Autoregulation of Nisin Biosynthesis in Lactococcus lactis by Signal Transduction

(Received for publication, July 7, 1995)

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The post-translationally modified, antimicrobial peptide nisin is secreted by strains of Lactococcus lactis that contain the chromosomally located nisin biosynthetic gene cluster nisABTCIR. When a 4-base pair deletion is introduced into the structural nisA gene (ΔnisA), transcription of ΔnisA is abolished. Transcription of the ΔnisA gene is restored by adding subinhibitory amounts of nisin, nisin mutants, or nisin analogs to the culture medium, but not by the unmodified precursor peptide or by several other antimicrobial peptides. Upon disruption of the nisK gene, which encodes a putative sensor protein that belongs to the class of two-component regulators, transcription of ΔnisA was no longer inducible by nisin. Fusion of a nisA promoter fragment to the promoterless reporter gene gusA resulted in expression of gusA in L. lactis NZ9800 (ΔnisA) only upon induction with nisin species. The expression level of gusA was directly related to the amount of inducer that was added extracellularly. These results provide insight into a new mechanism of autoregulation through signal transduction in prokaryotes and demonstrate that antimicrobial peptides can exert a second function as signaling molecules.

Nisin is an antimicrobial peptide (1-3) widely used in the food industry as a safe and natural preservative. The ribosomally synthesized precursor peptide undergoes extensive post-translational modification, which includes dehydration of serine and threonine residues and the formation of thioether bridges (β-methyl)lanthionines, resulting in five ring structures named A, B, C, D, and E (Fig. 1B). Peptides containing these characteristic modified residues are named lantibiotics (4). Eleven genes organized in a cluster have been implicated to be involved in the complex biosynthesis of nisin, i.e. nisABTCIPKFG (Fig. 1A) (5-11). Of these genes, nisE encodes the nisin A precursor peptide of 57 amino acid residues; nisB and nisC encode putative enzymes involved in the post-translational modification reactions (based on homology to genes found exclusively in other lantibiotic gene clusters); nisT encodes a putative transport protein of the ABC translocator family that is probably involved in the extrusion of modified precursor nisin (7,9); nisP encodes an extracellular subtilisin-like protease involved in precursor processing (8); nisI encodes a lipoprotein involved in the producer self-protection against nisin (9); and nisFEG encodes putative transporter proteins that have also been implied in immunity (11). A schematic representation of the post-translational events yielding mature nisin A is shown in Fig. 1B. Nisin Z is a natural variant of nisin A that contains an asparagine residue at position 27 instead of the histidine residue found in nisin A (12). Both nisin A- and nisin Z-producing strains are common in nature, and both structural genes (nisA and nisZ) have been cloned (5, 6, 13).

The proteins encoded by nisR (8) and nisK (10) have shown to be involved in the regulation of nisin biosynthesis (8,10). NisR is a response regulator, and NisK is a sensor histidine kinase which belong to the class of two-component regulatory systems (14-16). When the genes nisABTCIR are present on a multicopy plasmid, production of fully modified precursor nisin is observed, indicating that overexpression of nisR alone is sufficient to activate transcription of nisA and obviously also of the biosynthetic genes downstream by partially reading through an inverted repeat sequence (Fig. 1A) (8). This observation is similar to the regulation of expression of iep and deqU genes in Bacillus subtilis, where overexpression of the response regulator activates transcription of the target genes (17), and to the case of overexpression of epiQ, which encodes a response regulator involved in the biosynthesis of the lantibiotic epidermin (18). When only the genes nisABTCI are present on a multicopy plasmid (pNZ9000) in Lactococcus lactis MG1614, no transcription of nisA is observed (9). Two gene products have been identified for the regulation of the biosynthesis of the related lantibiotic subtilin (19), which also belong to the class of two-component regulators, i.e. SpaR, the response regulator, and SpaK, the sensor histidine kinase (20,21). Upon disruption of either of these genes, subtilin production was abolished, indicating the involvement of these gene products in subtilin biosynthesis (20). The regulation was shown to be growth-phase dependent, but an inducing signal was not identified (20,21).

While the structure and function of two-component regulators have been studied in great detail (14-16), the nature of the inducing signal has remained unclear in many cases. It is demonstrated here that fully modified nisin can induce the transcription of its own structural gene as well as of the downstream genes by limited read-through, via signal transduction, by acting as the extracellular signal for the sensor histidine kinase NisK.

MATERIALS AND METHODS

Strains and Plasmids—L. lactis strains MG1614 (22), NZ9700 (a nisin-producing transconjugant containing Tn5276) (23), and NZ9800...
Fig. 1. A, organization of the nisin gene cluster. Established (nisAIPRKFEG) and putative (nisBCT) functions of the gene products have been indicated. P denotes a mapped promoter, and IR denotes an extensive inverted repeat sequence that could act as a rho-independent terminator (7). B, schematic outline of the biosynthesis of nisin A. Rings are labeled A–E. Asterisks indicate residues that will be modified. The black arrow indicates processing of the N-terminal Met residue, while the small white arrow indicates processing of the leader peptide by the action of NisP (8). Dha, dehydroalanine; Dhb, dehydrobutyryl. 
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(a derivative of N29700 in which the nisA gene has been exchanged by replacement recombination with a modified nisA gene containing a 4-bp \(^3\) deletion in the prointrinsic-encoding part (\(\Delta{\text{nisA}}\)) and which is therefore no longer able to produce nisin A) have been described previously (9). L. lactis strains were cultivated without aeration at 30 °C in M17 broth (Difco) supplemented with 0.5% (w/v) glucose (GM17) or succinate (mg500). DNA of erythromycin-resistant strains (24), media were supplemented with 10 \(\mu\)g/ml chloramphenicol. Expression plasmids pNZ9010 and pNZ2013 (9, 25), containing the \(\text{nisA}\) and \(\text{nisZ}\) genes, respectively, under control of the efficient lac promoter, were introduced into L. lactis strain N29800, leading to the production of nisin A or nisin Z in similar amounts as in L. lactis with pMT1 and pUPS1. For a host strain for cloning experiments, E. coli strain MC1061 (26) was used.

The \(\text{nisA}\) promoter region including part of the \(\text{nisA}\) gene was isolated as a 1442-bp BglII-Ecd136I fragment from plasmid pNZ9000 (8). This fragment was cloned into pNZ273, containing the promoterless gusA gene (24), which had been digested with BglII and SacI, generating plasmid pNZ28003. Part of the upstream promoter region was deleted by digesting pNZ28003 with BglII and Hth111I. These sites were made blunt by Klenow polymerase and ligated, generating plasmid pNZ28008, which eventually contained a 312-bp \(\text{nisA}\) promoter fragment in front of the gusA gene. Another part of the \(\text{nisA}\) promoter region, including the full \(\text{nisA}\) gene and the first part of the \(\text{nisB}\) gene, was isolated as a BglII-Muni fragment from plasmid pNZ9000. This fragment was cloned into pNZ273 (24), which had been digested with BglII and EcoRI, generating plasmid pNZ28002. A 1442-bp BglII-Ecd136I promoter fragment was deleted in pNZ28002, generating pNZ28000. The resulting plasmid was named pNZ9135 and was used as a plasmid backbone for site-directed mutagenesis of the \(\text{nisA}\) promoter.

**DNA Techniques and DNA Sequence Analysis—Restriction enzymes and other DNA-modifying enzymes were purchased from Life Technologies, Inc. or U. S. Biochemical Corp. and used as recommended by the manufacturer. The DNA sequence of the \(\text{nisA}\) region was determined on the double-stranded plasmid DNA with the HindIII primer by the chain termination method (27). Transcription analyses of the \(\text{nisA}\) and \(\Delta{\text{nisA}}\) genes were performed by isolation of RNA from L. lactis strains N29700 and N29800, Northern blotting, and subsequent hybridization with a radiolabeled nisA probe as described previously (9). RNA isolation was performed 2 h after induction of a culture with an A\(_{600}\) of 0.5. RNA (20 \(\mu\)g) was loaded in each lane, and the amounts were estimated by comparing the intensity of the 16 S and 23 S RNA bands.

Inactivation of Chromosomal \(\text{nisK}\) and \(\text{nisB}\) Genes by REPLACEMENT—The chromosomal copy of the \(\text{nisK}\) gene was inactivated by introduction of an erythromycin resistance gene (28) into the open reading frame of the chromosomal \(\text{nisK}\) gene by homologous recombination. This was accomplished by cloning a 5.5-kilobasepair EcoRI fragment, containing the full \(\text{nisK}\) gene including the 5'-flanking region, into pUC19 vector, which had been modified by cloning a 4.4-kilobasepair BglII-EcoRI fragment, containing nisB and surrounding regions from the nisin gene cluster, into a BamH1-EcoRI-digested pUC19 vector, which harbored an additional erythromycin resistance marker, as has been described previously (9). The deletion was made by removing an internal HpaI fragment from the nisB gene and subsequent ligation. The resulting plasmid was named pNZ9135 and was used for transformation of L. lactis N29700. Following transformation, erythromycin-resistant colonies were obtained that had integrated the plasmid by recombination of the plasmid with one of the flanking regions of the deleted fragment. After growing for 200 generations in the absence of erythromycin and plating, a colony was obtained that was sensitive to erythromycin. This has apparently been caused by a second recombination event involving the flanking region on the other side of the deletion than the side of the first recombination event, resulting in the replacement of nisB with \(\Delta{\text{nisB}}\) on the chromosome. The configuration of the desired construct was confirmed by polymerase chain reaction analysis of the \(\text{nisB}\) region with the deletion and by Southern analysis of BglII-digested chromosomal DNA. The desired strain was called N29700.\(\Delta{\text{nisB}}\).

Production, Purification, and Characterization of Nisin Mutants—Mutants of nisin Z were produced as described previously (25). The primers used for site-directed mutagenesis of the nisZ gene were as follows: 5'-CAAGTGCTACACACCGTGACAGATTCCGCT-AGTAC-3' (1W), 5'-CACACCGCTTTCAGTTTCTGATG-3' (T2S), 5'-CACCAAGCATCAAAATTCTGCTATGTAACCCC-3' (S3T), and 5'-AACAGGAGCCTGGGTTGTAACAT-3' (M17W) (mutated nucleotides are indicated in boldface). All mutants were purified to homogeneity, and the structures of the modified residues were confirmed by one- and two-dimensional \(^1\)H NMR (25). It was established that T2S nisin Z has a dehydrouracil residue at position 2, S3T nisin Z has a \(\beta\)-methylalanine ring between residues 3 and 7 instead of a lanthionine, and M17W nisin Z contains a Trp residue at positions 1 and 17, respectively. Precursor nisin Z, containing the subtilin leader peptide (nis-Z), was obtained as described before (30). Purified lactococcin 481 was isolated as described previously (31, 32). Purified Pep5 (33) was obtained from Dr. H.-G. Sahl (Bonn, Germany). Unmodified precursor nisin A was obtained from the laboratory of Dr. G. Jung (Tübingen, Germany). Preparations of subtilin (19) and lactococcin A (34) consisted of culture supernatants of producing strains, which were confirmed to possess substantial antimicrobial activity in agar diffusion assays. Antimicrobial activities against L. lactis MG1614 were determined essentially as described before for Micrococcus flavus (25). L. lactis was cultured in GM17 broth at 30 °C with an initial A\(_{600}\) of 0.025, and outgrowth was measured when the culture without nisin had reached an A\(_{600}\) of 0.8.

**\(\beta\)-Glucuronidase Assays—Lactococcal cells (1 ml) were harvested at 1.5 h after induction with nisin (or mutants or fragments) or other antimicrobial species and adjusted to 10 \(\mu\)g/ml with Na\(\text{H} \)PO\(_4\), pH 7.0, 10 \(\mu\)g/ml \(\beta\)-mercaptoethanol, 0.2% (w/v) Triton X-100, and 100 mM p-nitro-\(\beta\)-glucurononic acid (CLONTECH, Palo Alto, CA). The mixture was incubated, and the increase in A\(_{405}\) was measured at 37 °C. Histochromic screening for \(\text{gus}\) positive colonies was performed by incubating 5-bromo-4-chloro-3-indolyl glucuronide (Research Organics Inc., Cleveland, OH) at a final concentration of 0.5 \(\mu\)l in GM17 plates (24). Purified nisin fragments (35–39) were obtained from Prof. T. Shiba (Protein Research Foundation, Osaka, Japan).

RESULTS AND DISCUSSION

**Transcription Analyses of NisA in the Presence and Absence of Nisin or Nisin Mutants**—The promoter sequence and the transcription start site of nisA have been identified, and a transcript of \(-260\) nucleotides has been demonstrated in L. lactis strain N29700, which contains Tn5276 (9). It has also been found that transcription of nisA is dependent on the integrity of nisA itself since a 4-bp deletion in the middle of the nisA gene (\(\Delta{\text{nisA}}\)) on the chromosome of L. lactis strain N29800 completely abolishes transcription of this gene (9). For further transcription analyses of the structural and biosynthetic genes, a series of isogenic lactococcal strains was used, including the nisin-producing N29700 and non-nisin-producing N29800 strains.

Northern blotting showed that in strain N29800, the transcript of nisA was absent, but after adding small amounts of

1 The abbreviation used is: bp, base pair(s).
against L. lactis by Use of the Nisin Promoter Fragment Fused to the Reporter Gene gusA—

Determination of the Induction Capacity of Nisin (Mutants) by Use of the Nisin Promoter Fragment Fused to the Reporter Gene gusA—To obtain a more quantitative assay of induction capacity and to investigate whether the nisA promoter could be used to regulate expression of heterologous genes in L. lactis, a nisin promoter fragment of 312 bp containing part of the nisA structural gene was fused to the promoterless reporter gene gusA of E. coli on plasmid pNZ273 (24). This construct, named pNZ8008, was used to transform strain NZ9800. The resulting strain was assayed for β-glucuronidase activity with and without induction by (mutant) nisins or other antimicrobial peptides. Without induction, β-glucuronidase activity could not be demonstrated, whereas wild-type nisin A and nisin Z effectively induced β-glucuronidase activity (Fig. 3). Moreover, the T2S and M17W nisin Z mutants were found to induce higher expression of gusA compared with wild-type nisin A and nisin Z, whereas the S3T and I1W nisin Z mutants were found to have lower induction capacity (Fig. 3). It was calculated that ≤5 molecules of the best inducer (T2S nisin Z) per cell are sufficient to activate transcription, which illustrates the high efficiency characteristic of signal transduction processes. Concordant with the transcription analyses, some of the antimicrobial peptides tested did not elicit induction of gusA expression (i.e. the unmodified synthetic nisin A precursor peptide, subtilin, lactacin 481, and lactococcin A). There is no direct relationship between antimicrobial activity of the nisin mutants against L. lactis strain MG1614 and their induction capacity (Table I). The difference in potency can be attributed to the observation that antimicrobial activity is dependent on pore-forming activity in membranes (40–42), while induction capacity is likely to be dependent on interaction (directly or indirectly) with NisK.

In further experiments, the nisin-producing strain NZ2700 with either plasmid pNZ273 (containing the promoterless gusA gene) or pNZ8008 (containing the nisA promoter fragment followed by the gusA gene) was used in an agar diffusion assay (8) to determine the amount of nisin produced. Fifty times lower nisin production and severely reduced immunity were observed when plasmid pNZ8008 was present compared with the situation where pNZ273 was present. This can be explained by titration of the response regulator NisR by the multicopy presence of the nisA promoter region containing the putative NisR-binding site.

Structural Requirements of the Inducer Molecule Tested by Use of Synthetic Nisin Fragments—More detailed insight into
the minimal structural requirements of the inducer molecule was obtained by using synthetic nisin A fragments (35–39) in the gusA reporter assay (Table I). The minimal requirement for retaining induction capacity (2% induction of that of nisin A) was the presence of residues 1–11 of nisin A, comprising the first two rings. Addition of the third ring enhanced induction (8–30% induction), whereas a severe decrease in induction was caused by deleting the N-terminal residues Ile-1 and dehydrobutyrine 2 (0–1% induction) (Table I). Fragments that contained rings B and C or rings D and E (for nomenclature of rings, see Fig. 1B) were not capable of acting as a signal effector. Thus, the most probable site of molecular interaction with the sensor protein NisK will be residues 1–11 of the nisin molecule.

Requirement of nisK Expression for Signal Transduction—The sequence of the nisK gene located on Tn5276 has been reported (43) and was found to be identical to that of nisK from L. lactis 6F3 (10). The chromosomal nisK gene was insertionally inactivated by introduction of an erythromycin resistance gene (28) into strain NZ9800, yielding strain NZ9805. As expected, transcription of ΔnisA was no longer inducible by any of the nisin species (Fig. 2, lanes 8 and 9). Nisin production in strain NZ9805 could not be restored by introduction of plasmid pNZ9010 (nisA) or pNZ9013 (nisZ), whereas it could be restored in strain NZ9800. Since the immunity level of strain NZ9805 is similar to that of strain MG1614 (0.01 μg/mL) (11), induction experiments were performed with amounts of nisin well below this level. Under these conditions, normal growth of the cells was observed. Strain NZ9805 was also transformed with pNZ8008, but after induction with 0.0005–0.0025 μg/mL of nisin A and no β-glucuronidase activity could be measured (<0.3 arbitrary unit), indicating at least 200 times lower expression than in strain NZ9800 containing pNZ8008, with the same inducer concentrations (Fig. 3). No polar effects of the nisK disruption on expression of the nisFEG genes downstream of nisK are expected since a promoter has been indicated in front of nisFEG (11). Moreover, the nisR and nisK genes have been integrated on the chromosome of strain MG1614 by replacement recombination, and the resulting strain was transformed by pNZ8008. In this strain, gusA expression was inducible by nisin species (data not shown), proving that only nisR and nisK are required for signal transduction. These results clearly demonstrate that NisK is essential in the signal transduction pathway and probably interacts directly with nisin itself.

Effects of Disruption of nisB on Transcription of nisA and Downstream Genes—An in-frame deletion in the nisB gene of L. lactis strain NZ9700, made by replacement recombination, abolished nisin production as well as transcription of nisA, demonstrating that a hampered biosynthesis of nisin abolishes transcription of nisA. In this case, transcription of nisA could be restored by addition of nisin to the cells (Fig. 2, lane 1), probably because of the presence of intact nisR and nisK genes, which have their own promoter. The transcription start site of nisRK was mapped by primer extension and shown to be an A nucleotide 26 nucleotides upstream of the start codon of nisR (position 2117 in the nucleotide sequence published in Ref. 8). To probe the influence of a large inverted repeat sequence located in the intergenic region between ΔnisA and nisB on expression of genes downstream of ΔnisA (Fig. 1A), another plasmid was constructed (pNZ8002) in which the nisin promoter fragment including anisA as well as the intergenic region and the first part of nisB was fused to the gusA gene. This plasmid was able to direct expression of gusA in strain NZ9800 only after induction with nisin species, albeit to an ~50-fold reduced level relative to gusA expression in pNZ8008 in strain NZ9800. When the nisin promoter fragment was removed from pNZ8002, yielding pNZΔ8002, β-glucuronidase activity was completely abolished, even in the presence of an inducer. These results show that expression of at least one downstream gene, i.e., nisB, is coregulated and is dependent on the presence of the nisA promoter. Most likely, expression of the other downstream genes nisTCIP limited read-through is also dependent on the nisA promoter since a significant increase in immunity levels, for which NisI is partially responsible (9), was found in the induced state relative to the uninduced state of strain NZ9800. Moreover, no apparent promoter sequences were found in front of any of the genes nisBTCIP.

Conclusion—We have demonstrated that transcription of nisA is autoregulated, not intracellularly by its direct trans-acting product, but extracellularly by the secreted and fully modified peptide via signal transduction by a two-component regulatory system. A model based on previous work (5–11) and on this study shows the possible sequence of events with regard to nisin biosynthesis and regulation (Fig. 4).

Mutants of nisin or precursors of nisin that have the leader peptide attached to the mature laitibiotic (second molecule shown in Fig. 1B) can also act as inducers, whereas other antimicrobial peptides are incapable of induction. The presence of the modified residues is of crucial importance for induction capacity, especially those present in the N-terminus of nisin. To our knowledge, this is the only report of peptides that can induce transcription of their own structural gene via signal transduction. Interestingly, a recent report on syndecan biosynthesis in mice, which plays a role in wound repair, describes the role of the antimicrobial peptide PR39 in induction of syndecan gene transcription (44), although the amount of inducer needed (0.5 μm) is at least a factor of 10,000 higher than for nisin (30 pm). This suggests that the role of antimicrobial peptides in nature might be broader than just the antagonistic action because in some cases these peptides can also act as signals for transcription activation of their own structural gene.

2 P. G. A. de Ruyter, O. P. Kuipers, and W. M. de Vos, unpublished data.
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or of other genes. There may be several evolutionary reasons for the autoregulation of nisin gene transcription via signal transduction, e.g. (i) to save energy by control of the integrity of the gene cluster since any dysfunctional biosynthetic gene will abolish inducer formation and thus expression of biosynthetic genes; (ii) to raise immunity levels in response to high nisin production by neighboring cells, in other words, to amplify the response to environmental signals; or (iii) to promote cell to cell communication that allows the production of antimicrobial peptides in high quantities in a concerted action, thereby decreasing the chance of resistance development in target organisms.

Acknowledgments—We thank Dr. T. Shiba, Dr. K. Fukase, and Dr. T. Wakamiya for the generous gift of synthetic nisin fragments; Dr. G. Jung and Dr. A. Biek-Sückinger for the generous gift of unmodified synthetic precursor nisin A; and Dr. H.-G. Sahli for the generous gift of PepS. We thank Roger Bongers and Patrick van den Bogaard for construction of several mutant nisin genes, Hans Koster for purifying and characterizing some of the mutants, and Lilian Vreede for determination of several mutant nisin genes, Hans Koster for purifying and characterizing some of the mutants, and Lilian Vreede for determination of several mutant nisin genes.

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