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Functional Analysis of Promoters in the Nisin Gene Cluster of *Lactococcus lactis*

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The promoters in the nisin gene cluster *nisABTCIPRKFEG* of *Lactococcus lactis* were characterized by primer extension and transcriptional fusions to the *Escherichia coli* promoterless β-glucuronidase gene (*gusA*). Three promoters preceding the *nisA*, *nisR*, and *nisF* genes, which all give rise to *gusA* expression in the nisin-producing strain *L. lactis* NZ9700, were identified. The transcriptional autoregulation of *nisA* by signal transduction involving the sensor histidine kinase NisK and the response regulator NisR has been demonstrated previously (O. P. Kuipers, M. M. Beerthuyzen, P. G. G. A. de Ruyter, E. J. Luesink, and W. M. de Vos, J. Biol. Chem. 270: 27299–27304, 1995), and therefore the possible nisin-dependent expression of *gusA* under control of the *nisR* and *nisF* promoters was also investigated. The *nisR* promoter was shown to direct nisin-independent *gusA* expression in *L. lactis* MG1363, which is a nisin-transposon- and plasmid-free strain. *L. lactis* NZ9800, which does not produce nisin because of a deletion in the *nisA* gene, containing the *nisF-gusA* fusion plasmid, gave rise to β-glucuronidase production only after induction by nisin. A similar regulation was found in *L. lactis* NZ3900, which contains a single copy of the *nisR* and *nisK* genes but no other genes of the nisin gene cluster. In contrast, when the *nisK* gene was disrupted, no β-glucuronidase activity directed by the *nisF* promoter could be detected even after induction with nisin. These results show that, like the *nisA* promoter, the *nisF* promoter is nisin inducible. The *nisF* and *nisA* promoter sequences have significant similarities and contain a conserved region that could be important for transcriptional control.

A great number of lactic acid bacteria and other gram-positive bacteria produce peptides that display antimicrobial activity (13). Because of their wide spectrum of activity, one class of these antimicrobial peptides, the lantibiotics, has received considerable attention in the last few years (5). Lantibiotics are posttranslationally modified peptides, containing dehydrated serine and threonine residues and thioether bridges. The most prominent lantibiotic is nisin, which is produced by several strains of *Lactococcus lactis* and is widely used as a food preservative (3).

In most strains of *L. lactis*, nisin production is encoded by large conjugative nisin-sucrose transposons (11, 22), and nucleotide sequences of several genes in nisin gene clusters have been determined (1, 5, 7–9, 12, 14, 17, 18, 25, 30). The 11 genes *nisABTCIPRKFEG* include those that, apart from the structural gene *nisA*, are involved in the intracellular posttranslational modification reactions (*nisBC*) (8, 17), export (*nisT*) (8, 17), and extracellular proteolytic activation (*nisP*) (30). In addition, the genes for two different systems involved in immunity to nisin are present in the nisin gene cluster, i.e., *nisI*, encoding a lipoprotein (9, 17), and *nisFEG*, encoding a putative ATP-binding cassette exporter that could be involved in nisin extrusion (25). Finally, the nisin gene cluster contains two regulatory genes, *nisR*, encoding a response regulator, and *nisK*, encoding a sensor histidine kinase. The NisR and NisK proteins have been shown to be involved in the regulation of nisin biosynthesis (9, 16, 17) and belong to the class of the two-component regulatory systems (15, 19, 27, 33).

Recently, it has been demonstrated that the transcription of *nisA* is autoregulated and requires intact *nisR* and *nisK* genes (16). The secreted and fully modified nisin peptide can extracellularly induce the transcription of its own structural gene via signal transduction by the NisR-NisK two-component regulatory system (16). Mutants of nisin or precursors of nisin that are completely modified can also act as inducers, whereas other antimicrobial peptides are incapable of induction (16). The promoter sequence and the transcription start site of *nisA* have been identified in *L. lactis* NZ9700 harboring the 70-kb conjugative transposon Tn*5276* (17). The large inverted-repeat sequence located between the *nisA* and *nisB* genes may act as a transcription terminator and could be responsible for limited read-through, since *nisB* expression is also dependent on the presence of the *nisA* promoter (16, 21). Most likely, expression of the other downstream genes *nisTCIP* is also dependent on the *nisA* promoter since no apparent promoter sequences were found in front of any of these genes that are partly overlapping (16).

Recently, we identified a promoter in front of the *nisRK* genes (16), but its regulation has not been analyzed. Most likely, a stem-loop structure at the end of *nisK* serves as a terminator of transcription. In addition, a putative promoter in front of the *nisFEG* genes was suggested recently, but no experimental data were shown (25). In order to study the transcriptional organization and regulation of the complete nisin gene cluster, gene fusions of putative promoter fragments with the promoterless β-glucuronidase gene (*gusA*) from *Escherichia coli* (20) were constructed and tested in various *L. lactis* strains. The transcription initiation sites of the *nisF* and *nisR* promoters were determined by primer extension, and their regulation was compared with that of the *nisA* promoter. The results indicate that the *nisR* promoter is nisin independent while the *nisF* promoter is controlled by nisin and subject to the same *nisRK*-dependent control as the *nisA* promoter.

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TABLE 1. *L. lactis* strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference
Strains		
MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	10
NZ9700	Nisin-producing transconjugant containing Tn*5276*	17
NZ9800	NZ9700 derivative, Δ*nisA*, non-nisin producer	17
NZ9850	NZ9800 derivative, Δ*nisK*	16
MG5267	MG1363 derivative, Lac$^+$, single chromosomal copy of *lac* operon	31
NZ3000	Δ*lacF*, derived from MG5267 by replacement recombination	29
NZ3900	NZ3000 derivative, *pepN*::*nisRK*	This work
Plasmids		
pNZ9107	pUC19 derivative containing the *nisP* and *nisR* genes	30
pNZ9201	pUC19 derivative carrying the *nisR* promoter region including the 3′ part of *nisP* and the 5′ part of *nisR*	This work
pNZ9570	pUC19 derivative containing the 3′ part of *nisP*; the intact *nisR*, *nisK*, *nisF*, and *nisE* genes; and the 5′ part of *nisG*	This work
pNZ124	Cm^r, 2.8 kb, pSH71 replicon	20
pNZ273	Cm^r, 4.7 kb, pNZ124 carrying the promoterless *gusA* gene from *E. coli* and translational stops in all reading frames	20
pNZ8008	Cm^r, 5.0 kb, pNZ273 derivative carrying the *gusA* gene fused to the *nisA* promoter	This work
pNZ8023	Cm^r, 5.2 kb, pNZ273 derivative carrying the *gusA* gene fused to the *nisR* promoter	This work
pNZ8024	Cm^r, 6.2 kb, derived from pNZ273 carrying the *gusA* gene fused to the *nisF* promoter	This work
pNZ84	Cm^r, pACYC184 derivative, nonreplicative in *L. lactis*	28
pNZ1104	Cm^r, pNZ84 derivative containing the *pepN* gene	28
pNZ9572	Cm^r, pNZ1104 derivative containing the 3′ part of *nisP* and the intact *nisR* and *nisK* genes inserted into the *pepN* gene	This work
pNZ9573	Cm^r, pNZ9572 derivative containing an erythromycin resistance gene	This work

[a] Cm^r, resistance to chloramphenicol; Lac$^+$, lactose-fermenting phenotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MC1061 (2) was grown in L broth-based medium with aeration at 37°C (23). The *L. lactis* strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown without aeration at 30°C in M17 (Merck, Darmstadt, Germany) broth containing 0.5% (wt/vol) glucose (GM17). If appropriate, the media contained chloramphenicol (10 μg/ml) or erythromycin (5 μg/ml).

DNA and RNA methodology, reagents, and enzymes. Isolation of plasmid DNA from *E. coli* and standard recombinant DNA techniques were performed according to the work of Sambrook et al. (23). Isolation of plasmid DNA from *L. lactis* was performed as described previously (32). DNA fragments were isolated from agarose gels by using the GlassMAX DNA Isolation Matrix System (BRL Life Technologies, Inc., Gaithersburg, Md.). Transformation of *L. lactis* strains was performed according to the work of Wells et al. (34). Nucleotide sequence analysis of double-stranded plasmid DNA was performed with an ALF automatic sequencer (Pharmacia Biotec) in combination with Autoread kits which include T7 DNA polymerase. Primer extension of the *nisF* and *nisR* promoters was performed by isolation of RNA from induced and uninduced cultures of *L. lactis* NZ9800 as described previously (17). Two oligonucleotides with the sequences 5′-CCTGCACCGTTAACTCC-3′ (primer 1) and 5′-CAAA ACTACGCAGCGTTGAAGTATC-3′ (primer 2), which are complementary to positions 247 to 264 of the *nisF* gene (in the nucleotide sequence published in reference 25) and to positions 2220 to 2244 of the *nisR* gene (in the nucleotide sequence published in reference 30), respectively, were used for the primer extension and for double-stranded DNA nucleotide sequencing of the *nisF* promoter (pNZ8024) and *nisR* promoter (pNZ8023) by the dideoxy chain-termination method (24), with [α-^{32}P]dATP. Restriction enzymes and other DNA-modifying enzymes were purchased from Gibco/BRL Life Technologies and used as recommended by the manufacturers. Oligonucleotides were purchased from Pharmacia.

Construction of plasmids. A schematic representation of the different fragments used in this study is shown in Fig. 1. The *nisA* promoter region including part of the *nisA* gene was cloned as a 0.3-kb *Tth*I-*Sst*I fragment in pNZ273 containing the promoterless *gusA* gene. The resulting plasmid, pNZ8008 (16), was used to transform *L. lactis* NZ9800 (Δ*nisA*), *L. lactis* NZ9850 (Δ*nisK*), and *L. lactis* NZ3900 (NZ3000 [29], with *nisRK* integrated on the chromosome).

The *nisR* promoter region (Fig. 1) was isolated as a 0.6-kb *Eco*RV-*Hin*dIII fragment from plasmid pNZ9107 (30) and cloned in pUC19 (35), digested with *Sma*I-*Hin*dIII, generating pNZ9201. Subsequently, the 0.6-kb fragment carrying the *nisR* promoter region was isolated as a *Eco*RI-*Hin*dIII (blunt) fragment and cloned in the promoter probe vector pNZ273 (20), which had been digested with *Pvu*II and *Eco*RI. The resulting plasmid, pNZ8023, contains the 0.6-kb *nisR* promoter fragment in front of the *gusA* reporter gene. All constructs were initially made in *E. coli* MC1061, and pNZ8023 was subsequently transformed into *L. lactis* strains. The authenticity of the insert of pNZ8023 was verified by restriction analysis and by sequence analysis of the relevant promoter sequences.

Plasmid pNZ9570 was constructed by cloning a 6-kb *Hin*dIII chromosomal DNA fragment from strain NZ9700 containing the 3′ part of the *nisP* gene; the intact *nisR*, *nisK*, *nisF*, and *nisE* genes; and the 5′ part of the *nisG* gene in pUC19 with *E. coli* MC1061. The *nisF* promoter region including the 3′ part of the *nisK* gene and the 5′ part of the *nisF* gene was isolated as a 1.5-kb *Nde*I-*Eco*RI fragment from plasmid pNZ9570. The *Nde*I site was made blunt by Klenow polymerase, and this fragment was cloned in pNZ273, which had been digested with *Pvu*II and *Eco*RI, generating plasmid pNZ8024. Plasmid pNZ8024 was used to transform *L. lactis* NZ9800, *L. lactis* NZ9850, and *L. lactis* NZ3900.

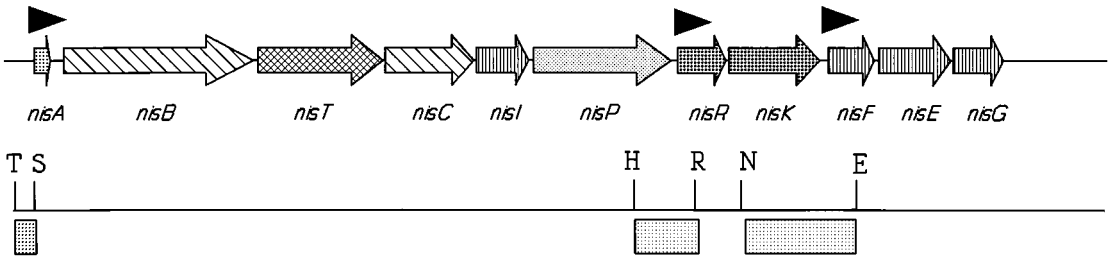

FIG. 1. Schematic representation of the organization of the nisin gene cluster, location of the nisin promoters, and fragments inserted in the *gusA* reporter plasmid pNZ273 (20). Relevant restriction sites are indicated: T, *Tth*I; S, *Sst*I; H, *Hin*dIII; R, *Eco*RV; N, *Nde*I; E, *Eco*RI.

TABLE 2. The phenotypes of several *L. lactis* strains containing nisin promoter fusion plasmids on plates containing X-Gluc

Plasmid (promoter)	Phenotype for *L. lactis* strains with and without nisin[a]								
	NZ9700 (Tn*5276*)	MG1363		NZ9800 (Δ*nisA*)		NZ9850 (Δ*nisK*)		NZ3900 (*nisRK*)	
	+	+	−	+	−	+	−	+	−
pNZ8008 (P_{nisA})	B	W	W	B	W	W	W	B	W
pNZ8023 (P_{nisR})	B	B	B	B	B	ND[b]	ND	B	B
pNZ8024 (P_{nisF})	B	W	W	B	W	W	W	B	W

[a] The colonies were grown in the presence (+) or absence (−) of nisin. Blue (B) or white (W) colonies appeared on the plates after overnight incubation at 30°C.

[b] ND, not determined.

Construction of strain NZ3900. A fragment containing the 3′ part of the *nisP* gene and the intact *nisR* and *nisK* genes was isolated as a 2.6-kb *Hin*dIII-*Hpa*I fragment from plasmid pNZ9570 in which the *Hin*dIII site had been made blunt with Klenow polymerase. This 2.6-kb fragment was cloned in plasmid pNZ1104, carrying the chromosomal *pepN* gene, digested with *Bst*EII and *Mlu*I, which had been made blunt with Klenow polymerase. The resulting plasmid, pNZ9572, contains an inactivated *pepN* gene in which the *nisR* and *nisK* genes are inserted in the direction opposite to that of *pepN* transcription. The erythromycin resistance gene from pIL253 (26) was introduced in the *Bam*HI sites of plasmid pNZ9572. The resulting plasmid was named pNZ9573, and this nonreplicating plasmid in *L. lactis* was used for transformation of *L. lactis* NZ3000 (29). Following transformation, erythromycin-resistant colonies in which the plasmid had been integrated by recombination with one of the flanking regions of the deleted *pepN* gene were obtained. One of these transformants with the expected single-copy integration was grown without erythromycin for 100 generations, and subsequently, erythromycin-sensitive colonies were screened for the absence of aminopeptidase N activation on plates as described previously (28). The resulting strain NZ3900 is *pepN* deficient and as a result of a second crossover event contains the *nisR* and *nisK* genes under control of their own promoter integrated in the *pepN* locus. The expected configuration of the *nisRK* genes in NZ3900 was confirmed by PCR analysis and Southern blotting.

β-Glucuronidase assays. Histochemical screening for β-glucuronidase activity by selecting for blue colonies was performed by including 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) at a final concentration of 0.5 mM in GM17 plates. For the quantitative β-glucuronidase assay, *L. lactis* cells were grown to the A_{600} of 0.5, induced with different concentrations of nisin A (hereafter named nisin; 0 and 0.1 to 5 ng ml^{-1}), and grown for another 90 min. Cells were harvested and resuspended in NaPi buffer (50 mM $NaHPO_4$, pH 7.0) to a final A_{600} of 2.0. The cells were permeabilized by adding 50 μl of acetone-toluene (9:1 [vol/vol]) per ml of cells and then incubating for 10 min at 37°C. Forty microliters of the extracts was used immediately in the β-glucuronidase assay by adding 950 μl of GUS buffer (50 mM $NaHPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100) and 10 μl of 100 mM para-nitrophenyl-β-D-glucuronic acid (Clontech Laboratories, Inc., Palo Alto, Calif.). The mixture was incubated at 37°C, and the increase in A_{405} (ΔA min^{-1}) was used to calculate the specific β-glucuronidase activity per optical density (at 600 nm) unit of cell density. When the molar absorption coefficient is used (ε-para-nitrophenyl-β-D-glucuronic acid = 18,000), the β-glucuronidase activity can be calculated in nanod-moles $minute^{-1}$ optical density $unit^{-1}$.

RESULTS

Development of promoter fusion vectors. Derivatives of pNZ273 that contain putative promoter inserts of the nisin gene cluster in front of the promoterless *E. coli* β-glucuronidase (*gusA*) gene (20) were constructed. Since translational stops are present in all three reading frames preceding *gusA* of the promoter probe vector pNZ273, translation initiates at the ATG start codon of *gusA*. Plasmids containing the fragments preceding the *nisR* gene (pNZ8023) and the *nisF* gene (pNZ8024) were used to transform *L. lactis* NZ9700, a nisin-producing strain containing the nisin-sucrose transposon Tn*5276* (17). In both cases, colony formation was accompanied by the development of a blue color on plates containing X-Gluc, indicating β-glucuronidase activity (Table 2). Because of their established promoter activity in *L. lactis* NZ9700, the plasmids pNZ8023 and pNZ8024 were subsequently used to transform several other *L. lactis* strains in order to compare the mode of control of the promoters with that of the nisin-inducible *nisA* promoter that drives expression of the *gusA* gene in pNZ8008 (16).

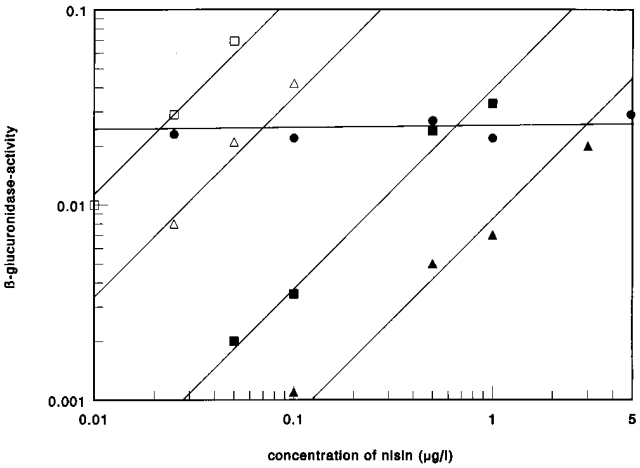

FIG. 2. Dose-response curves of *gusA* expression in cell extracts of *L. lactis* NZ9800 or *L. lactis* NZ3900 directed by the *nisA* promoter, *nisR* promoter, or *nisF* promoter induced with several concentrations of nisin. The β-glucuronidase activity is shown as specific activity per optical density (at 600 nm) unit. The standard errors are less than 20% for each value. Symbols: ■, NZ9800 (P_{nisA}); ●, NZ9800 (P_{nisR}); ▲, NZ9800 (P_{nisF}); □, NZ3900 (P_{nisA}); △, NZ3900 (P_{nisF}).

Plasmids pNZ8023 and pNZ8024 were first introduced in *L. lactis* MG1363, which is free of plasmids and of nisin genes, in order to check whether the *nisR* and *nisF* promoters depend upon an intact nisin operon or nisin itself for their activity. *L. lactis* MG1363 harboring pNZ8023 showed blue colonies on plates containing X-Gluc with and without nisin, in contrast to *L. lactis* MG1363 cells harboring pNZ8008 and pNZ8024, which both yielded only white colonies (Table 2). Cell extracts of the MG1363 transformants harboring pNZ8008 or pNZ8024 did not show any detectable β-glucuronidase activity in the absence or presence of nisin.

Expression of *gusA* under control of the *nisA*, *nisF*, and *nisR* promoters in *L. lactis* NZ9800. The plasmids pNZ8023 and pNZ8024 were also introduced in *L. lactis* NZ9800, which contains the transposon Tn*5276* but does not produce nisin because of a deletion in the *nisA* gene (17). Subsequently, β-glucuronidase expression was analyzed with and without induction by nisin, to allow a comparison with pNZ8008 introduced in NZ9800. Cells of *L. lactis* NZ9800 harboring pNZ8024 showed no blue colonies on plates containing X-Gluc (Table 2). However, blue colonies were formed when nisin was present in the plates. *L. lactis* NZ9800 cells harboring pNZ8023 showed blue colonies on plates containing X-Gluc, irrespective of the addition of nisin (Table 2).

The induction by nisin and the promoter efficiency were determined as a function of the external nisin concentration (Fig. 2). The highest β-glucuronidase activity was reached with the *nisA* promoter in the presence of 3 μg of nisin $liter^{-1}$. Concentrations higher than 3 μg of nisin $liter^{-1}$ influenced the growth rate slightly, probably because the immunity level of the strain was not high enough to cope with these amounts of nisin. The amount of β-glucuronidase activity directed by the *nisF* promoter correlated with the addition of different concentrations of nisin as a linear dose-response relationship (Fig. 2). At the same inducing concentration of nisin, the β-glucuronidase activity directed by the *nisF* promoter was lower

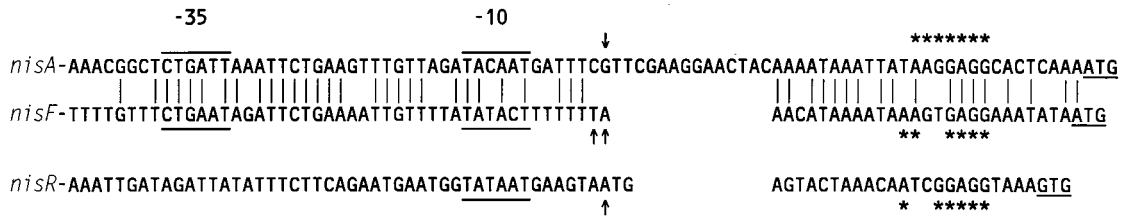

FIG. 3. Comparison of the *nisA*, *nisR*, and *nisF* promoter sequences. Arrows indicate the transcription initiation sites mapped by primer extension. The main start site of the *nisF* promoter was mapped on nucleotide 116 (numbering according to the work of Siegers and Entian [25]) with primer 1. The start site of the *nisR* promoter with primer 2 was mapped on nucleotide 2117 (numbering according to the work of van der Meer et al. [30]). The −35 and −10 sites and the start codons are underlined. Asterisks show the ribosome binding sites. A gap was introduced in the *nisF* and *nisR* sequences to show the homology around the ribosome binding sites, in the region preceding the ATG start codons (*nisA* and *nisF*) or GTG start codon (*nisR*).

than that found with the *nisA* promoter, suggesting a stronger transcription initiation efficiency of the *nisA* promoter (Fig. 2). The β-glucuronidase activity directed by the *nisR* promoter was similar in the presence and in the absence of nisin (Fig. 2). This indicates that the *nisR* promoter is constitutive under the conditions used and is not regulated by nisin.

Primer extension mapping of the *nisF* and *nisR* transcript. To locate transcription initiation sites, the putative *nisF* and *nisR* promoters, isolated in cloning experiments with a vector based on the *gusA* reporter gene, were characterized by primer extension analysis (Fig. 3). The primer extension experiment to map the *nisF* promoter was performed with total RNA obtained from *L. lactis* NZ9800 (Δ*nisA*) containing pNZ8024 and an oligonucleotide primer complementary to the coding strand of the *nisF* gene. In the presence of nisin, two extension products, differing by only one nucleotide in size, were detected, but no transcript was found in the absence of nisin (data not shown). Assuming that the most intense band is the main primer extension product, transcription of the *nisF* gene initiates at the T residue 28 bases upstream of the ATG start codon. The transcription start of the *nisR* gene was identical in both the presence and the absence of nisin, as was shown by using total RNA of strain NZ9800 and an oligonucleotide primer complementary to the coding strand of the *nisR* gene. The start site was mapped on an A residue 26 bases upstream of the GTG start codon. The *nisR* and *nisF* promoter sequences and the mapped transcription start sites were compared with the sequence of the *nisA* promoter, and striking similarities between the *nisF* and the *nisA* promoters were found (Fig. 3).

The requirement of the *nisR* and *nisK* genes in the regulation of the promoters of the nisin gene cluster. The chromosomal *nisK* gene in strain NZ9800 was insertionally inactivated by introduction of an erythromycin resistance gene, which yielded strain NZ9850 (16). *L. lactis* NZ9850 was used to study the requirement for an intact *nisK* gene for signal transduction via the two-component NisR-NisK system and to determine whether the β-glucuronidase expression of pNZ8024 is regulated via signal transduction. It has been shown that transcription of the Δ*nisA* gene, containing a 4-bp deletion, in NZ9850 was no longer inducible by nisin (16). In addition, no β-glucuronidase activity could be determined with the *nisA-gusA* fusion plasmid pNZ8008 introduced in *L. lactis* NZ9850. Plasmid pNZ8024 was also introduced in *L. lactis* NZ9850, but no β-glucuronidase activity could be demonstrated in the absence and in the presence of nisin. This clearly shows that the *nisF* promoter, like the *nisA* promoter (16), requires an intact *nisK* gene.

Expression of *gusA* under control of the *nisA* and *nisF* promoters in *L. lactis* NZ3900 was determined in order to verify whether the *nisR* and *nisK* genes are the only genes needed in the signal transduction pathway. *L. lactis* NZ3900 contains a single copy of the *nisR* and *nisK* genes under control of their own promoter in the *pepN* locus of *L. lactis* NZ3000 lacking Tn*5276*. The plasmids pNZ8008 and pNZ8024 were also introduced in *L. lactis* NZ3900. The β-glucuronidase activity directed by the *nisA* and the *nisF* promoter, respectively, in this strain was detected only after induction with nisin. Considerably lower concentrations of nisin (0.01 to 0.1 μg liter^{-1}) were required to induce *gusA* expression in strain NZ3900 to the same level as in strain NZ9800 (Fig. 2). Moreover, the same concentration of nisin was also found to induce higher expression in NZ3900 with the *nisA-gusA* fusion than with the *nisF-gusA* fusion as in strain NZ9800. The regulatory control in strain NZ3900 was similar to that in strain NZ9800 containing pNZ8008 or pNZ8024 (Fig. 2). This indicates that regulation of both the *nisA* promoter and the *nisF* promoter is dependent on the *nisR* and *nisK* genes.

DISCUSSION

The promoters in the nisin gene cluster *nisABTCIPRKFEG* were characterized by primer extension and transcriptional fusions to the promoterless β-glucuronidase reporter gene *gusA* of *E. coli*. Recently, it has been demonstrated that transcription of *nisA* is autoregulated by the fully modified nisin peptide via signal transduction by a two-component regulatory system. This signalling pathway depends on the presence of a *nisR* gene, encoding a response regulator, and an intact *nisK* gene, encoding a sensor protein (16).

The expression of *nisBTCIP* is likely to be dependent on the *nisA* promoter by limited read-through from *nisA* caused by the inverted repeat located between the *nisA* and *nisB* genes (16, 21). The promoter in front of the *nisRK* genes was mapped and was shown to give rise to *gusA* expression in the nisin-producing strain NZ9700 harboring the *nisR-gusA* fusion plasmid pNZ8023. This plasmid was also introduced in the non-nisin-producing *L. lactis* strain NZ9800 (Δ*nisA*) and in *L. lactis* MG1363 (no nisin genes). Quantitative β-glucuronidase assays revealed the same activity in all the strains, indicating nisin-independent expression of the *nisR* gene, and probably also of the *nisK* gene, because the genes are overlapping and no transcription terminator is present at the end of *nisR*. The continuous and nisin-independent production of the sensor (NisK) and regulator (NisR) ensures the availability of the proteins involved in signal transduction.

Another promoter was identified in front of the *nisFEG* genes, which are involved in development of immunity to nisin. Furthermore, it is demonstrated that expression of the *nisF* gene and most likely also of the partly overlapping *nisEG* genes (25) is controlled by a nisin-inducible promoter. The transcription initiation site of the *nisF* promoter was mapped in *L. lactis*

NZ9800, and a transcript was found only in the presence of nisin. Extracts of cells of NZ9800, containing pNZ8024 or the *nisA-gusA* fusion plasmid pNZ8008, induced with increasing concentrations of nisin, showed increasing β-glucuronidase activities, indicating a linear dose-response relationship (Fig. 2). No β-glucuronidase activity could be detected without induction. This shows that the *nisF* promoter is regulated in the same way as the *nisA* promoter. However, at the same inducing concentration of nisin the β-glucuronidase activity directed by the *nisF* promoter was lower than that found with the *nisA* promoter, which could indicate a higher transcription initiation efficiency of the *nisA* promoter.

The requirement of other genes of the nisin gene cluster in the regulation cascade, initiated by nisin, was analyzed by using several strains. In *L. lactis* MG1363, only the *nisR* promoter showed activity but no activity was found directed by the *nisA* and *nisF* promoter, which indicates that the regulated promoters need other nisin genes for their transcription activation. In *L. lactis* NZ9850 (Δ*nisK*), no β-glucuronidase activity was found to be directed by the *nisA* or *nisF* promoter in the absence or presence of nisin. This shows the requirement of an intact *nisK* gene for signal transduction, resulting in activation of both promoters.

L. lactis NZ3900, which contains a single copy of the *nisRK* genes on the chromosome, harboring either plasmid pNZ8008 or pNZ8024, showed β-glucuronidase activity only in the presence of nisin. Strain NZ3900 does not contain the known immunity genes *nisI* or *nisFEG*. However, this does not lead to a growth problem caused by a low level of immunity, because the concentrations of nisin needed for induction are far below the MIC (14 μg liter^{-1}). A linear dose-response curve was found in NZ3900, which strongly indicates that regulation of both the *nisA* and the *nisF* promoters is similar and dependent on the regulator NisR and the sensor NisK. However, the response in strain NZ3900 is 30-fold higher with the same inducer concentration, compared with the response in strain NZ9800 (Fig. 2). This observation may be a consequence of the fact that in strain NZ9800 are present immunity proteins (NisI, NisF, NisE, NisG) which can bind or react with the nisin molecules in the medium, thereby lowering the amount of nisin available for interaction with the sensor NisK. Strain NZ3900, without the immunity proteins, is a more sensitive strain to use for the induction by nisin and is for that reason an attractive strain to use for inducible high-level gene expression.

The sequence of the *nisF* promoter shows significant similarities to that of the *nisA* promoter. Most lactococcal promoters contain the canonical −35 and −10 consensus sequences, although the sequences that are bound by the main lactococcal transcription factor σ^{39} have not been identified yet (6). It has been suggested that a consensus promoter is characterized by −35 TTGACA and −10 TATAAT sequences that are spaced by an average of 17 nucleotides. Controlled promoters usually show a larger spacing between the canonical sequences (6). The *nisA*, *nisR*, and *nisF* sequences are aligned with respect to the conserved −35 and −10 sequences and the transcriptional initiation sites (Fig. 3). The atypical *nisF* promoter sequence shows a −35 region that, like the *nisA* −35 region, starts with CTG and is located 20 bp upstream of the −10 region, reminiscent of features of other lactococcus-controlled promoters (6). The promoter sequences of *nisA* and *nisF* contain a partially conserved region which could be involved in the transcriptional control function (Fig. 3).

The sequence of the *nisR* promoter region contains a distinct TATAAT box but no clear canonical sequence 17 bp upstream of the −10 box. However, a possible −35 sequence can be found 21 or 25 bp upstream of the −10 sequence, but this would be very large spacing considering the constitutive character of the *nisR* promoter under the tested conditions. There is no significant homology between the *nisA* and *nisR* promoters (Fig. 3). The *nisR* promoter seems to be a relatively strong promoter, since the β-glucuronidase activity obtained with the *nisR* promoter is similar to that obtained with the *nisA* promoter after induction with 0.5 μg of nisin liter^{-1} (Fig. 2). The fact that *nisK* contains a nonoptimal ribosome binding site (9) and that *nisR* starts with GTG (30) might lead to a lower translation efficiency and to moderate protein production of NisR and NisK in wild-type nisin-producing strains.

The autoregulated *nisA* and *nisF* genes are controlled in a nisin concentration-dependent manner. The question arises why the *nisFEG* genes are not transcriptionally linked to the *nisABTCIP* genes in such a way that they are coregulated. One reason could be that a higher expression level of the *nisFEG* genes is required to obtain sufficient immunity levels for the high amounts of nisin produced by wild-type cells (>10 mg liter^{-1}). The observed organization also has the advantage for the cells of being able to rapidly increase immunity levels in response to increasing nisin concentrations and amplifying the response to environmental signals. We have demonstrated that this form of quorum sensing occurs naturally in lactococci (reference 16 and this paper) and can be applied to the construction of nisin-inducible gene expression systems (4).

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