were washed twice in M17 medium pH 4.8. Nisin production was followed by measuring nisin activity in samples taken every 30 min from the culture. Nisin activity associated with the growth supernatant and with the cells was measured by extracting nisin attached to the cell surface using growth medium with low pH. Low pH has been shown to release nisin adsorbed *in vitro* to cell surfaces (Yang *et al.*, 1992). The results showed (Fig. 5) that nisin activity could first be detected in the growth supernatant when the cells were entering the exponential growth phase, with a maximum at the late exponential growth phase, similar to results previously reported (Buchman *et al.*, 1988; Engelke *et al.*, 1994; de Vuyst & Vandamme, 1992, 1993). The maximal nisin production rate (3000 IU ml⁻¹ per OD₆₀₀ unit h⁻¹) was observed during the fifth hour at the end of the exponential growth phase. However, during the first 4 h of growth, when only minor nisin activity could be detected from the growth supernatant, the specific nisin production rate was remarkably high (2000–2500 IU ml⁻¹ per OD₆₀₀ unit h⁻¹). Analysis of the nisin activity extracted from the cells showed (Fig. 5) that nisin produced during the first few hours of growth was adsorbed on the surface of the cells. When the pH of the growth supernatant fell below pH 5.5 the accumulation of nisin on the cell surfaces stopped and the cells started to release the adsorbed nisin, most likely due to the marked increase of nisin solubility below pH 5.5 (Liu & Hansen, 1990). At this stage of growth the amount of nisin in the supernatant started to increase significantly. The cells adsorbed only low amounts of nisin during the later stages of growth. The overall nisin production correlated with the sum of the nisin amount measured separately from the growth supernatant and the cells. The nisin immunity assay measured how many of the cells in a certain growth phase were immune to nisin and to what extent. The results showed (Fig. 5c) that the cells were approximately equally immune during all stages of growth.

DISCUSSION

The transcripts of the *nisZBTCIPRKFE* gene cluster

Northern blot results combined with RT/PCR and recent results (Ra & Saris, 1995) showed that one long *nisZBTCIPRK* mRNA from the *nisZ* promoter is trans-

cribed. The *nisB* gene and the six genes downstream of it were expressed at approximately the same level as the structural gene. Thus the inverted repeat between *nisA/Z* and *nisB* is not likely to function as a terminator as Steen *et al.* (1991) have suggested. The loop structure could function as a signal for the internal processing of the *nisZBTCIPRK* transcript at the intergenic gap between *nisZ* and *nisB*. The primer extension mapping of the *nisF* promoter and the size of the transcript detected using a *nisE* probe showed that *nisFEG* form one operon. Thus the putative transcription terminator downstream of *nisK* is functional (Engelke *et al.*, 1994; Immonen *et al.*, 1995).

Previous results have suggested the existence of a *nisR* promoter (O. P. Kuipers, unpublished results in de Vos & Simmons, 1994). However, we could not verify this putative *nisR* promoter by primer extension analysis. In addition, from RNA of the LAC16 and LAC46 strains, which have almost undetectable *nisZ* promoter activity, no hybridization signals could be detected with a *nisK* probe. Therefore, if the *nisRK* genes are transcribed from another promoter than the *nisZ* promoter, then the activity of this promoter is so low that it cannot be detected by Northern analysis. After nisin induction of strain LAC16 a strong *nisK*-specific signal was visualized in Northern analysis. The size of the signal was larger than the expected size of a *nisRK* transcript. Thus the major part of the transcription of the *nisRK* genes seems to be regulated by the *nisA/Z* promoter. These results showed that the *nisZBTCIPRK* gene cluster consists of at least two operons, *nisZBTCIPRK* and *nisFEG*, resulting after RNA processing in three transcripts (*nisZ*, *nisBTCIPRK* and *nisFEG*).

Regulation of the nisin operons

Antisense-RNA inhibition of the structural nisin gene and inactivation of the *nisT* gene resulted in loss of transcription of both operons. Addition of nisin to the mutant cells incapable of nisin production restored the transcription of the *nisZBTCIPRK* and *nisFEG* operons. Nisin induction also increased the level of nisin immunity and that of the NisI protein. Using increasing amounts of nisin for induction resulted in increasing levels of immunity. These results showed that nisin biosynthesis and immunity are autoregulated by mature nisin from outside the cell. Cells in the stationary growth phase still had some adsorbed nisin on their surfaces. If such cells are transferred to fresh growth medium, the adsorbed nisin can immediately start a new cascade of amplification. This, in addition to the low amount of nisin needed for induction, could explain why the cells of the nisin producer were immune in the very early growth stages. If there is no nisin present due to peptidase activity, the initial inducer has to be synthesized from transcripts originating from uninduced *nisZ* promoter activity. Northern blot analysis was not sensitive enough to detect this activity, but by Western analysis (Fig. 4, lane 4) the very low level of NisI produced by uninduced strain LAC16 was detected.

From the very early stages of growth to the stationary

phase only minor variation in the level of nisin immunity and the specific nisin production rate was observed. This further indicated that the cells were already almost fully induced during the very early growth stages. Only if nisin production is disrupted, as in the mutant strains LAC16 and LAC46, does the autoregulatory circuit close and transcription of the nisin operons stop. In the stationary phase the nisin titre started to decrease even though the level of nisin immunity and transcription was still high. This could be a result of the release of non-specific proteolytic enzymes during cell lysis (de Vuyst & Vandamme, 1992).

NisR (a response regulator) and NisK (a sensor histidine kinase), together forming a two-component regulator encoded by the first nisin operon, are needed for the expression of nisin (van der Meer *et al.*, 1993; Engelke *et al.*, 1994) and are likely to be involved in the signal transduction. The structure and function of many other two-component regulators are well characterized (Albright *et al.*, 1989), but the inducing signal is unclear in most cases. We showed that active nisin can induce the transcription of its own gene and the genes downstream of it. Nisin also induced the transcription of the *nisFEG* operon involved in nisin immunity. This indicates that antimicrobial peptides have a more general role than just the antagonistic action. As shown in this study, nisin can also act as a signal for transcriptional activation, not only for its own structural gene but also for other genes and operons. An evolutionary reason for the autoregulation via signal transduction could be to increase immunity levels in response to high nisin production by neighbouring cells. The signal transducing system may also be used for communication between cells in order to achieve synchronized production of nisin, resulting in efficient killing of competing bacteria. The nisin promoters in combination with the NisR/NisK two-component regulatory pair are good candidates for the development of protein expression systems for use in food with an ideal regulation, because the inducer is generally regarded as safe and the nisin promoters can efficiently be turned on and off.

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